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RESEARCH ARTICLE

Targeting Gut Microbiota Health in Aged Rats Through the Potent Strategy of Probiotics Supplementation During Intermittent Fasting

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ABSTRACT

Gut health in aging populations, including animal models, is a critical area of research due to the decline in microbial diversity with age. Maintaining gut health has important implications for overall health and longevity. This study aimed to evaluate the interdependent effects of SCD Probiotics and intermittent fasting (IF) on gut microbiota (GM) in 24-month-old male Sprague-Dawley rats, a well-established model for aging research. The experiment involved four groups: a control, IF-only, probiotics-only, and a combination of IF and probiotics. The metagenomic analysis of cecum contents for IF and SCD Probiotics groups has shown increased Shannon and Simpson diversity of alpha index values and improved ratios for Firmicutes to Bacteroidetes. High-Performance Liquid Chromatography (HPLC) analysis of short-chain fatty acids (SCFAs) revealed significant changes in the probiotics-only and combined IF with SCD Probiotics groups, particularly with acetic and propionic acids. The results indicate that combining SCD Probiotics with IF produces interdependent benefits, improving bacterial diversity and SCFAs profiles. These findings suggest that SCD Probiotics with intermittent fasting could be a promising strategy to enhance gut health in aging populations, with potential applications in veterinary health.

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INTRODUCTION

Recent research has emphasized the critical role of gut microbial diversity in influencing the aging process, with studies uncovering notable connections between microbial composition and aging in humans (Bana and Cabreiro, 2019).In animal health research, significant health implications on age-related microbial shifts in companion animals and livestock have been reported (Mondo *et al.*, 2020). A study on dairy calves found that aging and illness, such as diarrhea, significantly influence gut microbiome composition, in which younger calves show dynamic microbial shifts critical for early-life health (Kim *et al.*, 2021). In dogs, age was identified as one of the key factors shaping the gut microbial environment, in which older dogs exhibiting microbial profiles potentially impact health outcomes (You and Kim, 2021). Additionally, research on cattle has shown that age-related changes in rumen bacteria and methanogens are associated with metabolic shifts, impacting feed efficiency and methane production (Liu *et al.*, 2017). Microbiome establishment is known to start during gestation and continues to form during delivery and after birth with diet and environmental factors (Ceylani *et al.*, 2018). The microbiome, consisting of 10^{13} to 10^{14} microorganisms, plays crucial roles in digestion, metabolism, and gut barrier integrity (Kumarappah and Senderovich, 2016). However, bacterial diversity decreases with age, which negatively impacts health, leading to metabolic dysregulation and neurological (Ceylani *et al.*, 2023; Teker *et al.*, 2024b). In aging animals, the aforementioned changes in gut microbiota are also associated with

declining immune function and metabolic health (Pilla and Suchodolski, 2019).

It is evidenced that probiotic bacteria have an important role in balancing and maintaining proper function for gut bacteria to prevent aging-related dysbiosis (Plaza-Díaz *et al.*, 2017; Baba *et al.*, 2024). The Food and Agriculture Organization (FAO) and World Health Organization (WHO) define probiotics as live microorganisms with health benefits on the host, provided the amounts are adequately maintained (Hill *et al.*, 2014). It is also recognized that both probiotics and prebiotics modulate gut microbiota and improve cholesterol and lipid profiles, detoxify mycotoxins, reduce blood pressure, and enhance glucose tolerance with positive effects on diabetes management (Sanders *et al.*, 2019; Teker *et al.*, 2024a, 2024b). Probiotic supplementation has also been explored in veterinary applications, particularly for improving gut health in aging animals (Yang and Wu, 2023; Atuahene *et al.*, 2024). A probiotic mix helped reduce intestinal permeability and inflammation by modulating the microbiota and promoting the integrity of the gut barrier, which helped mitigate aging-related leaky gut and inflammation (Ahmadi *et al.*, 2020). In swine, probiotics have been used to maintain a healthy gut by balancing microbiota, improving nutrient utilization, and reducing digestive disorders, demonstrating the broad application of probiotics in animal health (Liao and Nyachoti, 2017). Furthermore, probiotics are utilized for the prevention of gastrointestinal disorders and for improving metabolic health in livestock (Fu *et al.*, 2023).

Intermittent fasting is known as partial or total food restriction for 16 to 24 hours. It can be utilized for therapeutic purposes or practiced socially or religiously (De Cabo and Mattson, 2019; Ceylani *et al.*, 2022). Recent studies have shown that IF has a role in the reduction of insulin resistance, regulating adipose tissue browning, preventing CNS autoimmunity, neuroinflammation and improving diabetes-related complications by altering the gut microbiota (Li *et al.*, 2021; Allahverdi, 2024). It also affects brain physiology, and cognitive function during aging (Silva *et al.*, 2020). We have recently shown the impact of IF on balancing the gut microbiota composition in rats (Teker and Ceylani, 2022). In this study, we explored the effects of SCD Probiotics during IF on gut microbiota in aged rats.

MATERIALS AND METHODS

Experimental Design: Male 24-month-old Sprague-Dawley rats (n=28) were used as model organisms in this study. The study consisted of four groups: control (n=7), intermittent fasting (IF) for 30 days (n=7), probiotics for 30 days (n=7), and probiotics with IF for 30 days (n=7). Rats in IF groups had restricted food access for 18 hours daily, with a 6-hour feeding window (9:00a.m. to 3:00p.m.), while water was available *ad libitum*. All rats were fed a standard rodent diet, and body weight, water, and food consumption were monitored regularly. The probiotic supplement (Essential Probiotics XI - 500 ml H.S. Code: 2206.00.7000), marketed by the SCD Probiotics company, containing *Bacillus subtilis, Bifidobacterium bifidum, Bifidobacterium lognum, Lactobacillus acidophillus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus plantarum, Lactococcus lactis, Saccharomyces cerevisiae*, and *Streptococcus thermophilus* species, was administered orally in doses of 3mL (1×10^8 CFU) each day. The probiotics were given in two doses of 1.5mL each after feeding (Ceylani, 2023). The animals were lightly sedated with ether and humanely euthanized one day following the completion of the experiment. Cecum contents were collected, frozen on dry ice, and stored at -80°C until processing within two weeks. The study was conducted under standard animal care with approval from the Saki Yenilli Experimental Animal Production and Practice Laboratory Ethics Committee (Approval No: 2021/05).

DNA Isolation, 16S rRNA V3-V4 Amplification, Library Preparation, and Sequencing: Genomic DNA was extracted from cecal content using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Cat. No. D6010). The concentration and purity of the extracted DNA were assessed fluorometrically using the Qubit system. V3-V4 regions of the 16S rRNA gene to be used for species determination were amplified with universal 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) primer sequences using SimpliAmp Thermal Cycler. The 16S rRNA V3-V4 amplicon libraries were prepared using Illumina's Nextera XT DNA Library Prep Kit (Cat. No. FC-131-1096), with indexing performed using the TG Nextera XT Index Kit v2 Set A (96 Indices, 384 Samples, Cat. No. TG-131-2001). PCR purification was carried out using AMPure XP beads (Beckman Coulter). Sequencing was performed on Illumina's MiSeq platform, generating paired-end (PE) reads of 2×150 bases. A minimum of 30.000 reads per sample was ensured. The PCR conditions were as follows: initial denaturation at 95°C for 10 minutes using a high-specificity (HS) enzyme, followed by 35 amplification cycles consisting of denaturation at 95°C for 45 seconds, annealing at 50-55°C for 45 seconds, and elongation at 72°C for 60 seconds. A final elongation step was performed at 72°C for 3 minutes. The reaction was then cooled to 4°C to complete the PCR process. The 16S rRNA V3-V4 amplicon libraries were constructed using the Nextera XT DNA Library Prep Kit (Illumina, Cat. No: FC-131-1096), with indexing performed using the TG Nextera XT Index Kit v2 Set A (96 Indices, 384 Samples; Cat. No: TG-131-2001). PCR purification was carried out using AMPure XP beads (Beckman Coulter). Sequencing was conducted on Illumina's MiSeq platform, generating paired-end (PE) reads of 2×150 bases. A minimum sequencing depth of 30,000 reads per sample was ensured. Metagenome sequencing was performed at Ficus Biotechnology (FicusBio), Ankara, Turkey (Ceylani and Teker, 2022).

Bioinformatics analysis of raw data: The raw sequencing data (FastQ files) underwent quality control to enhance the accuracy of microbial diversity analysis and to remove sequencing artifacts, including low-quality and contaminated reads. Quality assessments and trimming, if required, were performed using FastQC v0.10.1. Subsequently, the Kraken metagenomic system was utilized to classify and cluster the sequence data into

operational taxonomic units (OTUs). Heatmaps were created with GraphPad Prism 10.0.1 (GraphPad Software, USA) software. All the sample raw reads have been deposited at NCBI under the BioProject ID PRJNA887213.

Shannon and Simpson's diversity indexes*:* Alpha diversity indices were calculated at the species level. Shannon's Equitability values ranged between 1.5 and 3.5, with higher values indicating a more even distribution of species. The Simpson indices were determined based on the abundance and evenness of OTUs, with Simpson's Diversity Index (1-D) ranging from 0 to 1, where a value of 1 represents complete evenness in the community (Kalamaki and Angelidis, 2020).

Short Chain Fatty Acids (SCFAs) analysis: For the determination of short-chain fatty acids, 1g of the cecum content obtained in the control and experimental groups was collected and transferred into 2mL Eppendorf tubes to which 1mL of distilled water was added. The supernatants of gaita samples were taken after centrifugation of samples at 14000 rpm for 5 min. Then, the supernatants were filtered through 0.22µm cellulose-acetate filters (Isolab). The samples were diluted three times with ultrapure water to prepare them for HPLC analysis. Organic acids in the sample were measured by HPLC Shimadzu 20A series equipped with a UV detector (Shimadzu FCV-10AT). Transgenomic ICSep ICE-COREGEL 87H3 (300mm × 7.8mm) ion-exchange column was used for analysis. Sulfuric acid (0.088M) was used as a mobile phase with a flow rate of 0.6mL/min. The oven temperature was adjusted to 35°C.

Statistics: Statistical analysis was presented as mean ± SEM. Alpha diversities and F/B ratios were compared between control (Cnt), intermittent fasting (Fst), probiotic (Prb), and probiotic with IF (FstPrb) groups using One-Way ANOVA and Unpaired T-test (one-sided p-value) in GraphPad Prism 10.1 (GraphPad Software, USA). Significance levels were denoted as *P<0.05, ***P<0.001, ****P<0.0001. Heatmap analysis of metagenomic counts for bacterial families, genera, and species was also conducted using GraphPad Prism 10.1.

RESULTS

Analysis of body weight, water intake, and feed consumption: The results showed a significant reduction in body weight in the intermittent fasting (IF) groups (Fig. 1A). However, the FstPrb group, which received probiotics along with IF, experienced less pronounced weight loss, suggesting that probiotics may have helped mitigate fasting-related weight loss. Additionally, an increase in feed consumption was observed in the fasting groups, likely due to an adaptation process (Fig. 1B). No significant differences in water consumption were observed (Fig. 1C).

Alpha diversity analysis and Firmicutes/Bacteroidetes (F/B) ratio: The study demonstrated significant changes in alpha diversity and the Firmicutes/Bacteroidetes (F/B) ratio across all groups. The Shannon diversity index showed substantial increases in the Fst, Prb, and FstPrb groups. Specifically, there were significant differences between Cnt and Fst (P<0.0001), Cnt and Prb (P=0.0069), and Cnt and FstPrb (P=0.0034). Additionally, comparisons between Fst and Prb (P<0.0001), and Fst and FstPrb (P<0.0001) were also significant, while the difference between Prb and FstPrb was not statistically significant (P=0.9382) (Fig. 2A). For the Simpson diversity index, significant differences were observed between Cnt and Fst (P=0.0092), Cnt and Prb (P=0.0325), and Cnt and FstPrb (P=0.0457). However, comparisons between Fst and Prb (P=0.7857), Fst and FstPrb (P=0.6526), and Prb and FstPrb (P=0.9944) showed no significant differences (Fig. 2B). The F/B ratio exhibited highly significant changes. There were significant differences between Cnt and Fst (P<0.0001), Cnt and Prb (P<0.0001), and Cnt and FstPrb (P<0.0001). Additionally, Fst vs. Prb (P<0.0001) and Prb vs. FstPrb (P<0.0001) showed marked differences, with the comparison between Fst and FstPrb also reaching significance (P=0.0003) (Fig. 2C).

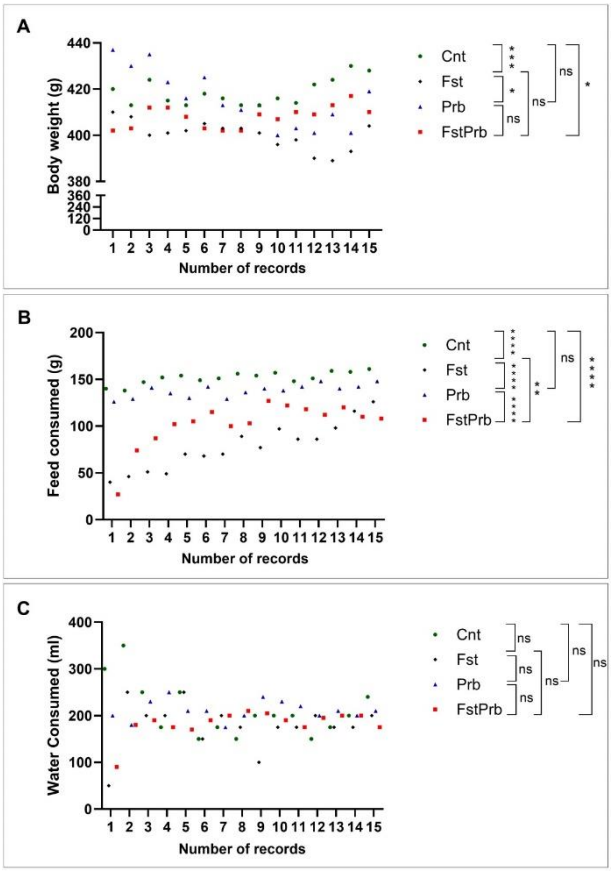

Fig. 1: The effects of intermittent fasting, SCD Probiotics supplementation, and SCD Probiotics supplementation during intermittent fasting on A) body weight, B) water, and C) food consumption. Cnt (control), Fst (intermittent fasting), Prb (SCD Probiotics) and FstPrb (SCD Probiotics supplementation during intermittent fasting).

Prevalent bacterial families, genera, and species: Intermittent fasting, probiotics, and probiotic supplementation during fasting led to significant changes in the predominant bacterial families (Table 1). The most notable impact was observed with probiotic supplementation. The Spirochaetaceae and Christensenellaceae families were particularly increased in

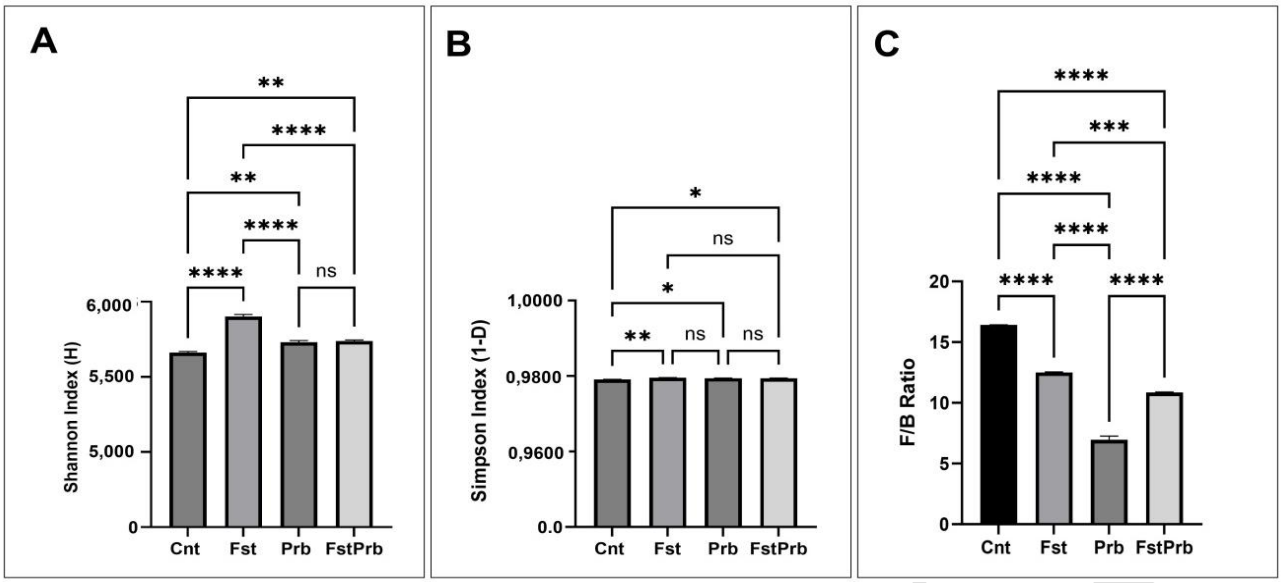

Fig. 2: The effects of intermittent fasting, SCD Probiotics supplementation, and SCD Probiotics supplementation during intermittent fasting on old rats' gut microbiota A) Shannon (H) and B) Simpson (1-D) indexes and C) Firmicutes to Bacteroidetes ratio (F/B ratio). Cnt (control), Fst (intermittent fasting), Prb (SCD Probiotics) and FstPrb (SCD Probiotics supplementation during intermittent fasting).

Table 1: Relative abundance of the top ten bacterial families and genera in control and intervention groups

	Top 10 Bacterial Families							
	Cnt	%	Fst	%	Prb	%	FstPrb	%
1	Lachnospiraceae	33.43	Lachnospira-ceae	33.58	Lachnospira-ceae	37.65	Lachnospira-ceae	35.27
2	Lactobacillaceae	20.87	Lactobacilla-ceae	14.02	Oscillospira-ceae	15.86	Lactobacilla-ceae	12.69
3	Oscillospiraceae	14.14	Oscillospira-ceae	12.64	Clostridia-ceae	5.20	Oscillospira-ceae	12.34
4	Clostridiaceae	3.23	Clostridiaceae	4.64	Prevotella-ceae	4.80	Clostridia-ceae	5.78
5	Bacillaceae	2.63	Bacillaceae	3.25	Spirochaeta-ceae	4.54	Bacillaceae	2.85
6	Muribaculaceae	2.25	Erysipelotricha-ceae	2.66	Lactobacilla-ceae	3.63	Turicibactera-ceae	2.66
7	Erysipelotricha-ceae	1.79	Peptostrepto-cocaceae	2.16	Bacillaceae	3.17	Muribacula-ceae	2.61
8	Coriobacteriaceae	1.49	Turicibacteraceae	1.94	Muribacula-ceae	2.24	Peptostrepto-coccaceae	2.07
9	Peptostreptoco-ccaceae	1.40	Prevotella-ceae	1.88	Eubacteria-ceae	0.97	Erysipelo-trichaceae	1.89
10	Prevotellaceae-	1.21	Muribacula-ceae	1.50	Christensenellaceae	0.96	Eubacteria-ceae	1.43
	Top 10 Bacterial Genera							
	Cnt	%	Fst	%	Prb	%	FstPrb	%
1	Lactobacillus	12.52	Ligilacto-bacillus	6.65	Ruminococcus	7.80	Rumino-coccus	6.02
2	Ruminococcus	9.12	Lacto-bacillus	5.72	Anaerostipes	5.77	Anaerostipes	5.92
3	Ligilactobacillus	4.78	Lachno-clostridium	5.06	Treponema	5.12	Clostridium	5.89
4	Blautia	4.51	Rumino-coccus	4.81	Clostridium	5.20	Lacto-bacillus	4.90
5	Intestinimonas	3.73	Clostridium	4.44	Roseburia	3.96	Lachno-clostridium	4.48
6	Lachnoclostridium	3.40	Anaerostipes	4.36	Lachnoclostridium	3.85	Ligilacto-bacillus	4.47
7	Clostridium	3.33	Bacillus	3.59	Prevotella	3.50	Intestini-monas	3.25
8	Anaerostipes	3.18	Blautia	3.58	Bacillus	3.54	Turicibacter	3.13
9	Coprococcus	3.07	Coprococcus	2.44	Intestinimonas	3.13	Blautia	3.05
10	Bacillus	2.81	Turicibacter	2.30	Blautia	2.51	Bacillus	2.99

The table shows the relative abundance of the top ten most dominant bacterial families and genera identified in the control and experimental groups. These percentages are calculated based on the total metagenomic reads within each respective group. Cnt (control), Fst (intermittent fasting), Prb (SCD Probiotics), and FstPrb (SCD Probiotics supplementation during intermittent fasting). The data provides a comparative insight into the microbial composition across groups, highlighting the influence of intermittent fasting and SCD Probiotics on gut microbiota diversity**.**

the probiotic-only group. Additionally, the Prevotellaceae family, which accounted for 1.21% of the control group, became more dominant with probiotics, rising to 4.80%. Notably, the Lactobacillaceae family, the second most dominant in all groups, decreased with probiotic use. In the FstPrb group, unlike the control group, the Eubacteriaceae family became dominant with probiotic supplementation, while the Turicibacteraceae and Muribaculaceae families became dominant only with IF. Fig. 3A compares the ten most prevalent bacterial families in the FstPrb group with their prevalence in other groups.

The prevalence of dominant genera was substantially changed and altered in all three groups as shown in Table 1. The top ten genera predominantly seen due to probiotic use varied greatly at different rates. Treponema, Roseburia and Prevotella were among the top ten most dominant genera. Ruminococcus and Anaerostipes continued to be the two most dominant genera in the FstPrb group with the use of probiotics. The strains of Turicibacter, which could only be found among the top ten most dominant genera with IF, had a higher incidence in the FstPrb group. It has been shown that probiotic supplementation alone cannot show the effect of supplementation combined with intermittent fasting. A comparison of the first ten bacterial genera predominantly found in the FstPrb group with the prevalence in other groups is shown in the heatmap Fig. 3B.

Intermittent fasting, probiotic supplementation, and their combination significantly impacted species diversity (Table 2). The most substantial changes were due to

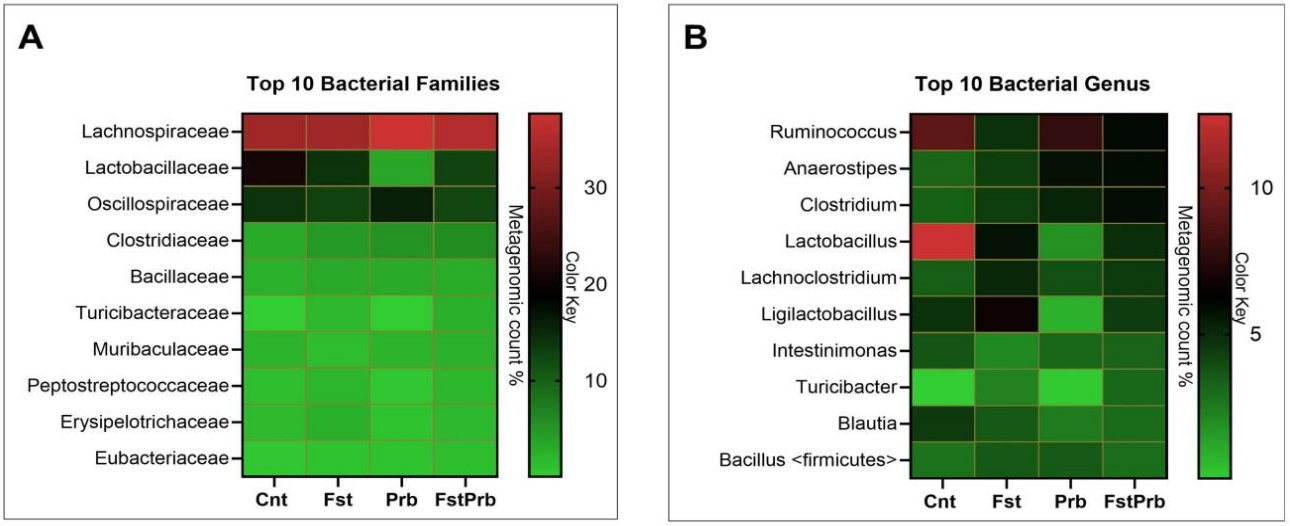

Fig. 3: Heatmap comparing the percentage abundance of the top ten most dominant A) families and B) genus of bacteria found in the FstPrb group (SCD Probiotics supplementation during intermittent fasting) with their respective abundance in other groups. The groups compared are: Cnt (control), Fst (intermittent fasting), Prb (SCD Probiotics), and FstPrb (SCD Probiotics with intermittent fasting). The bacterial abundance was determined using metagenomic analysis, and the heatmap illustrates the relative distribution of these dominant taxa across the groups. This figure allows for a visual comparison of how probiotic supplementation during intermittent fasting influences the microbiota composition relative to another group.

Table 2: Relative abundance of the top twenty bacterial species in the control and intervention groups

	Top 20 Bacterial Species							
	Cnt	%	Fst	%	Prb	%	FstPrb	%
1	*Ruminococcus sp.* JE7A12	8.70	*Anaerostipes hadrus*	3.17	*Anaerostipes hadrus*	3.11	*Intestinimonas butyriciproducens*	4.65
2	*Intestinimonas butyrici-producens*	5.64	*Intestinimonas butyriciproducens*	3.07	*Intestinimonas butyrici-producens*	3.01	*Anaerostipes hadrus*	3.60
3	*Collinsella aerofaciens*	3.56	*Flavonifractor plautii*	2.68	*Ruminococcus sp.* JE7A12	2.46	*Anaerostipes caccae*	3.35
4	*Anaerostipes hadrus*	3.08	*Ruminococcus sp.* JE7A12	2.65	*Flintibacter sp.* KGMB00164	2.22	*Coprococcus catus*	3.13
5	*Lactobacillus acetotolerans*	2.93	*Coprococcus catus*	2.56	*Bacillus velezensis*	2.16	*Butyrivibrio fibrisolvens*	3.08
6	*Candidatus Saccharimonas aalborgensis*	2.79	*Flintibacter sp.* KGMB00164	2.55	*Flavonifractor plautii*	2.04	*Flintibacter sp.* KGMB00164	2.97
7	*Coprococcus catus*	2.67	*Butyrivibrio fibrisolvens*	2.53	*Butyrivibrio fibrisolvens*	1.76	*Bacillus-Velezensis*	2.53
8	*Bacillus velezensis*	2.52	*Bacillus velezensis*	2.44	*Treponema pedis*	1.72	*Ruminococcus champanellensis*	2.27
9	*Blautia sp.* LZLJ-3	2.26	*Faecalibaculum rodentium*	2.26	*Ruminococcuschampane-llensis*	1.53	*Flavonifractor-Plautii*	2.03
10	*Enterococcus faecalis*	2.11	*Clostridium hylemonae*	2.12	*Anaerostipes caccae*	1.10	*Rumino-coccus sp.* JE7A12	1.91
11	*Ruminococcus torques*	2.04	*Anaerostipes caccae*	2.08	*Roseburia intestinalis*	1.10	*Clostridium hylemonae*	1.61
12	*Rutheni-bacterium lactatiformans*	1.85	*Lachno-clostridium phocaeense*	1.99	*Anaerotignum propionicum*	1,09	*Romboutsia-ilealis*	1.60
13	*Faecali-bacterium prausnitzii*	1.66	*Romboutsia ilealis*	1.72	*Coprococcus catus*	1.08	*Anaerotignum propionicum*	1.56
14	*Butyrivibrio fibrisolvens*	1.40	*Ruminococcus champanellensis*	1.71	*Treponema succinifaciens*	1.06	*Ruminococcus torques*	1.53
15	*Bacillus amylolique-faciens*	1,40	*Bacillus amylolique-faciens*	1.64	*Clostridium hylemonae*	1.03	*Lachnoclostridiumphyto-fermentans*	1.47
16	*Ruminococcus champane-llensis*	1.39	*Ruminococcus torques*	1.57	*Massilistercora timonensis*	1.00	*Fibrobacter succinogenes*	1.41
17	*Christen-senella minuta*	1.35	*Acutalibacter muris*	1.40	*Ruminococcus bicirculans*	0.89	*Anaerocolumna cellulosilytica*	1.23
18	*Clostridium hylemonae*	1.33	*Anaerotignum propionicum*	1.37	*Oscillibacter valericigenes*	0.88	*CandidatusSaccharimonas aalborgensis*	1.20
19	*Clostridium scindens*	1.22	*Methylomusa anaerophila*	1.35	*Christensenella minuta*	0.86	*Faecalibaculum rodentium*	1.14
20	*Faecali-baculum rodentium*	1.20	*Candidatus Saccharimonas aalborgensis*	1.19	*Herbinix Luporum*	0.85	*Anaerocolumna sedimenticola*	1.13

The table presents the relative abundance of the top twenty most dominant bacterial species identified in each group, including the control and intervention groups, expressed as a percentage of the total metagenomic reads within that group. Cnt (control), Fst (intermittent fasting), Prb (SCD Probiotics), and FstPrb (SCD Probiotics supplementation during intermittent fasting). This data provides a detailed comparison of microbial composition across groups, illustrating the impact of intermittent fasting and SCD Probiotics on gut microbiota diversity.

probiotics, with species like *Treponema pedis*, *Roseburia intestinalis*, and *Ruminococcus bicirculans* becoming dominant. IF also caused notable shifts, with species such as *Lachnoclostridium phocaeense* and *Bacillus amyloliquefaciens* predominating only in the Fst group. In the FstPrb group, IF suppressed the probiotic effect, with unique species like *Lachnoclostridium phytofermentans* and *Faecalibaculum rodentium* becoming dominant. Species such as *Faecalibaculum rodentium* and Romboutsia ilealis remained dominant across both Fst and FstPrb groups. Additionally, species like *Flavonifractor plautii* and *Anaerostipes caccae* were dominant in all groups except the control. The heatmap comparisons of the top twenty species in the FstPrb group against other groups are shown in Fig. 4.

Short-chain fatty acids: In this study, it was concluded that every experimental group demonstrated an augmentation in comparison to the control group. Notably, acetic acid experienced a substantial escalation specifically within the SCD Probiotics group, as depicted in Fig. 5A. In addition, probionic acid showed an increase, with the most pronounced difference being detected in the SCD Probiotics group (Fig. 5B).

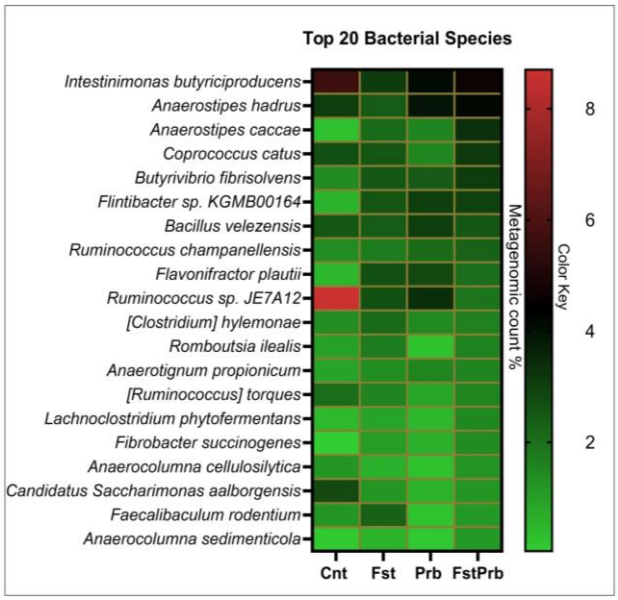

Fig. 4: Heatmap comparing the percentage abundance of the top ten most dominant species of bacteria found in the FstPrb group (SCD Probiotics supplementation during intermittent fasting) with their respective abundance in other groups. The groups compared are: Cnt (control), Fst (intermittent fasting), Prb (SCD Probiotics), and FstPrb (SCD Probiotics with intermittent fasting). The bacterial abundance was determined using metagenomic analysis, and the heatmap illustrates the relative distribution of these dominant taxa across the groups. This figure allows for a visual comparison of how probiotic supplementation during intermittent fasting influences the microbiota composition relative to other groups.

While there was no statistically significant variation in SCFAs within the IF group compared to control, a marked augmentation was noted when it was appraised in conjunction with SCD Probiotics. Furthermore, a congruent trend was observed for both butyric and isovaleric acids (Fig. 5C-D). However, isocaproic and hexanoic acids only exhibited a significant amplification within the IF group (Fig. 5E-F).

DISCUSSION

The findings of this study demonstrate that the combined effects of SCD probiotics and intermittent fasting produce significant improvements in gut microbiota diversity and short-chain fatty acid profiles in aged rats. These results align with previous research that highlights the role of both probiotics and IF in modulating gut health, suggesting that this complementary may offer a potent strategy for mitigating age-related dysbiosis and promoting overall gut health. Consistent with previous studies, weight reduction was observed in the fasting groups (Templeman *et al.*, 2020) while less reduction was observed in the probiotic group and FstPrb group compared to other probiotic studies (Cerdó *et al.*, 2019). This result suggests that probiotic supplementation may affect metabolism during the fasting period as a stabilizing effect of the process (Jung *et al.*, 2013).

Currently, a clear definition of a healthy microbiota, aside from showing diversity in gut microbiome, is lacking. Shannon and Simpson indices, commonly used to assess species diversity, were employed to quantify gut microbiota diversity in this study. Amplified sequence data is used for these evaluations (Ceylani and Teker, 2022). Although various factors influence gut microbiota (GM), species diversity is primarily affected by dietary choices. Intermittent fasting has been shown to significantly enhance species diversity, a finding supported by recent studies (Teker and Ceylani, 2022). SCD Probiotics also increase GM diversity (Ceylani, 2023). These findings suggest practical applications for improving gut health in aging companion animals, where maintaining microbial diversity is crucial for disease prevention (Lee *et al.*, 2022 ; Barathan *et al.*, 2024). Previously, probiotic supplementation in dogs with diarrhea showed improvements in microbial diversity and reduction in pathogens, suggesting that probiotics can restore balance in the gut microbiome in sick or aging animals (Torkan *et al.*, 2014; Xu *et al.*, 2019a). The reduced species diversity in the FstPrb group compared to the Fst group suggests that probiotics influence GM diversity. Similarly, IF appears more impactful on Simpson's value, with SCD Probiotics modulating this effect. The reduced species diversity observed in the combined group (IF + SCD Probiotics) compared to the IF-only group was unexpected. This suggests that while intermittent fasting alone significantly boosts diversity, adding probiotics may cause competitive interactions among microbial species, reducing overall diversity. The probiotic strains in SCD Probiotics likely promote the growth of specific bacteria, limiting others and thus lowering diversity. This interaction could be more pronounced in fasting conditions, where the gut environment is altered. Understanding these effects is crucial in both human and veterinary applications, where both probiotics and dietary interventions are used to improve gut health in aging animals and humans (Pilla and Suchodolski, 2019).

Firmicutes and Bacteroidetes strains constitute over 90% of the gut microbiota, while Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia are present in smaller quantities. The Firmicutes to Bacteroidetes (F/B) ratio is often linked to health and various clinical conditions (Magne *et al.*, 2020), where higher ratios are associated with dysbiosis, increasing from infancy to maturity. We have previously reported that IF stabilizes the F/B ratio (Teker and Ceylani, 2022), which we have observed again in this study. Although most probiotic groups in SCD Probiotics belong to the Firmicutes phylum we observed an increase in Bacteroidetes and a decrease in Firmicutes. The survival challenges at stomach acid and bile might be an effective factor (Cheng *et al.*, 2021). In livestock and companion animals, the F/B ratio is also a marker of gut health, and probiotics have been used to help balance these bacterial groups to prevent gastrointestinal diseases (Koo *et al.*, 2019; Stojanov *et al.*, 2020). Probiotics containing *Lactobacillus* and *Bifidobacterium* strains improved gut health in elderly dogs by increasing beneficial bacteria while reducing harmful bacteria, thus improving immune factors and digestive health (Xu *et al.*, 2019b).

Short-chain fatty acids like butyrate, propionate, and acetate are produced by gut microbiota. Those fatty acids have a crucial impact on intestinal health, metabolic regulation, energy production, and intestinal permeability,

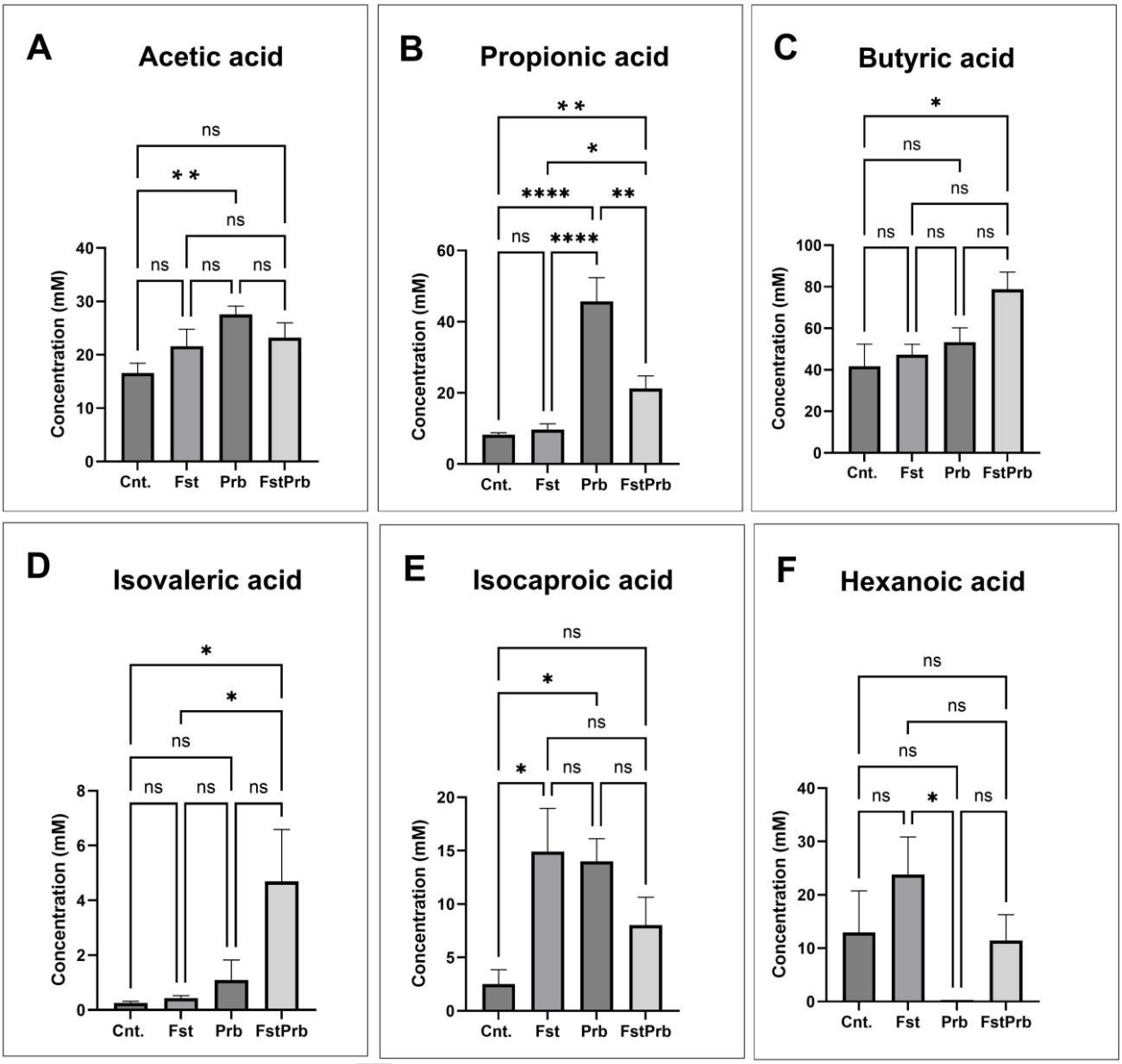

Fig. 5: The effects of intermittent fasting, SCD Probiotics supplementation, and SCD Probiotics supplementation during intermittent fasting on old rats' short chain fatty acids (SCFAs) A) Acetic acid, B) Probionic acid, C) Butyric acid, D) Isovaleric acid, E) Isocaproic acid and, F) Hexanoic acid. Cnt (control), Fst (intermittent fasting), Prb (SCD Probiotics) and FstPrb (SCD Probiotics supplementation during intermittent fasting).

and have hormonal regulation effects (Nagpal *et al.*, 2018). In this study, all experimental groups showed increased SCFA production. Significant increases were observed in acetic acid, propionic acids, butyric acid, and isovaleric acids in the SCD Probiotics group. However, a significant increase in isocaproic and hexanoic acids was only noted in the IF group. Those changes might suggest potential interconnected effects of these interventions. The role of SCFAs is well recognized in veterinary science as they are among the key metabolic byproducts that regulate gut health and energy metabolism in animals (He *et al.*, 2022; Liu *et al.*, 2023).

Microbiota established in the gut, are known to be highly dynamic, adapting quickly and reshaping in response to environmental factors, diet, and age (Ji *et al.*, 2020). Species-level diversity changes are important and inevitably decline during aging (Mangiola *et al.*, 2018). In this study, we observed that IF and SCD Probiotics differently affected dominant gut species. Even though both interventions are considered beneficial, IF had a more pronounced effect on gut microbiota diversity compared to SCD Probiotics. Although it is known that probiotic supplementation has a role in the stabilization of the gut microbiota (Nawab *et al.*, 2018: Wang *et al.*, 2021), our data showed that the combination of IF and SCD Probiotics appears more advantageous for maintaining a healthy gut microbiota. Maintaining a balanced gut microbiota through dietary interventions such as probiotics supplementation and IF could also be beneficial for aging animals, helping prevent dysbiosis-related diseases and promoting overall health.

Conclusions: The combination of SCD Probiotics and intermittent fasting provides synergistic benefits for gut microbiota in aged rats, enhancing bacterial diversity and improving the Firmicutes to Bacteroidetes ratio. This study highlights the novel interplay between these interventions, offering potential therapeutic strategies for addressing age-related dysbiosis. These findings have implications for human health and veterinary applications,

particularly in improving gut health in aging companion animals and livestock.

The study is limited by its short-term observation period, which restricts insights into long-term effects, and the use of a rat model, necessitating validation in larger animal models and human trials. Furthermore, the impact of different probiotic strains requires further exploration to identify optimal combinations for specific outcomes. Future research should focus on these areas to fully realize the potential of SCD Probiotics and IF for managing age-related gut dysbiosis.

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