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An exploratory 12-week pilot study of gut microbiota and body-weight patterns during sodium nitrite exposure and *Lactobacillus plantarum* 299v supplementation in Wistar rats

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Abstract

Background Sodium nitrite (E250) is a widely used additive in processed meat products and has been reported to disrupt the gut microbiota and exert carcinogenic effects via N-nitroso compounds. This exploratory pilot study examined body-weight and longitudinal gut-microbiota patterns during sodium nitrite exposure and *Lactobacillus plantarum* 299v supplementation in adolescent Wistar rats.

Methods Sixty-four adolescent Wistar rats were allocated to Control, sodium nitrite (SN), *Lactobacillus plantarum* 299v (LP), or combined SN + LP groups for 12 weeks. Body weight was assessed in the full cohort. The exploratory microbiota component comprised 48 serial fecal samples from eight selected rats, with one male and one female from each treatment group. V3–V4 16S rRNA amplicons were sequenced on an Illumina iSeq 100 using a 2 × 150-bp paired-end configuration. Read 1 (R1) sequences were processed using the single-end DADA2 workflow, and microbiota profiles were analyzed descriptively.

Results Body-weight trajectories varied across groups and sexes in the full cohort. The descriptive relative-abundance profiles varied across sampling times. The sodium nitrite profile showed higher relative Proteobacteria abundance at several post-baseline time points, while longitudinal ordination trajectories overlapped substantially.

Conclusions The exploratory findings describe patterns across sampling times but do not establish group-level treatment effects or probiotic attenuation of nitrite-associated changes. Confirmation in adequately powered studies with independent cage-level replication is required.

Clinical trial number Not applicable.

Keywords Sodium nitrite, *Lactobacillus plantarum*, Gut microbiota, Pilot study, Wistar rats

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Background

Nutrition is a fundamental requirement for growth and sustaining life, and the nutrients consumed are critical for the continuity of metabolic processes and overall health [1]. However, modern lifestyles have led to significant changes in eating habits, with processed foods that are practical and easily accessible largely replacing traditional foods [2, 3]. This change has also led to increased exposure to food additives. Food additives are substances added to foods for technological purposes such as extending shelf life, improving taste, texture, or appearance [4].

One such additive, sodium nitrite ($NaNO_2$, E250), is widely used as a preservative, color stabilizer, and flavor enhancer, particularly in processed meat products such as sausages, ham, and bacon [5, 6]. Sodium nitrite contributes to food safety by inhibiting the growth of *Clostridium botulinum* [5]. It also gives cured meat its characteristic pink color and delays lipid oxidation, thereby extending shelf life [7]. However, the use of sodium nitrite is controversial due to potential health risks. Specifically, its potential to react with naturally occurring secondary amines in meat to form N-nitroso compounds (e.g., nitrosamines) is a concern [8, 9]. These compounds are known to possess carcinogenic, mutagenic, and teratogenic properties [9] and have been suggested to increase the risk of developing certain cancers [10]. Furthermore, it is known that sodium nitrite can cause methemoglobinemia at high doses, thereby inhibiting oxygen transport [11, 12]. For these reasons, safe intake levels for sodium nitrite have been established by organizations such as the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [13], and its use is restricted by legal regulations in many countries [14].

Recent studies have increasingly focused on the effects of dietary components and food additives on the gut microbiome. The gut microbiota is a complex ecosystem comprising diverse microorganisms and is vital to host digestion, metabolism, immune function, and overall homeostasis [15–17]. While the balance of the microbiota (eubiosis) is critical for maintaining health, disruption of this balance (dysbiosis) has been linked to various health issues such as inflammatory bowel diseases, obesity, diabetes, cardiovascular diseases, and even neurological disorders [18, 19]. Factors such as diet, antibiotic use, and exposure to environmental toxins can significantly affect the composition and function of the microbiota [20–22]. There is evidence that sodium nitrite may also cause adverse changes in the gut microbiota [23, 24]. A limited number of animal studies have shown that exposure to sodium nitrite affects microbial diversity and causes changes in the relative abundance of certain bacterial phyla (e.g., Firmicutes, Bacteroidetes, Verrucomicrobia, Proteobacteria) and genera (e.g., *Akkermansia*, *Turicibacter*, *Mucispirillum*, *Parasutterella*) [23–26].

The use of probiotics to mitigate or prevent the potential adverse effects of dysbiosis in the gut microbiota is becoming increasingly widespread. Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits on the host [27]. *Lactobacillus plantarum* is a lactic acid bacterium found in many fermented foods. It is well adapted to the human gastrointestinal tract and has Generally Recognized as Safe (GRAS) status [28, 29]. *L. plantarum* is known to have antimicrobial, antioxidant, antimutagenic, and immunomodulatory effects [30, 31]. Its positive effects on gut health are particularly noteworthy. *L. plantarum* supplementation has been shown to modulate the gut microbiota in animal models and some human studies, increase the number of beneficial bacteria (e.g., *Bifidobacterium*, *Lactobacillus*), inhibit the colonization of pathogens, strengthen intestinal barrier function, and reduce inflammation [32–34].

The *L. plantarum* 299v strain (DSM 9843) was selected because it is a human intestinal isolate with documented capacity to survive gastrointestinal transit and adhere to human intestinal epithelial cells. These strain-specific characteristics provided a biological rationale for evaluating its potential to modify longitudinal microbiota profiles during sodium nitrite exposure, without presupposing an established therapeutic effect against dysbiosis [35, 36].

The combined effects of sodium nitrite and *L. plantarum* 299v on longitudinal gut-microbiota profiles remain poorly characterized. To our knowledge, this combination has not been evaluated in an adolescent rat model. This exploratory pilot study therefore examined gut-microbiota and body-weight patterns during exposure to sodium nitrite at the acceptable daily intake and supplementation with *L. plantarum* 299v. We hypothesized that sodium nitrite exposure would alter microbiota composition and that supplementation might modify the observed longitudinal profiles.

Methods

Ethical approval

This study was approved by the Ankara University Local Ethics Committee for Animal Experiments (Approval Date and Number: 13.04.2022 / 2022-8-82; Extension Date and Number: 05.04.2023 / 2023-7-65). All animals were housed and maintained according to local and national guidelines. This study is reported in accordance with the ARRIVE guidelines 2.0 [37].

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Laboratory animals and housing conditions

In the study, a total of 64 Wistar Albino rats, consisting of 32 male and 32 female rats, aged six weeks (corresponding to the adolescent stage [38]), were obtained from the Ankara University Faculty of Medicine Laboratory Animals and Research Laboratory. Animal housing, intervention procedures, sample collection, and euthanasia were conducted at the same facility. The rats were housed in polycarbonate cages under controlled conditions (22 ± 2 °C temperature, 60 ± 5% relative humidity, 12-h light/12-h dark cycle) throughout the experiment (12 weeks). The duration of acclimatization before the intervention was not recorded. At study initiation, each treatment group was housed in two sex-specific cages, with eight males in one cage and eight females in the other. Thus, the study initially included eight cages in total. At Week 4, each cage was divided into two cages of four rats to maintain adequate space, resulting in four cages per treatment group and 16 cages overall. All rats were provided with standard pellet feed and drinking water *ad libitum* (free access). Drinking water was changed daily at the same time.

Experimental design and groups

The experiment was conducted over 12 weeks, from February to May 2023. Rats were allocated to four experimental groups. The method used to generate the allocation sequence was not recorded. Each group comprised 16 animals (8 males and 8 females). Because the interventions were administered through shared drinking water, the cage was the experimental unit for treatment exposure, whereas the individual rat was the unit of observation and analysis. As this was an exploratory pilot study, no formal a priori sample-size calculation was performed, and the sample size was not based on a prespecified primary outcome. The four groups were as follows:

Group 1 (control)

Rats were fed standard pellet feed and given normal drinking water.

Group 2 (sodium nitrite, SN)

Rats were fed standard pellet feed and given drinking water containing sodium nitrite ($NaNO_2$, E250). The sodium nitrite dose was targeted to correspond to the acceptable daily intake (ADI) level determined by JECFA (0.07 mg/kg body weight/day) [13]. Based on weekly body weight and daily water consumption measurements, the average sodium nitrite intake at the end of 12 weeks was calculated to be 0.071 ± 0.0084 mg/kg/day in male rats and 0.072 ± 0.0106 mg/kg/day in female rats.

Group 3 (L. plantarum, LP)

Rats were fed standard pellet feed and given drinking water supplemented with *L. plantarum* 299v (DSM 9843), obtained from a commercial probiotic preparation (Probest Digestive, Abdi Ibrahim Pharmaceutical Industry and Trade Inc.). The target bacterial dose was at least 1.0×10^8 colony-forming units (CFU)/mL.

Group 4 (sodium nitrite + L. plantarum, SN + LP)

Rats were fed standard pellet feed and given drinking water supplemented with both the sodium nitrite dose used in Group 2 and the *L. plantarum* 299v dose used in Group 3. In this group, the average sodium nitrite intake at the end of 12 weeks was calculated to be 0.069 ± 0.0083 mg/kg/day in male rats and 0.075 ± 0.0111 mg/kg/day in female rats. The target *L. plantarum* dose was at least 1.0×10^8 CFU/mL.

At the end of the 12-week experimental period, all rats were euthanized under deep anesthesia induced with ketamine (90 mg/kg) and xylazine (10 mg/kg), administered intraperitoneally. A surgical plane of anesthesia was confirmed by loss of pedal withdrawal reflex and absence of response to noxious stimuli before euthanasia was completed by exsanguination via cardiac puncture. This procedure was performed in accordance with institutional animal care guidelines and international recommendations for the euthanasia of laboratory rodents.

L. plantarum 299v was administered in drinking water. Because the water was replaced every 24 h, bacterial viability and concentration were assessed over the same period to verify maintenance of the target dose. The procedures are described below.

Preparation and viability count of *L. plantarum*

The *L. plantarum* 299v (DSM 9843) strain was propagated on de Man, Rogosa and Sharpe (MRS) agar. Dehydrated MRS was dissolved in distilled water to a concentration of 68.2 g/L, autoclaved at 121 °C for 15 min, cooled to 45–50 °C, and then poured into sterile Petri dishes (~ 12.5 mL each). The pH of the prepared medium at 25 °C was recorded as 5.7 ± 0.2.

Colonies grown on MRS were collected with a sterile loop and used to create a master stock in Ringer's solution (NaCl 2.25 g/L; KCl 0.105 g/L; anhydrous $CaCl_2$ 0.06 g/L; $NaHCO_3$ 0.05 g/L). Nine hundred microliters of Ringer's solution were placed in each of seven 2 mL microtubes, and sequential 1:10 dilutions were performed (100 µL culture + 900 µL Ringer's solution, with brief vortexing at each step).

Fifty microliters from each dilution were spread evenly onto two separate MRS agar plates, which were incubated at 37 °C for 24 h. Samples in Ringer's solution were stored at – 80 °C until extraction. Colony counts were

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performed after 24 h, and CFU/mL values were calculated using the formula [39].

$$N = \frac{C}{V \times (n_1 + 0.1 \times n_2) \times d}$$

N: Number of microorganisms in 1 g or 1 mL.
C: Total number of microorganisms in all Petri dishes counted.
V: Volume (mL) transferred to the Petri dishes counted.
n_1: Number of Petri dishes used in the first dilution.
n_2: Number of Petri dishes used in the second dilution.
d: The dilution ratio of the more concentrated of the two consecutive dilutions counted.

For *L. plantarum* 299v added to drinking water, the water was renewed every 24 h to maintain a target minimum dose of $\geq 1.0 \times 10^8$ CFU/mL. In this verification experiment, viable counts confirmed that *L. plantarum* was present at 2.09×10^8 CFU/mL in the drinking water after 24 h. This measurement was performed to confirm that the target dose was maintained across a 24-h renewal interval and was not repeated for each cage or each week.

Sample collection and measurements

Body weight and water consumption

The body weights of the rats were measured and recorded weekly after fecal sample collection. Water consumption was monitored by measuring the amount of water remaining in each cage daily.

Fecal samples

Fecal samples were collected weekly throughout the 12-week study. For microbiota profiling, samples collected at baseline (T0) and at Weeks 1 (T1), 2 (T2), 4 (T4), 8 (T8), and 12 (T12) were analyzed from eight selected rats, comprising one male and one female from each treatment group. This yielded 48 analyzed longitudinal samples. The selection method for the microbiota subset was not recorded. The selected male and female initially represented the respective sex-specific cages within each treatment group. Following the cage division at Week 4, longitudinal sampling continued from the same animals. Consequently, only one of the two post-division cages for each sex-by-treatment combination was represented in the microbiota dataset. Each treatment group therefore contributed two biological subjects, and each sex-by-treatment profile was represented by one rat. Repeated samples were not treated as independent biological replicates. For body-weight and descriptive microbiota analyses, the individual rat was the unit of measurement and analysis. Serial fecal samples were treated as repeated observations within rat. The cage was the experimental unit for treatment exposure and the unit of water-consumption measurement.

DNA extraction

DNA was isolated from the selected fecal samples using a modified phenol–chloroform extraction method [40]. Cell walls were disrupted using lysozyme and proteinase K. Proteins were removed with phenol–chloroform–isoamyl alcohol, and DNA was precipitated using isopropanol and sodium chloride before ethanol washing. DNA concentration was measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA). Samples were adjusted to 5 ng/µL and stored at – 20 °C until sequencing.

16S rRNA gene sequencing

The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide [41]. Briefly, DNA samples adjusted to 5 ng/µL were amplified using KAPA HiFi HotStart ReadyMix and primers specific to the V3–V4 region. The forward primer was 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′, and the reverse primer was 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. Polymerase chain reaction (PCR) products were purified using AMPure XP (Agencourt) magnetic beads. Subsequently, sample-specific barcodes (indexes) were added using the Nextera XT Index Kit (Illumina) (Index PCR) and purified again using AMPure XP beads. The expected V3–V4 insert was approximately 460 bp. Amplicon PCR products were approximately 550 bp after the addition of primer overhangs, and the final indexed libraries were approximately 630 bp, as assessed by 1.5% agarose gel electrophoresis. Libraries were pooled in equal proportions and supplemented with approximately 10% PhiX because of the low sequence diversity of 16S amplicons. Sequencing was performed on an Illumina iSeq 100 using a 2 × 150-bp paired-end configuration [42]. Because the V3–V4 insert exceeded the combined 300-bp read length, read 1 (R1) and read 2 (R2) lacked sufficient overlap for reliable merging. Downstream processing was therefore performed using R1 reads only. Samples collected at T0, T1, T2, and T4 were sequenced in one run, whereas samples collected at T8 and T12 were sequenced in a second run on the same instrument.

Bioinformatics analysis

Raw sequencing data were processed using Quantitative Insights Into Microbial Ecology 2 (QIIME 2, v2021.11) [43]. R1 reads were processed using the single-end DADA2 workflow [44]. Quality filtering and denoising were followed by chimera removal and amplicon

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sequence variant (ASV) inference. Taxonomic assignment of ASVs was performed using the Greengenes reference database [45]. No rarefaction was applied to the taxonomic composition or ordination analyses. Within each sample, taxon counts were normalized to relative abundances. Genus-level relative-abundance profiles were Hellinger-transformed for principal component analysis (PCA). Bray–Curtis dissimilarities for principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) were calculated from the relative-abundance profiles. For hierarchical clustering, group-by-week mean phylum-level relative-abundance profiles were clustered using the unweighted pair-group method with arithmetic mean (UPGMA) based on Bray–Curtis dissimilarities. Ordinations were calculated separately within each sex. Consecutive observations from each rat were connected in chronological order. The ordination analyses were descriptive, and no group-level inferential tests were performed.

Statistical analyses

Body-weight analyses included all 64 rats. Body-weight trajectories were analyzed using a linear mixed-effects model with group, week, sex, and their interactions as fixed effects and animal identity as a random intercept (weight ~ group × week × sex + (1 | animal_id)). Week was treated as a categorical factor. Model assumptions were assessed using residual diagnostics. Post hoc p values were adjusted using the Benjamini–Hochberg (BH) procedure. Confidence intervals for contrasts against Control were adjusted using the Bonferroni procedure. These intervals do not correspond directly to the BH-adjusted p values. The animal-level random intercept accounted for repeated measurements within rats but not dependence between cage-mates. Each rat was an analytical unit rather than an independent treatment replicate. At study initiation, cage identity was confounded with the sex-by-treatment combination. Body-weight contrasts, p values, and confidence intervals were therefore interpreted as exploratory and did not provide confirmatory evidence of treatment effects. The BH adjustment did not address cage-level dependence. Because microbiota profiling included only two rats per treatment group and one rat per sex-by-treatment profile, taxonomic composition and ordination trajectories were analyzed descriptively. No group-level inferential tests were performed for microbiota outcomes. Cage-level variation was not modeled in the microbiota analysis because the subset contained only one animal per sex-by-treatment profile and did not provide independent cage-level replication. No a priori inclusion or exclusion criteria were specified for animals, samples, or outcome data points. No animals or samples were excluded from the reported analyses. Investigators were aware of group allocation during intervention administration, outcome assessment, and data analysis. No blinding was used. Analyses were conducted in R version 4.5.1 [46] using the lme4 and emmeans packages. A two-sided threshold of $p < 0.05$ was used for exploratory body-weight contrasts.

Results

Body weight increased progressively in all groups over the 12-week period (Fig. 1). At baseline, body weights differed across groups within each sex. Patterns differed by sex. In males, the treated groups, particularly LP, generally had higher values than Control from baseline onward. In females, Control generally had the highest values, while the treated groups had lower values from baseline onward. Several within-sex pairwise contrasts had BH-adjusted p values below 0.05 at individual time points, most frequently at Weeks 4, 8, and 12. Contrasts against Control are shown in Fig. 1b. Differences among the treated groups were smaller, and their p values did not consistently fall below this threshold after multiplicity correction.

The 48 archived R1 FASTQ files contained 502,872 reads, corresponding to a mean input depth of 10,477 ± 6,380 reads per sample. The final ASV-derived taxonomic profiles contained 233,968 sequence counts, corresponding to a mean post-processing depth of 4,874 ± 3,631 counts per sample and an overall retention of 46.5%. The mean number of detected ASVs was 57 ± 38 per sample. At the phylum level (Fig. 2a), relative Proteobacteria abundance was generally higher at later sampling points across all groups, although the timing of the observed shifts varied. In the SN group, Proteobacteria rose rapidly from baseline and remained high thereafter, with prominent elevations at Weeks 2 and 12. A similar early surge was also evident in Controls by Week 2, followed by fluctuations between Proteobacteria and Firmicutes. In the LP group, the community resembled Control at early time points but shifted sharply toward Proteobacteria at Weeks 8–12. The SN + LP group displayed a pronounced increase in Proteobacteria at Week 8, and high relative abundance remained evident at Week 12. Reciprocal decreases in Firmicutes and Bacteroidetes accompanied these shifts.

At the genus level (Fig. 2b), *Lactobacillus* increased markedly and dominated the LP group profile around Week 8. Composition at Week 12 was more variable. Early relative increases in *CF231* and *Bifidobacterium* were also observed. The SN profiles were heterogeneous over time, with no single genus consistently dominant. In the SN + LP group, *Lactobacillus* increased to moderate-to-high relative abundance at Weeks 8–12 but remained lower than in the LP group at Week 8. *Peptostreptococcus* remained low at most sampling points.

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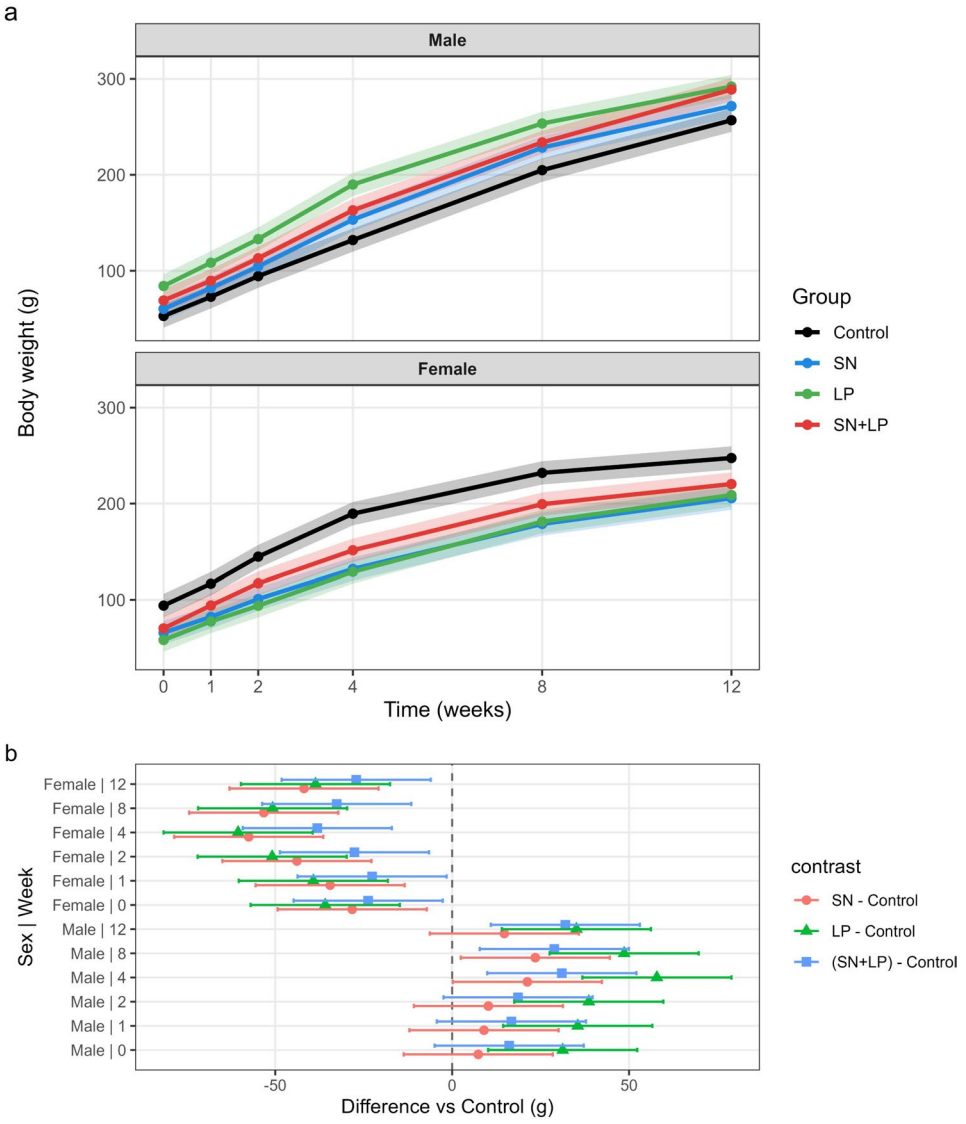


Fig. 1 Longitudinal analysis of body weight by sex and treatment group over 12 weeks. **a** Model-based estimated marginal means with 95% confidence intervals (CIs) from a linear mixed-effects model, weight ~ group × week × sex + (1 | animal_id), with week treated as a categorical factor. Panels are stratified by sex. **b** Pairwise contrasts vs. Control (estimate ± Bonferroni-adjusted 95% CI) shown for each week and sex. The dashed vertical line denotes no difference. Positive values indicate higher weight relative to Control, whereas negative values indicate lower weight. Treatments were administered through shared drinking water at cage level. The model did not account for dependence between cage-mates. The CIs should therefore be interpreted as exploratory

Heatmap clustering based on Bray–Curtis dissimilarity showed a predominantly temporal pattern (Fig. 3). Most Week 8–12 profiles from Control, LP, and SN + LP were located in one branch, whereas many Week 0–2 profiles were located in another. Week 4 profiles occurred in both branches. The SN profiles formed a subcluster that included Weeks 8 and 12 and showed higher relative Proteobacteria and lower relative Firmicutes. These observations are descriptive and reflect relative-abundance data.

PCA, PCoA, and NMDS ordinations were generated separately for males and females (Fig. 4). Each treatment-profile trajectory connected six serial observations from one rat. The trajectories overlapped at multiple sampling points across all three ordination methods. NMDS stress values were 0.138 for males and 0.126 for females.

Discussion

Sodium nitrite and *L. plantarum* are used in the food industry for different purposes. Sodium nitrite is used as a preservative and color stabilizer in meat products, whereas *L. plantarum* is used for its probiotic properties. However, the long-term effects of these two agents on the gut microbiota and their potential combined effects remain incompletely understood. This exploratory study

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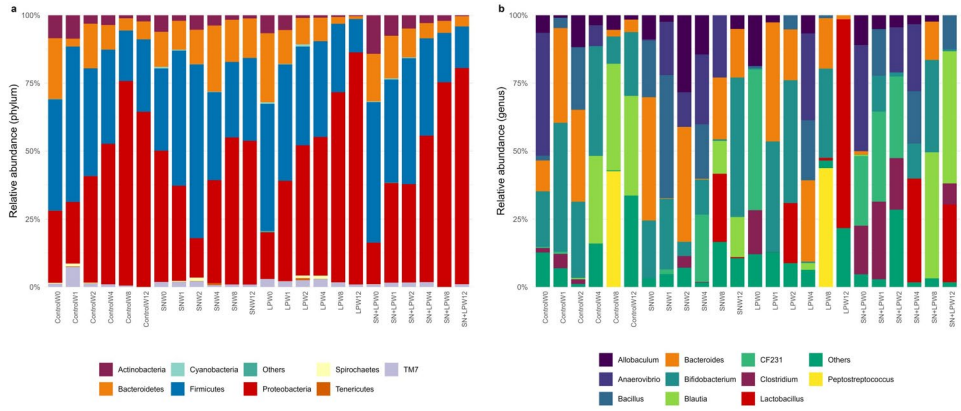


Fig. 2 Gut microbiota composition dynamics at phylum and genus levels across treatment and time. Stacked bar plots show mean relative abundance (%) by treatment group and week. Each bar represents the mean of the two sequenced rats, one male and one female, within the corresponding treatment group at that time point. **a** Phylum-level composition across the 12-week intervention. **b** Genus-level composition across the same time points. Minor taxa are grouped as "Others." These descriptive profiles should be interpreted cautiously because each treatment group included only two sequenced rats

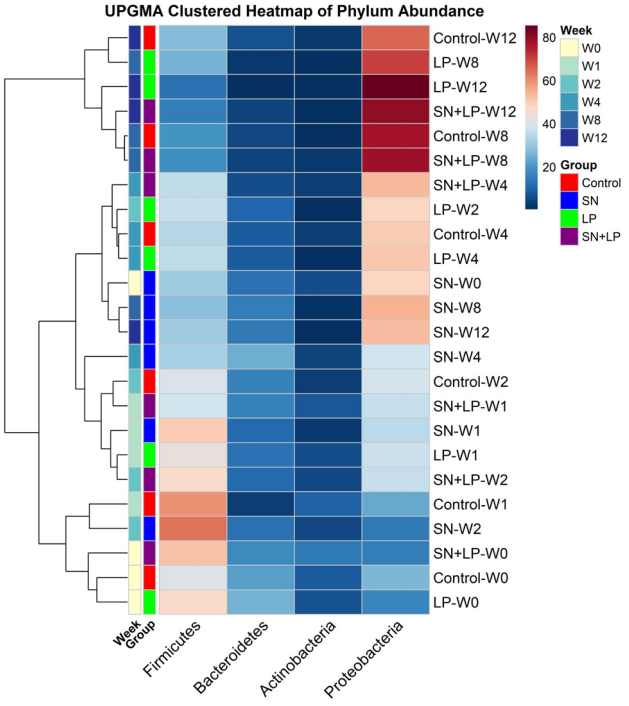


Fig. 3 Hierarchical clustering of gut microbiota at phylum level across groups and intervention weeks. UPGMA hierarchical clustering heatmap of group-by-week mean phylum-level profiles. Each profile represents the mean relative abundance of the two sequenced rats, one male and one female, within the corresponding treatment group at that time point. Bray–Curtis dissimilarities and UPGMA clustering were calculated from the complete phylum-level relative-abundance profiles. The heatmap displays the four most abundant phyla for readability. Profiles were organized predominantly by week. Most Week 8–12 profiles from Control, LP, and SN + LP occurred in one branch, whereas many Week 0–2 profiles occurred in another. Week 4 profiles occurred in both branches. The late-stage SN profiles formed a subcluster with higher relative Proteobacteria and lower relative Firmicutes. Interpretations are descriptive and reflect relative abundances

evaluated body-weight trajectories in the full cohort and longitudinal microbiota profiles in an eight-rat subset during sodium nitrite exposure at the ADI level and *L. plantarum* 299v supplementation over 12 weeks. Both components describe patterns across sampling times and do not establish confirmatory treatment effects.

Expected growth-related body-weight gains were observed in all groups over 12 weeks (Fig. 1). In males, the LP group generally had higher values than Control from baseline onward, and the SN and SN + LP groups also exceeded Control at several mid-to-late time points. In females, Control generally had the highest values, whereas the three intervention groups were lower from baseline onward. Several within-sex pairwise comparisons had BH-adjusted p values below 0.05 at individual time points. These contrasting trajectories suggest differences in the observed body-weight patterns between sexes, although the study did not assess the mechanisms underlying these differences.

This pattern regarding body weight is partly consistent with and partly divergent from findings reported in the literature following sodium nitrite exposure. For example, Fareed et al. reported significant weight loss in male albino rats after 8 weeks of nitrite treatment compared with controls ($p < 0.05$) [47]. Varzandi et al. reported that the administration of 50 mg/L nitrite for 3 months in obese rats with type 2 diabetes reduced weight gain, though the difference was not statistically significant ($p > 0.05$) [48]. Zhang et al. demonstrated that dose-dependent nitrite administration significantly modulated body weight in both male and female mice by the second week ($p < 0.05$) [49]. Similarly, Salama et al. reported a significant weight decrease in rats receiving 80 mg/kg/day nitrite for 12 weeks ($p < 0.05$) [50], and Helal et al. showed that the percentage of weight gain was

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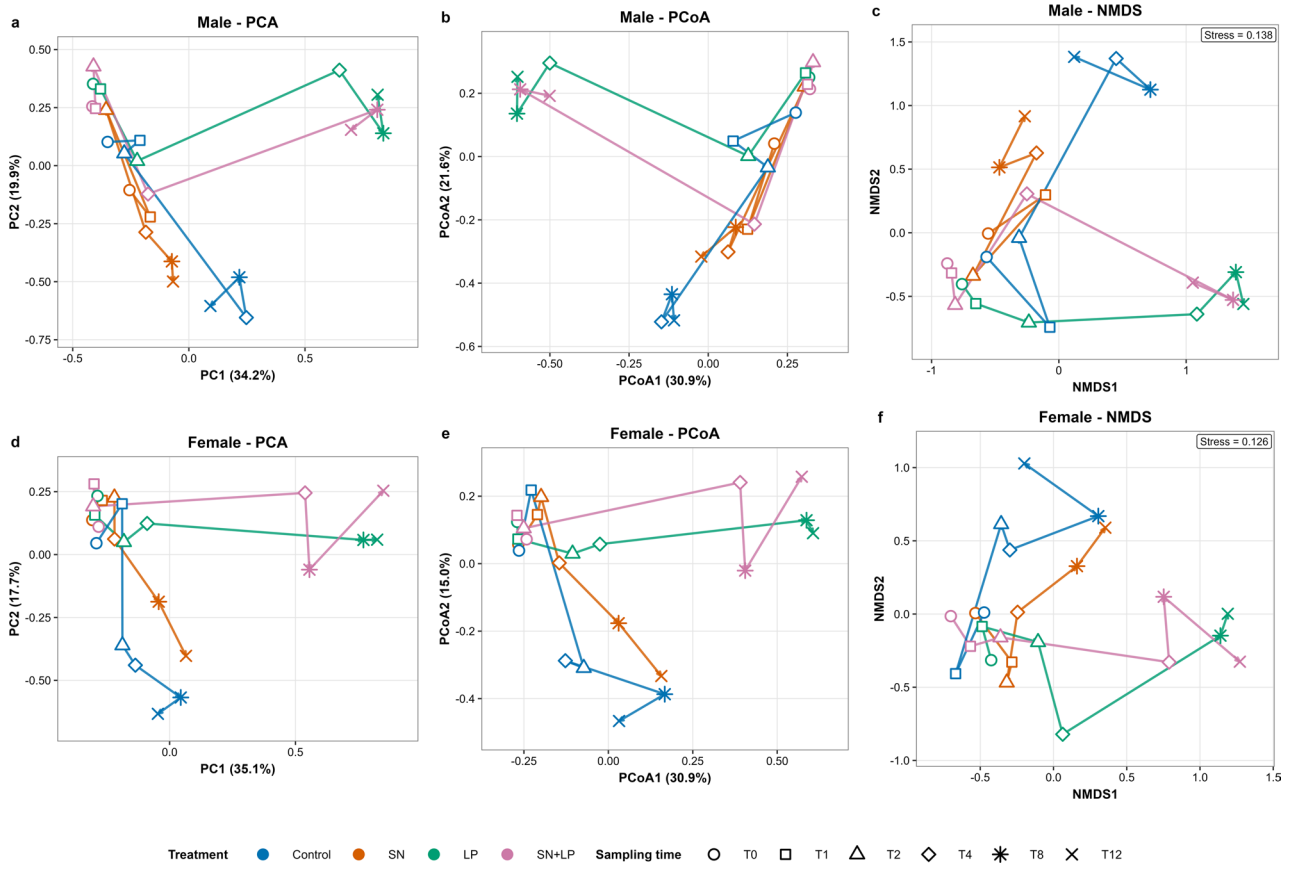


Fig. 4 Descriptive longitudinal ordinations of genus-level gut microbiota profiles by sex. Ordinations were calculated separately for males and females. The panels show the following ordinations: **a** PCA in males, **b** PCoA in males, **c** NMDS in males, **d** PCA in females, **e** PCoA in females, and **f** NMDS in females. PCA was calculated from Hellinger-transformed relative abundances, while PCoA and NMDS were based on Bray–Curtis dissimilarity. Points represent six serial samples from one rat per sex-by-treatment profile. Lines connect consecutive sampling times in chronological order. Colors denote treatment groups, and point shapes denote sampling times. NMDS stress values are shown within the corresponding panels. No confidence ellipses or group-level inferential tests were applied

significantly lower at 3 and 6 months ($p < 0.05$) [51]. In contrast, Ghanbari et al. detected higher birth weights in some offspring groups following parental and prenatal nitrite exposure ($p < 0.05$) [52]. The higher body weights observed in males in the LP group and the lower values observed in females in the treatment arms must be interpreted in light of baseline weight differences and the lack of independent cage-level replication.

Published evidence on the effects of *L. plantarum* on weight regulation is heterogeneous. In the cafeteria diet model, *L. plantarum* IMC 510 was shown to reduce final body weight by 12% ($p < 0.05$) [53]. In rats fed a high-fat diet, *L. plantarum* S9 administration reduced body-weight gain by 23.35% ($p < 0.05$) [54]. In mice, the ZJUFT17 strain was associated with a significant decrease in weight gain over 6 weeks ($p < 0.05$) [55]. The ATCC 14917 strain also suppressed weight gain in a dose-dependent manner ($p < 0.05$) [56]. However, the male LP group was already heavier than Control at baseline, so its higher absolute body weights cannot be interpreted as evidence of increased weight gain attributable to LP.

At the phylum level, the SN group showed high relative Proteobacteria abundance at several post-baseline time points. Its Week 8 and Week 12 profiles occurred within the same subcluster (Figs. 2a and 3). In the LP group, the Week 12 profile was dominated by Proteobacteria. In the SN + LP group, the increase in relative Proteobacteria was particularly pronounced at Week 8 (Fig. 2a). At the genus level, the LP group showed a marked relative increase in *Lactobacillus* around Week 8, together with early relative increases in *CF231* and *Bifidobacterium*. The SN group showed more heterogeneous late-period profiles. The SN + LP group showed a moderate relative increase in *Lactobacillus* at Weeks 8–12 (Fig. 2b). Hierarchical clustering showed a predominantly temporal organization. Most Week 8–12 profiles from Control, LP, and SN + LP occurred in one branch, whereas many Week 0–2 profiles occurred in another. This early-to-late separation coincided with the boundary between the

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two sequencing runs and therefore cannot be attributed exclusively to temporal change. Week 4 profiles occurred in both branches. The late-stage SN profiles formed a subcluster with higher relative Proteobacteria and lower relative Firmicutes (Fig. 3).

The phylum Proteobacteria includes numerous opportunistic taxa, and increased relative abundance of this phylum has been discussed as a potential marker of community disturbance [57, 58]. However, phylum-level relative abundance cannot establish expansion of specific pathogens or infer metabolic or inflammatory consequences. Likewise, the detection of taxa associated in other studies with butyrate production, fiber metabolism, or microbial cross-feeding does not demonstrate that these functions occurred in the present animals [59–61].

Across PCA, PCoA, and NMDS, the treatment-profile trajectories overlapped at multiple sampling points in both sexes (Fig. 4). This overlap did not reveal a consistent pattern of treatment separation. However, each sex-by-treatment profile was represented by one rat, so the ordinations cannot support group-level inference.

Nagpal et al. showed that nitrite increased the relative abundance of the phylum Verrucomicrobia and the genera *Turicibacter* and *Akkermansia*. They also reported reduced alpha diversity, while beta diversity indicated treatment-dependent compositional differences [24]. Xu et al. reported that low (0.15 g/L) and high (0.3 g/L) doses affected different genera (e.g., *Bifidobacterium*, *Clostridium XIVa*, *Parasutterella*, *Falsiporphyromonas*, *Alloprevotella*, *Acetatifactor*, *Coprococcus*), with *Akkermansia* decreasing at high doses [23]. Guéraud et al. showed that reducing nitrite levels in processed meats decreased *Candidatus Soleaferrea* and *Romboutsia* while increasing *Eisenbergiella*. Complete nitrite removal caused significant changes in taxa such as Peptococcales and Peptostreptococcales–Tissierellales [62]. In *APC*^{min} mice, consumption of nitrite-containing frankfurters reduced microbial diversity and was associated with a decrease in *Bacteroides* and an increase in Firmicutes [26]. It has also been reported that additive mixtures (E250 + E211+E202) deepen dysbiosis in the context of *Nod2* deficiency, with a decrease in Firmicutes/Clostridiales and an increase in Proteobacteria [25]. Collectively, these studies indicate that the direction and magnitude of nitrite-associated microbiota changes may depend on dose, duration, dietary matrix, host genetics, and baseline microbiota.

In the LP profile, the relative abundance of *Bifidobacterium* was higher at early time points, while a marked relative increase in *Lactobacillus* was observed before the Proteobacteria-rich profile at Week 12 (Fig. 2a–b). Some models have reported increased Proteobacteria following *L. plantarum* administration. In BALB/c mice, Proteobacteria increased and Bacteroidetes decreased with *L. plantarum* NWAFU-BIO-BS29 ($p < 0.05$) [63]. In C57BL/6J mice, the *L. plantarum* FBT215 strain similarly increased Proteobacteria ($p < 0.05$) [64]. The *L. plantarum* ZDY2013 strain was also associated with increased Proteobacteria and decreased Bacteroidetes over three weeks [65]. Conversely, other studies have reported different compositional responses to *L. plantarum*. In the dextran sulfate sodium-induced colitis model, *L. plantarum* increased the Firmicutes/Bacteroidetes ratio and enriched bifidobacteria, thereby reducing pathogens [66]. The *L. plantarum* 1201 strain regulated the Firmicutes/Bacteroidetes ratio and increased *Bifidobacterium* and *Lactobacillus* [32]. The *L. plantarum* NA136 strain suppressed pathogen growth and promoted *Allobaculum*, *Lactobacillus*, and particularly *Bifidobacterium* [67]. The *L. plantarum* FRT4 strain increased microbial diversity and the abundance of *Bacteroides*, *Parabacteroides*, *Anaerotruncus*, *Alistipes*, *Intestinimonas*, *Butyricicoccus*, and *Butyricimonas* [68]. The *L. plantarum* Lp01 strain increased beneficial bacteria and reduced Proteobacteria [69]. Strain, administration route, dose, intervention duration, dietary context, and host physiology may contribute to the heterogeneous compositional patterns reported across studies. The present study did not assess mechanisms that could explain these differences.

The combined treatment arm showed a phylum-level profile that was closer to SN while partially overlapping with LP. Firmicutes predominated initially, followed by a shift toward Proteobacteria at later time points (Fig. 2a–b). These profiles describe temporal compositional variation but do not establish probiotic attenuation or functional recovery.

Genus-level analysis showed that *Lactobacillus* increased markedly in the LP group around Week 8, whereas no single genus was consistently dominant in the SN group. The SN + LP group showed a mixed profile with a moderate relative increase in *Lactobacillus* at Weeks 8–12 (Fig. 2b). These genus-level patterns reflect relative taxonomic composition only. They do not demonstrate community rebalancing or functional change.

Nitrosative stress, local pH, microbial cross-feeding, and mucosal immune responses may influence microbiota composition [23, 24, 57, 60, 61, 70]. These mechanisms were not measured in the present study and remain speculative.

Body-weight data showed that the LP group generally had higher values than Control in males from baseline onward. The SN and SN + LP groups also exceeded Control at several mid-to-late time points. In females, Control generally had the highest values, whereas the intervention groups were lower at multiple time points (Fig. 1). These sex-specific body-weight patterns were not directly linked to microbiota composition in the present analyses. No host–microbiota correlation, metabolomic,

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or inflammatory-marker analyses were performed. Accordingly, the microbiota profiles should be considered a descriptive component of the overall biological response. They should not be interpreted as a mechanistic explanation of the separately analyzed body-weight trajectories.

Limitations

The full cohort comprised 64 rats, whereas the microbiota component included eight selected rats, with one male and one female from each treatment group. The six serial samples from each rat did not constitute independent biological replicates, and each sex-by-treatment profile was represented by one animal. The findings therefore describe individual longitudinal profiles and cannot support confirmatory group-level inference. This limited biological replication also restricts generalizability and increases sensitivity to individual variation. Cage effects could not be separated from treatment and sex effects in the microbiota component. At study initiation, each sex-by-treatment combination was represented by a single cage, and only one of the two corresponding post-division cages was represented after Week 4. Cage-level variation therefore remains a potential source of confounding.

The body-weight model did not account for dependence between cage-mates, which may have underestimated standard errors and produced overly narrow confidence intervals. The division of cages at Week 4 did not create independent replicates of the initial treatment allocation. Baseline body weights also differed across groups, so subsequent differences in absolute weight cannot be attributed solely to treatment. Body-weight comparisons should therefore be considered exploratory observations within this cohort.

Furthermore, the administration of nitrite via drinking water created uncertainty regarding dose accuracy because the actual intake of individual animals could only be derived from cage-level consumption. Therefore, equal and targeted intake by each animal could not be confirmed. Administration of *L. plantarum* in drinking water rather than by gavage may also have introduced variation related to the water matrix and bacterial viability. Although a 24-h verification experiment confirmed a concentration of 2.09×10^8 CFU/mL under the tested conditions, probiotic concentration and viability were not monitored repeatedly in the drinking bottles of individual cages during the 12-week intervention. Day-to-day and cage-to-cage variation in the delivered dose therefore cannot be excluded, and its contribution to the observed taxonomic profiles cannot be determined.

Because R1 alone was used, the sequence available for ASV inference and taxonomic classification was shorter than a merged V3–V4 sequence. This choice may have reduced discrimination among closely related taxa and influenced ASV-level richness and dissimilarity estimates [71]. Accordingly, the taxonomic findings were interpreted at the phylum and genus levels, and no species-level conclusions were drawn. Platform- and read-length differences should also be considered when comparing these profiles with other 16S rRNA datasets [72]. Variation in post-processing sequencing depth among samples provides an additional reason for interpreting the longitudinal microbiota profiles descriptively. Samples collected at T0–T4 and T8–T12 were sequenced in separate runs. Consequently, run-associated variation cannot be distinguished from temporal change between these sampling periods.

Only relative taxonomic abundances were measured. Absolute bacterial loads, metabolite concentrations, short-chain fatty acids, microbial gene functions, intestinal barrier markers, and inflammatory outcomes were not assessed. Consequently, the data cannot establish changes in microbial activity, metabolic output, pathogenic burden, or host physiological mechanisms.

Conclusions

This exploratory 12-week pilot study characterized body-weight trajectories in the full cohort and longitudinal gut-microbiota profiles in a subset of eight rats. Body-weight trajectories varied across groups and sexes. The microbiota subset showed heterogeneous variation across sampling times in the relative abundance of dominant taxa and substantial overlap among longitudinal ordination trajectories.

These descriptive pilot profiles do not establish that *L. plantarum* 299v attenuated or reversed sodium nitrite-associated changes. Given the microbiota subsample of two rats per group, reliance on relative abundances, potential cage confounding, dose variability inherent in drinking-water delivery, and sequencing-depth constraints, the findings are hypothesis-generating. Confirmation requires larger studies with independent cage-level replication, absolute microbial quantification, and functional measurements such as short-chain fatty acids and inflammatory markers.

Abbreviations

ADI	Acceptable daily intake
ASV	Amplicon sequence variant
BH	Benjamini–Hochberg
CFU	Colony-forming unit
CI	Confidence interval
GRAS	Generally Recognized as Safe
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LP	Lactobacillus plantarum 299v
MRS	de Man, Rogosa and Sharpe
NMDS	Non-metric multidimensional scaling
PCA	Principal component analysis
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
QIIME	Quantitative Insights Into Microbial Ecology

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SN Sodium nitrite
UPGMA Unweighted pair-group method with arithmetic mean

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Author contributions
ED, AK, MK, ADD and VCÖ conceived and designed the study. ED performed the formal analysis and investigation with support from ÖÇ and TNB. ED drafted the manuscript. ED and AK critically revised the manuscript. AK supervised the study. All authors read and approved the final manuscript.

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Data availability
The datasets generated and/or analyzed during the current study are available at the following persistent link: https://doi.org/10.5281/zenodo.17793971.

Declarations

Ethics approval and consent to participate
This study was approved by the Ankara University Local Ethics Committee for Animal Experiments (Approval Date and Number: 13.04.2022 / 2022-8-82; Extension Date and Number: 05.04.2023 / 2023-7-65). All animals were housed and maintained according to local and national guidelines. No additional consent was required beyond institutional animal ethics approval.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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