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RESEARCH ARTICLE

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Timing of chocolate intake affects hunger, substrate oxidation, and microbiota: A randomized controlled trial

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Abstract

Eating chocolate in the morning or in the evening/at night, may differentially affect energy balance and impact body weight due to changes in energy intake, substrate oxidation, microbiota (composition/function), and circadian-related variables. In a randomized controlled trial, postmenopausal females (n = 19) had 100 g of chocolate in the morning (MC), in the evening/at night (EC), or no chocolate (N) for 2 weeks and ate any other food *ad libitum*. Our results show that 14 days of chocolate intake did not increase body weight. Chocolate consumption decreased hunger and desire for sweets ($P < .005$), and reduced *ad libitum* energy intake by ~300 kcal/day during MC and ~150 kcal/day during EC ($P = .01$), but did not fully compensate for the extra energy contribution of chocolate (542 kcal/day). EC increased physical activity by +6.9%, heat dissipation after meals +1.3%, and carbohydrate oxidation by +35.3% ($P < .05$). MC reduced fasting glucose (4.4%) and waist circumference (−1.7%) and increased lipid oxidation (+25.6%). Principal component analyses showed that both timings of chocolate intake resulted in differential microbiota profiles and function ($P < .05$). Heat map of wrist temperature and sleep records showed that EC induced more regular timing of sleep episodes with lower variability of sleep onset among days than MC (60 min vs 78 min; $P = .028$). In conclusion, having chocolate in the

Abbreviations: BMI, body mass index; EC, chocolate in the evening/at nigh; FID, flame ionization detector; MC, chocolate in the morning; N, no chocolate; PCA, principal component analysis; RQ, respiratory quotient; SCFAs, short-chain fatty acids; T2DM, type 2 diabetes mellitus; VAS, visual analog scale.

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morning or in the evening/night results in differential effects on hunger and appetite, substrate oxidation, fasting glucose, microbiota (composition and function), and sleep and temperature rhythms. Results highlight that the "when" we eat is a relevant factor to consider in energy balance and metabolism.

KEYWORDS

circadian, chocolate, energy balance, glucose control, microbiota

1 | INTRODUCTION

Food timing is a relevant factor in weight control.[1] We and others have shown that not only "what" but also "when" we eat can impact obesity and weight loss.[2-5] Several biological mechanisms could explain the causal relationships between late eating and adverse cardiometabolic health, including changes in basal energy expenditure, diet-induced thermogenesis, substrate oxidation, glucose metabolism, wrist body temperature pattern, food intake concurrent with elevated melatonin signaling, and phase shifts of peripheral clocks including those in adipose tissue.[6]

The circadian system keeps us in synchrony with the 24-hour day. Meal timing can influence circadian rhythms[7] and eating a high energy and high sugar food, such as chocolate, either at night or in the morning may have a different effect on the circadian system, the peripheral clocks of different organs and tissues, and consequently on body weight and metabolism. Eating at the "wrong" time could be a determining factor for the loss of synchrony between the circadian system and different metabolic processes affecting energy and adipose tissue metabolism and the obesity risk.[8-11]

Milk chocolate has a name for contributing to weight gain due to its high fat, sugar and caloric content. Chocolate eating habit has been associated with long-term weight gain[12] in a dose-dependent manner,[13] especially in postmenopausal females[12] who are particularly vulnerable to weight gain.[14] Nevertheless, a meta-analysis of randomized clinical trials has shown that chocolate supplementation during several weeks (from 2 to 24 wk. depending on the trial) does not change body weight or body fat distribution.[15] Furthermore, non-conclusive data on the impact of chocolate on health pertain to glycemic control.[16] Most of these clinical trials have been performed with dark chocolate (35-85% of cocoa), while milk chocolate, with less cocoa content (~10-35%), is still the basis of most popular candy bars and sweet treats. More importantly, none of these studies have considered the timing at which the chocolate is consumed.

To our knowledge, there are no studies in humans considering the timing of chocolate intake and the effect on sleep or circadian-related variables such as those derived from the daily rhythms of temperature or activity, which have been used to assess circadian health in different populations.[17-19] Recently, we demonstrated that the timing of eating may change the daily rhythms of diversity and abundance of microbiota, with opposite patterns between early or late eating.[20]

We hypothesize that having a high-energy and high-sugar food such as chocolate during a short-term period of two weeks in the morning or in the evening/at night, may affect energy balance and differentially impact body weight or body fat distribution due to changes in energy intake, substrate oxidation, sleep- and circadian-related variables, or microbiota composition and their metabolic activity. To address this question, 19 postmenopausal females completed a randomized controlled cross-over trial of *ad libitum* food intake with either 100 g of chocolate (~33% of their daily energy intake) in the morning (within 1 hr after waking time) or at evening/night (within 1 h before bed time) as compared to no chocolate intake, with a duration of two weeks for each intervention and washout periods in between, for a total duration of 9 weeks.

2 | SUBJECTS AND METHODS

2.1 | Subjects

Nineteen postmenopausal Caucasian females aged 52 ± 4 years were studied, with initial weight of 65.5 ± 10.5 kg, body mass index (BMI) of 25.0 ± 3.7 kg/m^2, and body fat of 32.7 ± 5.9%, from a Mediterranean area (Spain, Murcia), who were following their usual dietary habits (*ad libitum*) (Trial Registration: Chocolate and Menopause Time Study (ONTIME-CHOC) [ClinicalTrials.gov: #NCT03949803]). General characteristics of the volunteers at baseline are presented in Supplemental Table S1. Inclusion criteria were females aged between 45 and 65 years, with menopause (absence of menstruation for at least 1 yr) who liked milk chocolate. Exclusion criteria were: BMI higher than 35 kg/m^2; endocrine, renal, hepatic, eating or psychiatric disorders; using any pharmacologic treatment or fiber supplements. All research methods and procedures were performed in accordance with the Helsinki Declaration of Human Studies and approved by the Ethical Committee of the University of Murcia (UMU). All volunteers signed an informed consent form.

2.2 | Experimental design

The study utilized a randomized, crossover design protocol (Supplemental Figure S1; Flow chart). Randomization was performed by the UMU staff with block size 3 in a balanced design (http://www.ramdomization.com).

All females were contacted by social media. From a total of 30 females, 24 consented to participate in the study (Supplemental Figure S1; Flowchart). Nineteen females performed the study and took 100 g of milk chocolate for 2 weeks within an hour of waking up together with breakfast (MC), 100 g of milk chocolate for 2 weeks within the hour before bedtime (EC), and no chocolate for 2 weeks (N), in a randomized order. There was a week of washout between each condition in which volunteers followed their usual dietary habits but without eating any chocolate (Supplemental Figure S1; Flowchart). Baseline measurements were taken at the beginning of the study. For microbiota composition and function, baseline was defined as the first day of each volunteer's first condition.

We chose 100 g of chocolate to achieve ~30% of habitual total daily caloric intake in the volunteers studied, based on a previous study performed in animal models, showing that a dosage of chocolate that comprised a 30% of total daily calories was able to entrain the circadian system[21] and based on the chocolate dosage in a previous study performed in humans that used 100 g of chocolate/day.[22]

The milk chocolate composition was 18.1 g cocoa, 31 g fat, 58.4 g carbohydrates (of which 57.5 g were sugar), 6.3 proteins, and 1.8 g fiber *per* 100 g of chocolate. It contained 215 mg of theobromine, 2.06 mg of caffeine, and 854 mg of total polyphenols (mainly epicatechin and catechin) *per* 100 g of chocolate. In the weeks of chocolate intervention (MC, EC), volunteers were allowed to have chocolate only during a 1-hour time window. Nevertheless, they were allowed to have another type of chocolate-free candy at any time. During the weeks of control (N) and washout, volunteers were not permitted to have any chocolate, but they were allowed to have any other chocolate-free sweets.

2.3 | General measurements

Volunteers underwent the following tests and measurements (Supplemental Figure S1A,B):

2.3.1 | Anthropometry

All measurements were performed at the same time in every condition (morning first hour after habitual awakening). Body weight was determined at baseline (Supplemental Figure S1A) and in three different moments (first day, 8th day and last day) of each condition (Supplemental Figure S1B), while volunteers were barefoot and wearing light clothes using a digital scale accurate to the nearest 0.1 kg.

Height was measured at baseline in the morning (Harpenden digital stadiometer [rank, 0.7-2.05]). Volunteers were positioned upright, relaxed, and with the head in the Frankfurt plane. BMI was calculated as weight (kg)/height^2 (m^2).

Total body fat (kg and %) was measured at baseline (Supplemental Figure S1A) and the last day of each condition (Supplemental Figure S1B) by bioelectrical impedance (TANITA MC 180 MA equipment, Tanita Corporation of America, Arlington Heights, IL, USA) together with waist circumference that was measured at the umbilicus level and hip circumference measured at the widest circumference over the greater trochanters.

2.3.2 | Dietary intake

Volunteers were instructed to eat *ad libitum* and to freely choose their meal timings (Supplemental Figure S1A,B).

Subjective measurements

Dietary intake was continuously recorded (dietary intake record, DIR) during the 7-day baseline (7-DIR) and during the 14 days of each condition (14-DIR). Volunteers recorded quantity and timing (clock times) for each meal and snack.

Objective determinations

At baseline and during the 14 days of each condition, time-stamped photographs were captured with a cell phone app.

Total energy intake, macronutrient composition, caffeine, and polyphenol content during the 14 days of each condition were analyzed with the nutritional evaluation software (Grunumur 2.0 8), both including and excluding the chocolate intake (Supplemental Table S2). The intake of several food groups and dietary scores were also estimated from the 14-day dietary record of each condition, including a Mediterranean Diet Score that is comprised of 8 food groups: (1) vegetables and greens; (2) pulses (lentils, beans, chickpeas, and peas); (3) fruit and nuts; (4) dairy products (milk, yogurt, and cheese); (5) cereals; (6) meat, meat derivatives, and eggs; (7) fish; and (8) wine, as previously described.[23,24]

2.3.3 | Hunger and sweets appetite (VAS)

Volunteers completed several 10-cm visual analog scales (VAS) before and after each meal at baseline and during each condition (during the last 3 days) (Supplemental

Figure S1A,B). Each day, volunteers completed a total of 8 hunger/appetite assessments during the N and MC, and 10 tests during the EC condition (the latter including before and after evening chocolate intake).

The questions intended to cover the full wake episodes, from the moment the volunteers get up until the moment they go to bed, in the following order: (1) Right at the time of getting up in the morning; (2) Just before breakfast (or taking the chocolate in the morning) with the food served on the table; (3) Immediately after eating breakfast or eating chocolate; (4) Before lunch with food being served; (5) Immediately after eating lunch; (6) Before dinner with food being served; (7) Immediately after finishing dinner; (8) In bed before sleep; (9) Just before eating the chocolate (EC); and (10) Immediately after eating the chocolate (EC).

2.3.4 | Wrist temperature and actimetry

Females wore a wristwatch during the last 7 days of each condition (N, EC, MC) on the non-dominant wrist to record body temperature (T) and activity (A) (Supplemental Figure S1A,B). This wristwatch integrated two different sensors:

1. Temperature sensor for determining wrist temperature rhythms (ThermochroniButton, DS1921H, Dallas, Maxim, Dallas, TX) programmed to collect information every 10 minutes.
2. Accelerometer sensor (G Acceleration Data Logger UA-004-64; Onset Computer, Bourne, MA, 169 USA) that records and monitors physical activity and body position rhythms every 30 seconds. Activity was calculated as degrees of change in X, Y, and Z axes.

The study was conducted between October and April, avoiding extreme environmental temperatures of Murcia in summer that could mask effects on wrist temperature.

2.3.5 | Sleep duration, number of awakenings, and siesta frequency and duration

Subjective measurements

Sleep onset and offset, sleep duration, number of awakenings, and siesta timing were self-reported during 7 days at baseline and 14 days during each condition (Supplemental Figure S1A,B).

Objective determinations

Sleep characteristics information, and siesta frequency and duration were obtained from the 7-day wrist temperature and actimetry recordings obtained by the wristwatch (Supplemental Figure S1A,B).

2.3.6 | Indirect calorimetry

Volunteers arrived in the indirect calorimetry room at the nutritional clinic in a fasting state and stayed there in a sedentary position, under the following controlled conditions: temperature 23°C and relative humidity 52 ± 2%. Indirect calorimetry (Oxicon Pro/Delta, VIASYS Healthcare, Germany) was performed in a calm state, after 30 minutes of initial rest on a reclining chair and, at the same time in the morning for every chocolate condition (MC and EC) and for control (N) (Supplemental Figure S1A,B). A face mask Triple V-highly precise (Oxycon Pro/Delta, VIASYS Healthcare, Germany) was used to capture gas exchange. Females were encouraged to keep quiet and immobile and to breathe normally during each measurement (20 minutes). Oxygen (O_2) consumption (mL/min) and carbon dioxide (CO_2) production (mL/min) were measured. The Respiratory Quotient (RQ) was calculated out of O_2 consumption (mL/min) and CO_2 production (mL/min). Energy expenditure values (kcal min^{-1}) were calculated according to the Weir equation. Carbohydrate (CHO; g min^{-1}) and fat oxidation (F; g min^{-1}) were calculated from the RQ and % of total energy was determined.

2.3.7 | Cortisol saliva determinations

Saliva for cortisol measurements was collected at baseline (on the penultimate day) and during each chocolate condition (on the penultimate day) (Salivettes, Sarstedt, Barcelona, Spain). Samples were collected before breakfast (9:00 h), lunch (14:00 h), and dinner (21:00 h) and kept at 4ºC until centrifuged. They were then aliquoted in cryotubes of 500 µL and stored at −80ºC until analysis. Cortisol was measured by radioimmune assay (IZASA, Barcelona, España).

2.3.8 | Fasting glucose

Fasting whole-blood glucose concentration was determined on the last day of each condition with reactive strips to measure glucose (Gluco-Men LX Plus +, Menarini diagnósticos S.A. Barcelona Spain).

2.3.9 | Microbiota

Fecal samples. Volunteers collected a stool sample in the morning on the first and penultimate day of each condition. Samples were stored at -80ºC until analysis

Analysis of microbiota composition by 16S rRNA amplicon sequencing

Total fecal DNA was isolated (MasterPure Complete DNA & RNA Purification Kit, Epicentre, Madison, WI) following the user's guide. We included a bead-beater step and enzyme incubation to increase DNA extraction as described previously (Garcia-Mantrana et al[68]). Total DNA concentration was measured (Qubit 2.0 Fluorometer, Life Technology, Carlsbad, CA, USA) and normalized to 5 ng/μL for 16S rRNA gene (V3-V4 region) amplification (Nextera XT Index Kit).

Amplicons were checked (Bioanalyzer DNA 1000 chip) and libraries were sequenced using a 2 × 300 pb paired-end run (MiSeq Reagent kit v3) (MiSeq-Illumina platform, Valencia, Spain). Controls during DNA extraction and PCR amplification were also included and sequenced. An open reference OTU picking method using 97% identity to the Greengenes 13_8 database was performed (QIIME pipelineversion 1.9.0). Singletons and OTUs with a relative frequency below 0.01 were removed. Sequences that could not be classified to domain level as Cyanobacteria, Chloroplasts and Rhizobiales, were removed. Relative abundances of specific bacteria and alpha-diversity indices (Chao1: richness and Shannon: diversity) were obtained.

Analysis of Short-Chain Fatty Acids (SCFAs) by GLC

A protocol was used to determine SCFAs in feces samples.[25] Briefly, feces samples were mixed with NaOH 1 N in a proportion of 1:1 (w/v) and lyophilized for 48 hours. One hundred milligrams of lyophilized sample was homogenized in a vortex with a mixture of formic acid (20%), methanol and 2-ethyl butyric acid (internal standard, 2 mg/mL in methanol) (1/4.5/1, v/v), exposed to ultrasounds for 5 minutes and centrifuged at 16110 ×*g* for 15 minutes at room temperature. The supernatant obtained was filtered (13 mm, 0.22 PTFE, VWR International, USA) and analyzed by GC-FID.

Chromatographic analysis was carried out using an Agilent 7890A GC system equipped with a flame ionization detector (FID) and a 7683B automatic injector (Agilent Technologies, USA). A fused-silica capillary column Nukol (Supelco, USA) of 30 m × 0.25 mm I.D., 0.25 μm coated was used to separate different SCFAs. The supplied carrier gas was Helium at a flow rate of 25 mL/min. The initial oven temperature was 80ºC and was kept constant for 5 minutes and then raised to 185ºC at a rate of 5ºC/min. Samples (2 μL) were injected in splitless mode with an injection port temperature of 220ºC. The flow rates of hydrogen, and air as makeup gas were 30 and 400 mL/min, respectively. The temperature of the FID was 220ºC and each analysis was run for 26 minutes.

SCFAs were identified by comparison with the retention times of authentic standards (Supelco, USA). Quantification was based on calibration curves constructed for a set of SCFAs standards. The concentration expressed as mmol/mg feces (fresh weight) of each SCFAs was calculated using linear regression equations ($R^2 = 0.99$) from the corresponding curves of standards. All chemicals were at least GC grade quality.

2.4 | Statistical methods

An analysis of variance (ANOVA) with repeated measurements was performed between the three conditions: having chocolate in the morning (MC), chocolate in the evening/at night (EC), and no chocolate (N) with post hoc comparisons. Data normality was confirmed by the Shapiro–Wilk and Kolmogorov–Smirnov tests (significance level $P > .05$). Acetate concentration was logarithmically transformed to accomplish normality. Fasting glucose analyses were adjusted by age and initial body weight. To eliminate the potential influence of the caffeine content of other components of the diet (that differed between conditions, Supplemental Table S2) we adjusted for the caffeine content for the different outcomes, and significance was maintained for the outcomes except for the waist circumference that lost significance and changed to a statistical trend ($P = .053$).

To obtain additional information about the relationships between bacterial groups and SCFAs, a principal component analysis (PCA) was performed, by varimax normalized rotation (Kleinbaum et al., 1988).[26] Microbiota was adjusted by the fecal sample of the first day of the volunteer's first condition. Accepting an alpha risk of 0.05 in a 2-sided test, for the current sample size (n = 19), we wanted to ensure that we would be able to detect any substantial body weight changes across the study protocol. For total body weight, we would have had a statistical power of 80.48%, 84.84%, 97.3%, to detect 1kg of change within each condition (morning chocolate, evening/night chocolate and control, respectively).

For the main outcome measures of interest, we tested if we had enough power to detect meaningful changes: for waist circumference we had a statistical power of 70.99%, 93.99%, and 90.66%, to detect 2 cm of change within each condition. For fasting glucose, CHO and lipids expenditure, we had a statistical power of 70.42%, 75.98%, and 63.73%, to detect changes of 4.7%, 34.55%, and 17.86% between morning and evening conditions. SPSS 20.0 software (SPSS, IBM, Madrid, Spain) was used for the analysis. A two-tailed *P*-value of $< .05$ was considered as statistically significant.

3 | RESULTS

3.1 | Females did not gain significant body weight with chocolate intake

Females consumed 100 g of milk chocolate every day during the 2-week intervention arms (extra 542 kcal). However,

despite these extra calories added by chocolate, they did not gain significant body weight when eating *ad libitum* within each condition (Figure 1). Of interest, females reduced waist circumference when having chocolate in the morning (Supplemental Figure S2; $P < .05$).

The following potential mechanisms may explain the lack of weight gain with chocolate intake:

3.1.1 | *Ad libitum* energy intake was reduced (Figure 2A)

While the volunteers had an increase of energy due to chocolate extra calories (extra 542 kcal) (represented in grey at the top of each bar) as compared to the non-chocolate condition (N) (represented in green color bar), they spontaneously reduced their *ad libitum* energy intake by 16% (296 ± 442 kcal/d) when eating chocolate in the morning (orange color bar) (MC), and to a lesser extent with chocolate at evening/night (blue color bar) (EC) (10%; 165 ± 404 kcal/d) ($P = .01$).

Regression models showed that there was a significant and positive correlation between the *ad libitum* energy intake during both chocolate conditions and body weight gain ($r = -0.64$; $P = .004$). Those females who better compensated the extra caloric intake of chocolate were those who gained less weight or lost more weight. When eating chocolate in the morning, volunteers decreased their daily cortisol levels as compared to when eating chocolate in the evening/night (Figure 2B,C) and *ad libitum* energy intake by reducing fat (−17 ± 7 g/d) and carbohydrate (−27 ± 17 g/d) intake as compared to control ($P < .05$) without significant changes in proteins $P > .05$ (data not shown). Females were less hungry when eating chocolate in the evening than in the morning or with no chocolate and had less desire for sweets when eating chocolate (in either the morning or evening) than with no chocolate (Figure 2D-G).

3.1.2 | Energy expenditure was increased through increased physical activity and heat dissipation (Figure 3)

Volunteers increased physical activity +6.9% when consuming chocolate at evening/night (EC) as compared to control (N) (42.3 ± 1.9 Δ°/min; 39.6 ± 1.8 Δ°/min; $P = .003$) and differences were significant during the night time and day time (Figure 3A-C). Females experienced a higher heat dissipation, as indicated by increased wrist temperature, while eating chocolate during the evening/night than when not eating chocolate, which was significant at night time and during the postprandial episodes after the three main meals of the day (Figure 3D-F).

3.1.3 | Changes in sleep- and circadian-related variables with chocolate intake

Heat maps of wrist temperature indicated that all participants maintained a day/night rhythm, with highest values during the sleep phase (Figure 3G). This was not shifted when ingesting chocolate in the morning (MC) or during the evening/night (EC) as compared with baseline. Nevertheless, Heat-map of wrist temperature visualization suggests that EC induced more regular timing of sleep episodes (Figure 3G) with lower variabilities of sleep onset among weekdays than in the morning condition (60 min vs 78 min, $P = .028$) ($P = .028$). Sleep fragmentation tended to be higher when the chocolate was consumed during the evening/night (Intraday variability; IV = 0.18 ± 0.17) than in the morning (IV = 0.14 ± 0.16) or without chocolate (0.15 ± 0.14). However, we did not find significant differences among conditions ($P = .086$). Eating chocolate in the morning (MC) was associated with an increased siesta frequency (self-reported) as compared to control (N) (4 ± 0.49 d/wk; 1 ± 0.21 d/wk; $P = .019$) with no significant differences between evening/night chocolate (EC) and control (N).

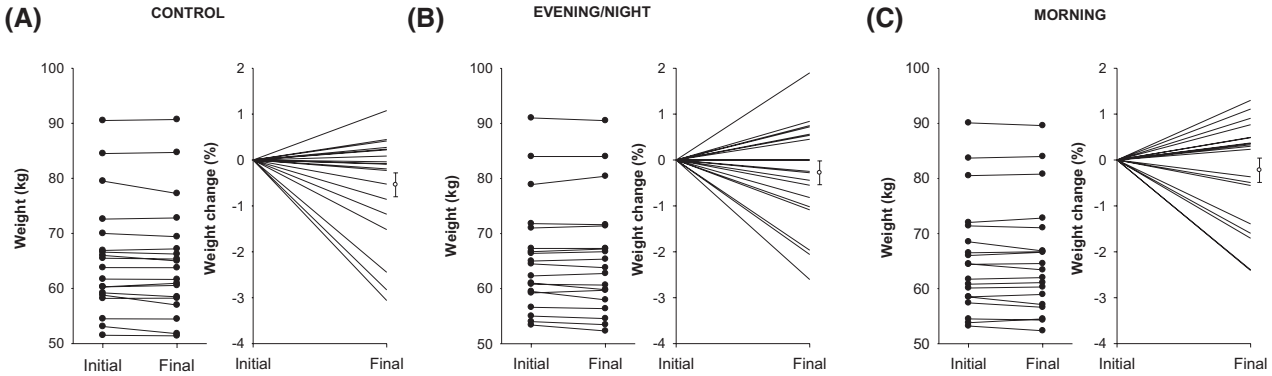

FIGURE 1 No changes in body weight within each condition of the experiment (A, no chocolate [control]; B, evening/night chocolate; and C, morning chocolate) ($P > .05$ for all figures). Absolute values in each female and percentage of change (right panels) within each condition

3.2 | Changes in substrate oxidation depended on the timing of chocolate intake (Figure 4A,B)

Eating chocolate in the morning (MC) induced lipid oxidation, 25.6% more than chocolate during the evening/night (EC) (Figure 4A), while chocolate intake during the evening/night (EC) induced carbohydrate oxidation, +35.3% more than morning chocolate (MC) (Figure 4B). No significant differences were found between both chocolate conditions (MC and EC) and control (N). No significant differences were found in basal metabolic rate (data not shown). Table 1 summarizes the differential effects of chocolate depending on the time of consumption.

3.3 | Chocolate in the morning decreased fasting glucose (Figure 4C,D)

Consumption of chocolate in the morning (MC) decreased fasting glucose by −4.4% with respect to control (Figure 4), while chocolate intake during the evening/night (EC) showed a significant increase of fasting glucose levels by +4.9 % as compared to morning chocolate (MC) (Figure 4D) (Table 1).

3.4 | Timing of chocolate intake differentially influenced microbiota profiles and function (Figures 5 and 6)

3.4.1 | Microbiota composition

We observed an impact of chocolate intake on microbiota composition and diversity (a trend) and a significant increase in the interindividual variability as compared to control (N) (Figure 5). We obtained increased abundances of Actinobacteria and reductions in the relative abundance of Firmicutes ($P = .003$) (Figure 5A,C), specifically in unclassified Lachnospiraceae genus (Firmicutes) ($P = .014$) (Figure 5D) and a reduction in microbial diversity (inverse Simpson index) (a trend; $P = .082$) (Figure 5B). *Methanobrevibacter* genus abundance also increased with chocolate intake during the evening/night (EC) ($P = .013$) (Figure 5E). In addition, chocolate intake increased the abundance of *Akkermansia* (a trend, $P = .09$), *Ruminococcus* ($P = .029$), and *Dorea* genus ($P = .020$), among others (Figure 5E).

3.4.2 | Microbiota function

Chocolate consumption during the evening/night (EC) significantly increased the production of SCFAs such as acetate and propionate and other minor fatty acids (isobutyrate, isovaleronate, and valeronate) as compared to no chocolate (control) (N), while morning chocolate levels of SCFA were in between (Figure 6A). The beneficial effect of chocolate intake was supported by the increased levels of *Ruminococcus* (Figure 5E), which associated with higher acetate levels ($P = .039$) (Figure 6C), and the decreased levels of Lachnospiraceae genus (Figure 5D), which associated with a reduction of acetate (P=0.026) (Figure 6C).

TABLE 1 Summary of differential effects of the timing of chocolate (as compared to the non-chocolate control condition) on body weight and body fat distribution, energy expenditure, energy intake, metabolic variables, microbiota abundance and function, and circadian-related variables

	Morning chocolate	Evening/night chocolate
Anthropometry		
Body weight	No effect	No effect
Waist circumference	↓	No effect
Energy intake		
Change in *ad libitum* energy intake	↓↓	↓
Appetite for sweets	↓	↓↓
Hunger	No effect	↓
Cortisol levels	No effect	No effect
Energy expenditure		
Physical activity at night	No effect	↑
Physical activity during the day	No effect	↑
Resting energy expenditure	No effect	No effect
Lipid oxidation	↑	No effect
Carbohydrate oxidation	No effect	↑
Skin heat dissipation	No effect	↑
Glucose metabolism		
Fasting glucose	↓	No effect
Microbiota		
Firmicutes (Lachnospiraceae)	↓↓	↓
Actinobacteria	↑↑	↑
Ruminococcus	↑	↑
Methanobrevibacter	No effect	↑
SCFA (acetate, propionate, other minorities)	↑	↑↑
Sleep and circadian-related variables		
More regular timing of sleep episodes	No effect	↑
Siesta frequency	↑	No effect

Note: The direction of the arrow indicates increase or decrease in the effect, two arrows refers to a more robust effect based in *P* values. "No effect" indicates no significant differences between conditions based in *P* values.

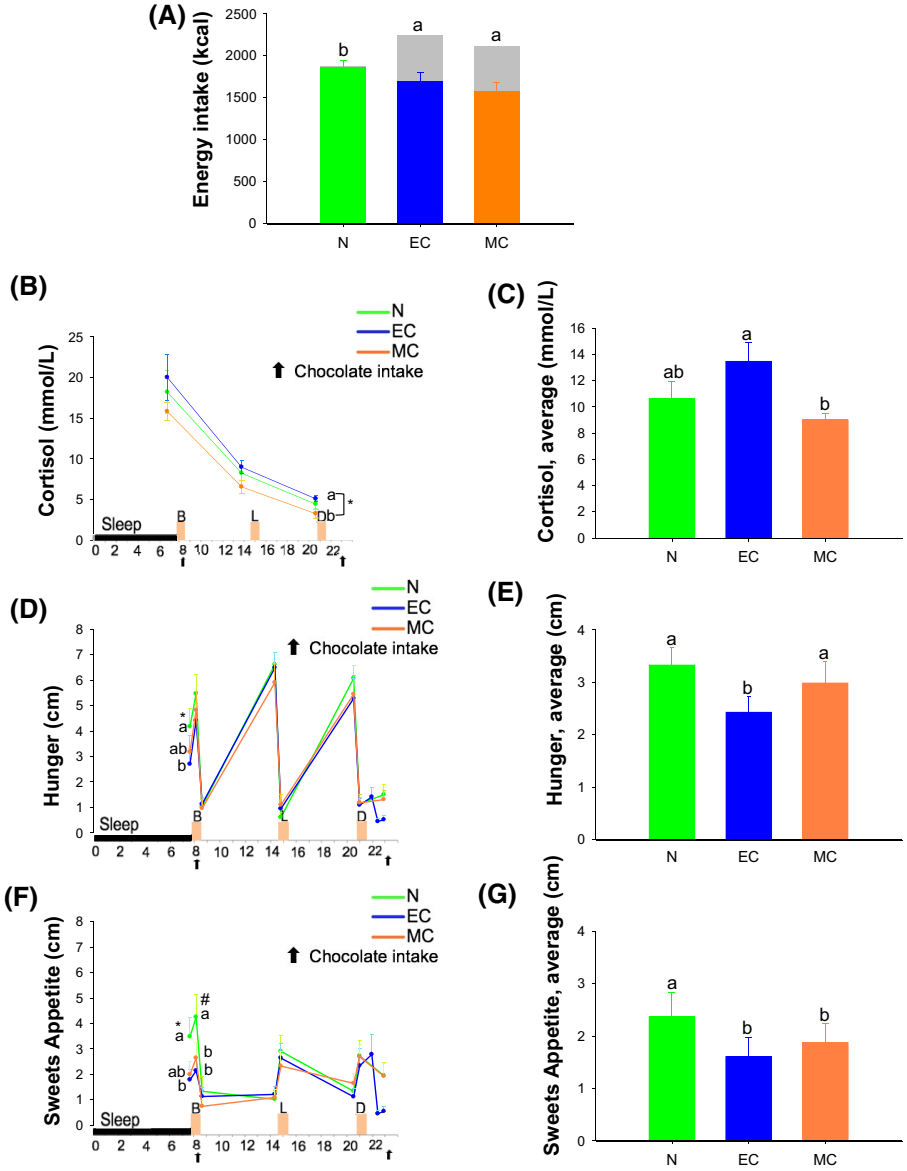

FIGURE 2 Significant differences in energy intake and related variables between No chocolate (N), Evening/Night Chocolate intake (EC), and Morning Chocolate intake (MC) (Average values of the 19 participants in each condition) ($P < .05$ for all figures). Different letters indicate significant differences between conditions after pairwise comparison test. A, *ad libitum* daily energy intake; color bars represent the *ad libitum* average daily energy intake during the two weeks of each condition of the experiment. Gray bars represent the *extra* energy intake from chocolate (542 kcal; 33% of average habitual daily energy intake). B, Cortisol values in saliva at different time points before breakfast (9:00 h), lunch (14:00 h), and dinner (21:00 h) measured on day 13 of each condition. The pink bars located vertically on the X axis represent the habitual timing of meals of the participants (average). B, breakfast; D, dinner; L, lunch. The black bars located horizontally on the X axis represent the habitual sleep timing and duration of the participants (average), while C, represents average values of cortisol in each condition (Mean and SEM). D, F, Data (2D, hunger and 2F, sweets appetite) (Mean and SEM) from visual analog scale (VAS) from different time points completed during the last 3 days of each condition: first dot to the left. (1) Just at the time of getting up in the morning; dot 2) Just before breakfast (or before having chocolate, MC) with the food on the table; dot 3) Immediately after eating breakfast or chocolate; dot 4) Before eating lunch, with food on the table; dot 5) Immediately after eating lunch; dot 6) Before dinner, with food on the table; dot 7) Immediately after eating dinner; dot 8) In bed, before sleep; dot 9) Just before eating chocolate, EC; and dot 10) Right after eating chocolate (EC). E,G, Daily average values of hunger and sweets appetite (Mean and SEM) from all the time points and the 19 volunteers studied in each condition.

3.4.3 | Associations between microbiota composition and function

A PCA was used to visualize the relationships between bacterial groups and the different SCFAs among the three conditions (Figure 6B, Supplemental Table S3). The information provided by the first two PCs allowed graphical separation of the different conditions (N, EC, and MC) (Figure 6B, panel 1). Differences were mostly marked by bacterial metabolites (SCFAs) produced by the microbiota, represented

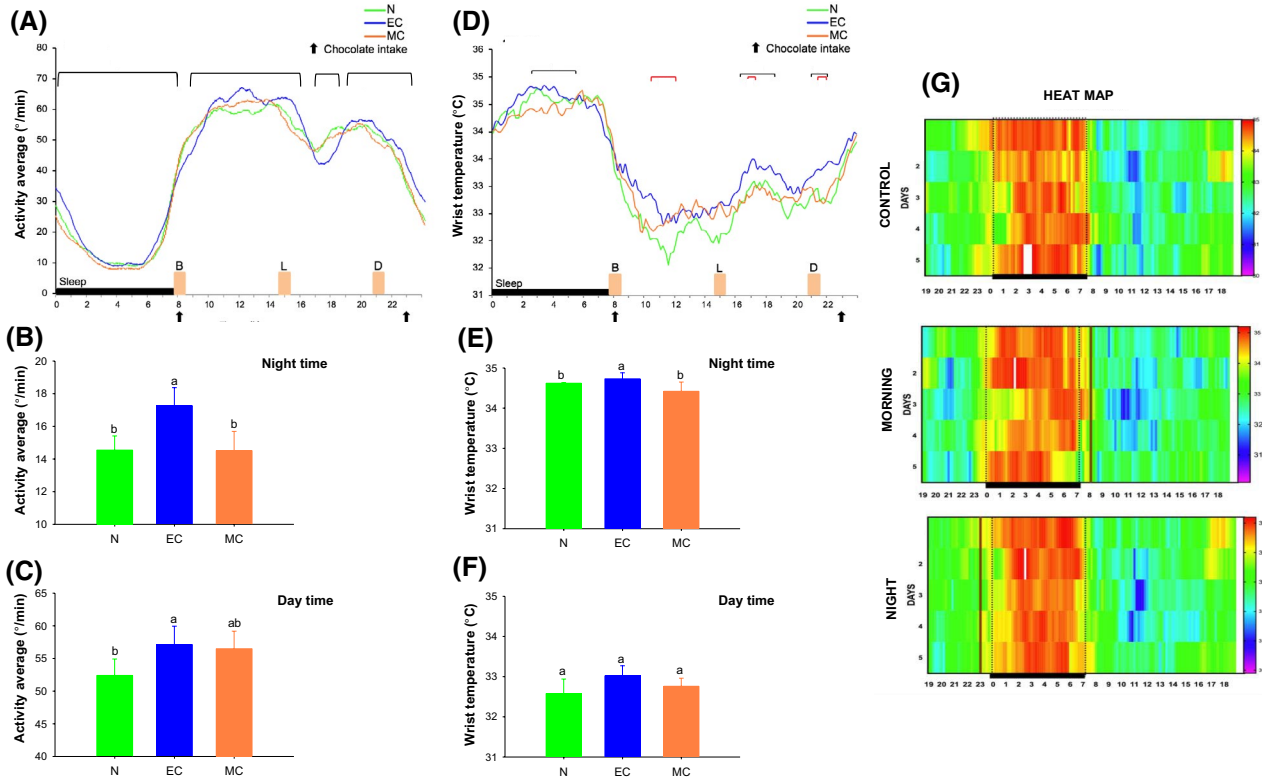

FIGURE 3 Significant differences in energy expenditure and related variables between No chocolate (N), Evening/Night Chocolate intake (EC), and Morning Chocolate intake (MC) (Average values of the 19 participants in each condition) ($P < .05$ for all figures). Different letters indicate significant differences between conditions after pairwise comparison test. A, Physical activity levels recorded by actimetry; D, Skin heat dissipation from wrist temperature records. Square brackets indicate the time slots where significant differences were observed by ANOVA repeated samples (ANOVA rm), $P < .05$. Black brackets indicate significant differences between evening/night and morning chocolate conditions. Red brackets indicate significant differences between evening/night and control chocolate conditions. The pink bars located vertically on the X axis represent the habitual timing of meals of the participants (average) N, no chocolate; EC, chocolate evening/night; MC, chocolate morning; B, breakfast; L, lunch; D, dinner. The black bars located horizontally on the X axis represent the habitual sleep timing and duration of the participants (average). B, Differences in average physical activity level between the different conditions during the evening/night; while C, represents daytime. E, Differences in average temperature between the different conditions during the evening/night; while F, represents daytime. G, The heat map of each condition, temperature values are represented in red for high temperature values, followed by orange, and yellow, green, and blue color for low temperature values

in pink color (M1 to M9) (Supplemental Table S3; Figure 6B, panel 2). In the PCA, *Ruminococcus* appeared in the 3th factor, together with Acetate and Acetate:Propionate ratio (Supplemental Table S3), which is consistent with the positive and significant correlation found between the abundance of *Ruminococcus* and acetate levels (Figure 6C).

4 | DISCUSSION

The postmenopausal females studied did not gain body weight with chocolate intake. Nowadays, the reported effects of chocolate intake on body weight are contradictory: while it has been shown that chocolate supplementation does not increase or reduce body weight, subsequent subgroup analyses following dose-response evaluations indicated that chocolate consumption (as compared to no chocolate) resulted in waist circumference reduction.[15] Congruently, experimental studies performed in adipose tissue from mice and in murine cell lines show that cacao decreases fat accumulation in adipocytes by decreasing lipogenic enzyme expression[27] while increasing lipolytic enzyme expression.[28]

Considering the so-called 3,500-calorie rule that estimates that increasing caloric intake by 500 kcal *per* day, or 3500 kcal *per* week would result in 1 lb of weight gain *per* week, these postmenopausal females should have gained more than 2 lb (~1 kg) in each chocolate intervention (2 wk × 7d × 542 kcal = 7588 kcal) if there would be no differences in *ad libitum* intake or energy expenditure.[28]

To investigate why volunteers didn't gain significant body weight, we explored different aspects of energy intake and expenditure. Results show that chocolate reduced *ad libitum* energy intake, consistent with the observed reduction in hunger, appetite and the desire for sweets shown in previous studies.[30]

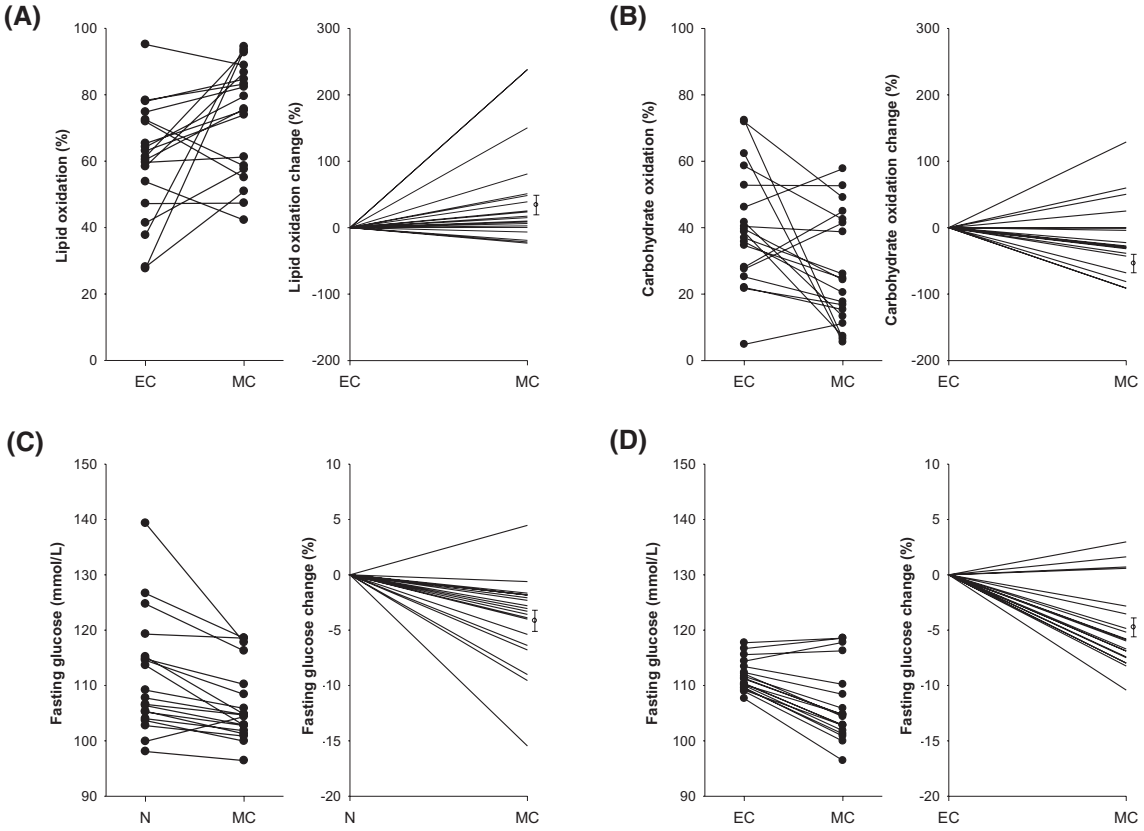

FIGURE 4 Significant differences in metabolic effects of timing of chocolate intake (No chocolate (N), Evening/Night Chocolate intake (EC), and Morning Chocolate intake (MC) ($P < .05$ for all figures). Absolute values in each female and percentage of change in lipid oxidation (A) and carbohydrate oxidation (B) and fasting glucose (C,D) in all conditions of the experiment

While the volunteers had an increase of energy intake due to chocolate's extra calories (extra 542 kcal) as compared to the non-chocolate condition, they spontaneously reduced their *ad libitum* energy intake by 16% when eating chocolate in the morning. This happened even though females consumed milk chocolate that has been shown to have less of an effect in decreasing appetite than dark chocolate.[31] Specific components of chocolate, such as epicatechin, may be accounting for this compensatory effect.[32] Indeed, participants had an increase in total polyphenols of 854.3 mg (mainly epicatechin and catechin) *per day* due to milk chocolate (100 g), while no differences were found in the other polyphenols content in the rest of the diet, when eating *ad libitum*. Furthermore, the adherence to the Mediterranean Diet and the intake of food groups rich in polyphenols in the *ad libitum* food intake were similar among the three conditions (Supplemental Table S2). Macronutrient composition of milk chocolate may also account for this caloric compensation. Indeed, when eating chocolate in the morning, volunteers decreased their *ad libitum* energy intake by reducing fat and carbohydrates as compared to control, and without significant changes in proteins. This concurs with the extra macronutrient intake from milk chocolate that is rich in fat (31 g) and carbohydrates (58.4 g), and low in proteins (6 g).

Results show that when eating chocolate, females were less hungry and had less desire for sweets than with no chocolate, especially when taking chocolate during the evening/night. Moreover, daily cortisol levels were lower when eating chocolate in the morning than at evening/night. Lower cortisol levels have been related to a lower stress-related appetite which may partly explain the better caloric compensation by the females when eating chocolate in the morning (Epel et al., 2001).[33] The decreased desire for sweets could be related to reduced cravings and improved diet satisfaction.[34] However, all these forementioned factors were not enough to fully compensate the extra energy derived from chocolate intake.

The other component determining energy balance besides energy intake is energy expenditure.[15,35] One mechanism to increase energy expenditure could be to spontaneously increase physical activity or body heat dissipation after a high caloric intake. Our results show that EC induced a rise in skin temperature at night, and in the postprandial period after breakfast, lunch (from 4 to 7 p.m.) and after dinner (9 to 10 p.m.) that may be related to an increased diet induced thermogenesis (DIT). Theobromine content in chocolate may be involved in these results, at least in the increase of body temperature at night, because theobromine increases heat production[36] and peaks in plasma around 2 hours after chocolate consumption while the half-life is 7 to 8 hours.[37,38]

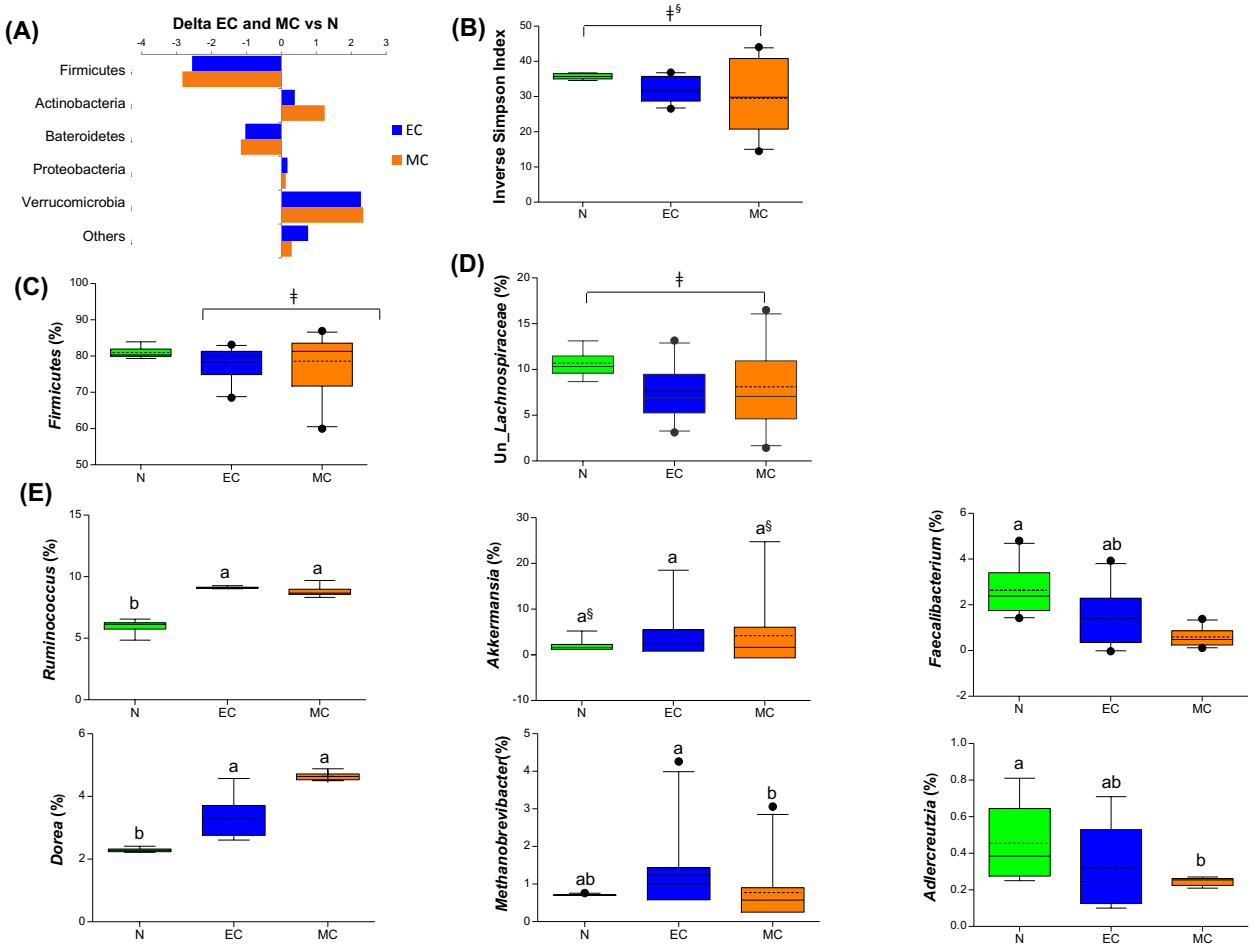

FIGURE 5 Microbiota composition and diversity in the chocolate intervention study. A, Significant changes in the predominant phyla in Evening/Night Chocolate intake (EC) and Morning Chocolate intake (MC) comparing with No chocolate (N)($P < .05$). B, Microbial diversity measured as inverse Simpson index. C, Changes in the relative abundance (%) of Firmicutes. D, Unclassified Lachnospiraceae genus in the feces samples at the different conditions. Values used were baseline-adjusted and grouped according different groups: N (control, no chocolate in green), EC (chocolate at night in blue), and MC (chocolate at morning in orange). Significant differences were obtained by ANOVA repeated measures [‡ represents $P < .05$ and ‡§ represents a trend $P < .09$], furthermore, differences between conditions are indicated in the graphs with the multiple pairwise comparisons (different letters represent significant differences and § represents a trend $P \leq .09$]. Boxes indicate the upper and lower quartiles, the dotted line within the box indicates the mean and the thin line indicates the median; the vertical lines indicate the 90-10 percentiles and the dots are outliers

Furthermore, morning chocolate induced lipid oxidation. These results may also be related to theobromine and other methylxanthines present in chocolate that have been shown to increase thermogenesis and lipid oxidation,[39] or to flavanols (epicatechin or catechin), other foods or beverages with similar contents of epicatechin or catechin that have been shown to increase fat oxidation.[40] As adipose tissue contributes to lipid oxidation,[41] these differences in substrate oxidation may be accounting for the decrease in waist circumference when chocolate was eaten in the morning. Literature shows that good weight loss responders have higher lipid oxidation rates than those experiencing weight relapse,[42] and reduced lipid to carbohydrate oxidation ratio represents the most important factor (more important than other behavioral and physiological factors) in regaining body weight following successful weight loss.[43] During menopause, females are prone to gain body weight; therefore, inducing lipid oxidation may be beneficial.

Evening/night chocolate induced carbohydrate oxidation as previously seen in studies where pre-sleep chocolate milk altered next-morning resting and exercise metabolism to favor carbohydrate oxidation.[44] Eating chocolate during the evening/night may increase endogenous carbohydrate reserves of glycogen in muscle and liver by the next morning, and carbohydrate utilization may be promoted because of the reduced capacity that the body has to store carbohydrates as compared to fat.[45] Therefore, chocolate intake at evening/night could be advisable for next morning performance during high intensity exercises or prolonged exercises and thus avoid hepatic and muscle glycogen depletion.

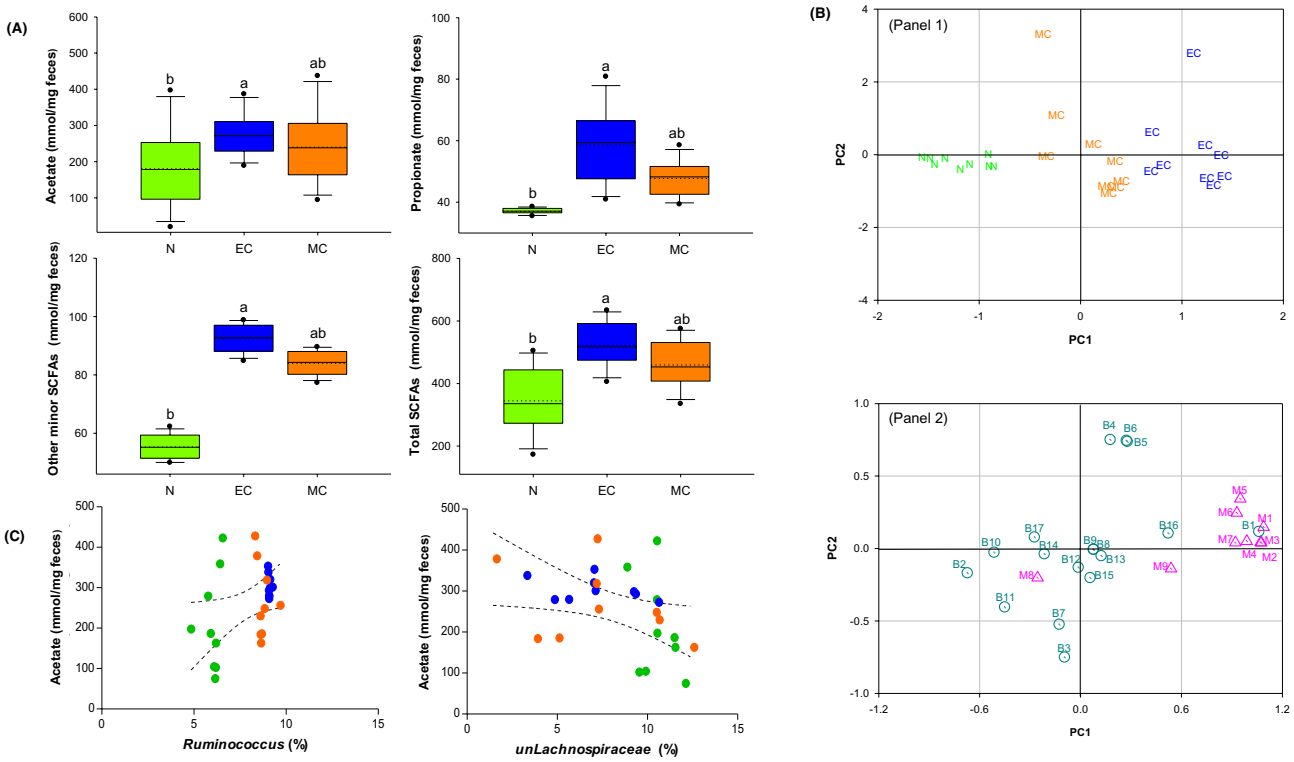

FIGURE 6 Significant differences in microbiota function between No chocolate, Evening/night, and Morning chocolate. Concentration of short-chain fatty acids (SCFAs) (Average values of the 12 participants in each condition). A, Significant differences ($P < .05$) between the different conditions, calculated with the pairwise comparison test, are indicated in the graphs with an asterisk (*).B, breakfast; D, dinner; EC, chocolate evening/night; L, lunch; MC, chocolate morning; N, no chocolate. Other minor SCFAs (isobutyrate, isovaleronate, and valeronate) and total SCFAs (acetate, propionate, isobutyrate, butyrate, isovaleronate, and valeronate). Significant differences between conditions are indicated in the graphs with the *P*-value of pairwise comparisons. Boxes indicate the upper and lower quartiles, the dotted line within the box indicates the mean and the thin line indicates the median; the vertical lines indicate the 90-10 percentiles and the dots are outliers (n = 12). B, Principal component analysis corresponding to the data of bacterial groups and short-chain fatty acids (SCFAs). (Panel 1) Distribution of the different conditions in the components: Control no chocolate in green, N; chocolate at evening/night in blue, EC; and chocolate at morning in orange, MC. (Panel 2) Variables in first (PC1) and second component (PC2) represented according to their factor loading. Valeronate, M1; Isovaleronate, M2; Other minor SCFAs (isobutyrate, isovaleronate, and valeronate), M3; Total SCFAs (acetate, propionate, isobutyrate, butyrate, isovaleronate, and valeronate), M4; Isobutyrate, M5; Propionate, M6; Butyrate, M7; Ratio acetate:propionate, M8; Acetate, M9; Methanobacteriaceae, B1; *Akkermansia*, B2; Firmicutes, B3; Adlercreutzia, B4; *Methanobrevibacter*, B5; Peptostreptococcaceae, B6; Invsimpson, B7; Streptococcaceae, B8; *Ruminococcus*, B9; Faecalibacterium, B10; *Dorea*, B11; Clostridiaceae, B12; *Streptococcus*, B13; Verrucomicrobiaceae, B14; UnLachnospiraceae, B15; UnClostridiaceae, B16; Eubacterium, B17. C. Associations between microbiota relative abundance at genus levels and specific SCFAs Pearson correlation with baseline-adjusted values of N (control, no chocolate in green), EC (chocolate at evening/night in blue) and MC (chocolate at morning in orange) between *Ruminococcus* and unclassified Lachnospiraceae and acetate levels in the fecal samples. The lines represent linear regression lines and the shaded areas show the 95% confidence interval

The relatively short duration of the intervention (28 days, including both morning and evening/night conditions) could be a factor to explain the absence of effects of chocolate on body weight. However, some studies have shown increases in body weight with chocolate interventions in studies with similar durations to ours[46-48] while others with longer interventions showed no effect,[49,50] or even opposite effects (ie, longer periods of chocolate interventions associated with body weight loss).[51-54] In a previous systematic review Kord-Verkaneh et al,[15] concluded that the duration of the intervention was not critical for the effects of chocolate on body weight, while if any, the positive effects on waist circumference were more evident with longer durations.

Heat-map visualization suggests that chocolate during the evening/night provides a time cue for more regular timing of sleep episodes than the non-chocolate condition which was demonstrated by the variability of sleep onset among days. Greater sleep irregularity has been previously correlated with greater obesity and fasting glucose, among other metabolic disturbances.[55] It would be expected that the consumption of this high amount of chocolate during the evening/night could cause sleeplessness due to the theobromine content. However, we did not find significant differences in the fragmentation of sleep, and we cannot deduce from our data that the sleep was disturbed. Moreover, having chocolate in the

morning was associated with an increased siesta frequency as compared to control. These changes may be contributing to the different metabolic effects of morning and evening/night chocolate intake, because it has been described that having siesta has a metabolic impact.[56,57]

Our results also show that chocolate in the morning decreases fasting glucose. Chocolate may improve glucose homeostasis by slowing carbohydrate digestion and absorption. Indeed, cocoa could reduce the rate and extent of macronutrient digestion by binding to and antagonizing digestive enzymes[35] which may help explain the previously reported inverse relation between chocolate intake and Type 2 Diabetes Mellitus (T2DM) incidence.[58,59]

In general, the "timing" of chocolate intake resulted in differential changes in microbiota profiles and function as shown in Figure 6A. These differences were mostly marked by metabolites (SCFAs) produced by the microbiota, indicating that SCFAs might be a good biomarker to explain, at least in part, the differences found among conditions. In addition to the effects on appetite, SCFAs have been associated with beneficial changes in intestinal permeability.[60-63]

Consistent with previous studies,[64,65] our study showed an impact of chocolate intake on microbiota composition and diversity and a significant increase in the interindividual variability as compared to control (no chocolate). Previously, dark chocolate intake had been associated with an increase of potential beneficial bacteria such as *Bifidobacterium* (Actinobacteria phylum) and inhibition of potential deleterious bacteria such as *Clostridium* group (Firmicutes phylum).[66,67] Similarly, our results with milk chocolate as compared with non-chocolate, showed increased abundances of Actinobacteria and reductions in the relative abundance of Firmicutes and more specifically in unclassified Lachnospiraceae genus. *Methanobrevibacter* genus abundance also increased with chocolate intake during the evening/night. A high intake of dietary fiber could increase the abundance of this bacteria[68] which tends to deplete in obesity.[69,70]

In addition, we observed that milk chocolate intake increased the abundance of *Akkermansia*, *Ruminococcus* and *Dorea* genus. A lower *Akkermansia muciniphila* abundance has been associated with obesity, and type 2 diabetes mellitus (T2DM),[71,72] while increased levels of *Ruminococcus* have been found after consumption of high carbohydrate diets with fiber.[73]

In terms of microbiota function, chocolate consumption during the evening/night significantly increased the production of SCFAs (such as acetate, propionate, isobutyrate, isovaleronate, and valeronate) as compared to no chocolate (control). It is known that modifications in the microbiota profile can change their metabolic activity producing and releasing different quantities of SCFAs in the intestinal lumen. This may be accounting for the significantly lower hunger and appetite reported by the females studied when eating chocolate during the evening/night.[60,74]

The study was limited to postmenopausal females. Therefore, further studies should be performed in men and younger females, to confirm these results. While our sample size and randomized, controlled, cross-over design ensured enough power to detect differences among chocolate conditions in the main outcomes, a larger sample size would have increased the power and would have allowed for more accurate estimation and differentiation between chocolate conditions. Furthermore, this study does not allow us to distinguish if the discovered effects are consequence of the rewarding effect of chocolate, specific chocolate components such as epicatechins, or the high energy and high sugar content of chocolate (nutritional composition). Further studies are needed to distinguish these aspects. It would be also interesting to conduct deep-phenotyping studies into the mechanisms underlying the effects of chocolate on glucose metabolism.

This randomized controlled trial study suggests that chocolate, in the morning or in the evening/night, in a narrow window of time (1 h), results in differential effects on hunger and appetite, substrate oxidation, fasting glucose, microbiota composition and function, and sleep and temperature rhythms. The intake of a rather high amount of chocolate (100 g) concentrated in a narrow (1 h) timing window in the morning could help to burn body fat and to decrease glucose levels in postmenopausal women.

5 | CLINICAL TRIAL REGISTRY NUMBER

Chocolate and Menopause Time (OMTIME-CHOC) study (ClinicalTrials.gov: #NCT03949803).

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CONFLICT OF INTEREST

The authors have no conflicts of interest relevant to this article to disclose. F.A.J.L.S. received speaker fees from Bayer Healthcare, Sentara Healthcare, Philips, Kellogg Company, Vanda Pharmaceuticals, and Pfizer.

AUTHOR CONTRIBUTIONS

T. Hernández-González conducted the clinical trial and collected the data, M. Garaulet and F.A.J.L. Scheer designed the study and conducted the clinical trial. R. González-Barrio, M.J. Periago, and M.C. Periago conducted the microbial composition and their metabolic activity analyses. J.A. Madrid and C. Escobar conducted the temperature analyses. All authors participated on writing the manuscript.

DATA AVAILABILITY STATEMENT

Raw sequencing data: NCBI-SRA BioProject PRJNA592909.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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