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Grape Seed Proanthocyanidins: A Potential Microbiome-Targeted Intervention for Healthy Aging in Rats

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Keywords: aging | dysbiosis | gut barrier function | microbiota | proanthocyanidins

ABSTRACT

Aging and age-related metabolic complications are global health issues that pose a serious threat to public health. Gut dysfunction and dysbiosis contribute to age-related health decline. Grape seed-derived procyanidins (GSPE) have shown efficacy in mitigating adaptive homeostasis decline in young animal models, but their impact on intestinal health and the gut microbiome in aged animals remains unexplored. Twenty-one-month-old female rats were treated with 500 mg GSPE/kg of body weight for 10 days. After 11 weeks, GSPE anti-aging potential was evaluated by measuring plasma lipopolysaccharide, gut integrity gene expression, ex vivo gut barrier function, myeloperoxidase activity, and fecal microbiome composition. GSPE shifted the microbiota toward a younger profile, even restoring lost strains in aged rats. Despite the presence of metabolic aging markers, there was minimal deterioration in gut barrier function. Neither ex vivo permeability tests, transcriptional analysis of barrier function, nor gut histology showed significant impairment in gut. Only jejunal myeloperoxidase activity was increased in aged rats and reduced by GSPE. Intestinal barrier function showed mild deterioration in this model of aged rats. GSPE improved the aging process by modulating the gut microbiome, suggesting its potential as a microbiome-targeted intervention for promoting healthy aging.

1 | Introduction

The intestine is the largest surface in the body [1]. The intestinal barrier must act as both a physical and biochemical barrier to ensure the absorption of nutrients but also avoid the translocation of macromolecules, including microbial components and pro-inflammatory agents [2, 3]. Several factors, such as diet, sedentary lifestyle, drug abuse, and aging, affect intestinal homeostasis and can cause intestinal disruption [1, 2, 4]. Consequently, the microbiota-derived antigens, especially lipopolysaccharide (LPS),

Abbreviations: GSPE, grape seed-derived procyanidins; TEER, transepithelial electrical resistance; TJ, tight-junction.

Marta Sierra-Cruz and Adrià Vilalta contributed equally to this study.

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can cross the intestinal barrier, reach the bloodstream, and trigger a systemic inflammatory response known as endotoxemia [1, 5]. High-fat/high-sugar diets have been widely demonstrated to cause intestinal dysfunction. These alterations encompass an elevation in intestinal permeability by disrupting tight-junction (TJ) proteins [6], modifications in gut microbiota composition, and ultimately, metabolic endotoxemia in animal models of diet-induced obesity in young rats [7–9]. However, the literature on the involvement of aging in intestinal dysfunction presents conflicting findings.

There is increasing evidence that the gut microbiome lies at the core of many age-associated changes and plays a role in longevity across species [10, 11]. The gut microbiome is responsible for several metabolically important functions like vitamin and short-chain fatty acid production, amino acid synthesis, bile acid biotransformation, and fermentation of nondigestible substrates [12, 13]. In addition, the gut microbiome also participates in immune-cell homeostasis and dynamics [14]. Consequently, dysbiosis of the gut microbiome has significant impacts on health due to the intricate symbiotic relationship humans have with their commensal bacteria.

While early studies demonstrated that significant changes occur in the gut microbiome from birth until 2–3 years of age, after which microbial populations remain relatively stable [15], recent evidence has begun to show how aging changes the gut microbiome, and how the gut microbial community can, in turn, influence aging [16, 17]. An added complexity is that while human aging can be a healthy process, aging can also be associated with physical frailty, increased inflammation, metabolic changes, and weakened immune responses [14], as well as various lifestyle alterations such as increased medication use, changes in diet, and reduced physical activity; all of which can independently affect the diversity and stability of the gut microbiota. In fact, an age-related decrease in gut microbiota diversity has been reported among the elderly, and this has been linked to an increase in the risk of different diseases [18].

Numerous strategies have been developed to prevent, improve, and/or delay the metabolic alterations associated with aging. Polyphenols constitute a very interesting field of study due to their previously demonstrated anti-oxidant, anti-inflammatory, and anti-aging properties [19]. Proanthocyanidins, a significant polyphenol group found in fruits and vegetables, are oligomeric and polymeric flavan-3-ols, primarily composed of (+)-catechin and (−)-epicatechin monomers. They constitute a prevalent group of phenolic compounds in the human diet [20]. Grape seed procyanidins, renowned for health benefits, provide a distinctive opportunity for reusing by-products in the agro-food industry, particularly during wine or grape juice production. Harnessing these procyanidins not only reduces by-product waste but also facilitates sustainable production of health-valuable ingredients.

We have previously shown that some doses of grape seed procyanidin extract (GSPE) have beneficial effects in young and aged rats by limiting their body weight increase and adiposity among other properties effective against metabolic syndrome [21–23]. We have shown that their effects on body weight and adiposity continue over the long term once the GSPE administration has finished [24, 25]. Indeed, 11 weeks after the treatment, GSPE maintained its effects on limiting visceral adipose tissue growth and ameliorating insulin sensitivity in aged rats [26]. GSPE also reverted inflammation, intestinal permeability, and metabolic endotoxemia in young animals under an obesogenic diet [21, 22, 27].

The effects of polyphenols over the microbiota modulation have been largely studied in the last decades. In past studies, GSPE treatment for 8 days was sufficient to produce changes in several taxonomic levels including the reduction of Firmicutes: Bacteroides ratio, usually associated with obesity and type II diabetes [28]. In certain obesity models, polyphenol administration has been shown to restore the Firmicutes: Bacteroidetes ratio with GSPE [29] or grape-derived polyphenols [30], thereby improving metabolic health.

The effect of GSPE and microbiota modulation appears to be associated with a prebiotic effect and the antimicrobial properties of the compounds found in the extract [31]. Further details and examples of how polyphenols and specifically GSPE modulate the microbiome can be found in several review articles [31–33].

Considering these GSPE effects over the long term, we hypothesize that its effectiveness in delaying the appearance of aging-related diseases might be through the modulation of host-microbiome interaction and ameliorating intestinal health. To address this, we analyzed microbiome composition, its influence on aging, and how GSPE could modulate these interactions.

2 | Experimental Section

2.1 | Proanthocyanidin Extract

The grape seed extract rich in proanthocyanidins (GSPE) was provided by *Les Dérivés Résiniques et Terpéniques* (Dax, France). According to the manufacturer, the GSPE used in this study (Batch number: 207100) contains a total procyanidin content of 76.9%, which consists of a mixture of monomers (23.1%), dimers (21.7%), trimers (21.6%), tetramers (22.2%), and pentamers (11.4%) of flavan-3-ols. A more detailed analysis of GSPE composition was previously done by Margalef et al. [34].

2.2 | Animal Model

In this study, a total of 34 outbreed female Wistar rats were used, 24 were 21 months old (weighing 300–350 g) and 10 were 2 months old (weighing 210–220 g) at the beginning of the experiment. Rats were acquired from Envigo (Barcelona, Spain). After 1 week of adaptation, the rats were individually caged in the animal quarters at 22°C with a 12-h light/12-h dark cycle and were fed ad libitum with a standard chow diet (Teklad 2014 Envigo, Barcelona, Spain) and tap water.

2.3 | Experimental Design

Aged animals were randomly distributed into the two experimental groups and were fed a standard chow diet ad libitum during the whole duration of the experiment. The experimental design is

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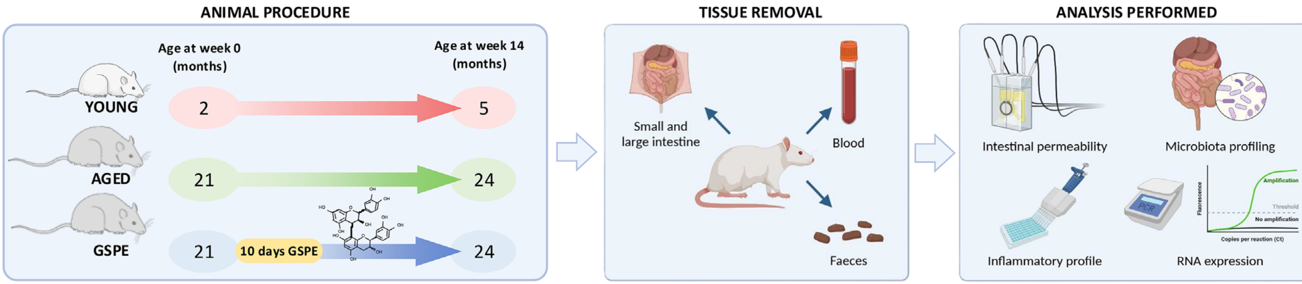

FIGURE 1 | Schematic diagram of the experimental design. All groups were adapted to the environment and to oral gavage during 1 week before the experiment started. Young: 2-month-old rats receiving standard chow diet; aged: 21-month-old rats receiving standard chow diet; GSPE: 21-month-old rats receiving standard chow diet and GSPE preventive treatment for 10 days.

shown in Figure 1. GSPE was administered at a dose of 500 mg/kg body weight. GSPE was dissolved in tap water and administered by oral gavage 1 h prior to the night cycle for 10 days. Animals not supplemented with GSPE received water as a vehicle. After the GSPE treatment, rats were maintained under normal housing conditions for 11 weeks.

All procedures were approved by the Animal Experimentation Commission of the Generalitat de Catalunya, Spain (Department of Territory and Sustainability, General Directorate for Environmental and Natural Policy, project authorization code: 10183).

2.4 | Blood and Tissue Collection

At the end of the study, animals were fasted for 12 h and euthanized by decapitation. The blood was collected using heparin (Deltalab, Barcelona, Spain) as anticoagulant. Plasma was obtained by centrifugation (1500 × g, 15 min, 4°C) and stored at −80°C until analysis. Both the small and large intestine were dissected. Five-centimeter segments of the duodenum and colon were used for Ussing chamber assays. The rest of the tissue was rapidly removed, snap-frozen in liquid nitrogen, and stored at −80°C.

2.5 | Microbiota Data Preparation

The feces from 24 animals (young, $n = 6$; aged, $n = 9$; and GSPE, $n = 9$) were collected from the large intestine after sacrifice, snap-frozen in liquid nitrogen, and stored at −80°C. The genomic bacterial DNA was obtained from 200 mg of feces with the DNAeasy PowerSoil kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Partial 16S ribosomal RNA gene sequences were amplified from 20 ng of extracted DNA using three primer pairs, which target the V3, V4 from 16S rRNA, and ITS1 regions. Equimolar pools (60 pM) of each fragment were combined to create the DNA library, which was subjected to a clonal amplification by an emulsion PCR.

After an Ion Sphere Particle enrichment process, samples were loaded onto 530 chips and sequenced using the GeneStudio S5 (Life Technologies, Carlsbad, California, USA). The raw single-end reads from Miseq were processed and analyzed using QIIME2 (version 2019.11 https://docs.qiime2.org/2020.11/) pipeline.

The raw sequences were run with the denoise-single method from DADA2 pipeline with default parameters and specific trimming and truncating parameters (–p-trim-left 0 and –p-trunc-len 200) for the quality control and denoising step. This step included filtering, trimming, denoising, dereplicating, and removing singletons and chimeric sequences. Taxonomy for the resulting Amplicon sequence variants (ASVs) was assigned using a Naïve-Bayes model trained with the Greengenes database (gg_13_8) at 99% homology. A total of 5 704 619 filtered variants from 24 samples were classified into 2914 features. These annotated variants were then collapsed at the genus taxonomic level (Level 6) and were further analyzed using MicrobiomeAnalyst.

2.6 | Ussing Chamber Experiment

At the end of the experiment, rat intestine segments of the duodenum and colon were immediately placed in cold oxygenated Krebs buffer (KRB), dissected to remove fat and muscular layers, and placed in a 0.237 cm^2 aperture Ussing chamber (Dipl.-Ing. Mussler Scientific Instruments, Aechen, Germany). A total of 19 rats were used in this experiment: young ($n = 6$), aged ($n = 6$), and G GSPE ($n = 7$). First, apical and basolateral Ussing chamber compartments, representing intestinal lumen and blood circulation, respectively, were bathed with 2 mL of fresh KRB buffer containing 10 mM glucose (Panreac, Barcelona, Spain) for stabilization during 15–20 min. Each gut segment was mounted in duplicate. Bathing solutions were at pH = 7.4 and continuously bubbled with an O_2/CO_2 (95%/5%) gas mixture and circulated in water-jacketed reservoirs kept at 37°C.

2.6.1 | Electrophysiological Evaluation

Intestinal integrity and permeability were evaluated ex vivo by measuring the transepithelial electrical resistance (TEER). We used a four-electrode system coupled to an external six-channel voltage/current clamp electronic unit (Dipl.-Ing. Mussler Scientific Instruments, Aachen, Germany) to monitor the electrophysiological parameters in each Ussing chamber. We used one pair of Ag/Cl electrodes for measuring the potential difference (PD) and another pair for the current passage. The spontaneous transepithelial PD was measured under open-circuit conditions after appropriate correction of fluid resistance. TEER (Ω cm^2) was calculated every 30 min from the transepithelial PD and the short-circuit current in accordance with Ohm's law.

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2.6.2 | Paracellular Transport of Fluorescently Labeled Dextran

A 110 mg/mL stock solution of 4 kDa-fluorescein isothiocyanate-dextran (FD4; TdB Consultancy AB, Uppsala, Sweden) was prepared in phosphate-buffered saline. FD4 was added apically to each Ussing chamber at a final concentration of 5.6 mg/mL and incubated for 30 min. The quantity of FD4 that crossed the basolateral compartment was measured by a PerkinElmer LS-30 fluorimeter ([Beaconsfield, UK] at λ_{ex} = 485 nm; λ_{em} = 540 nm) and compared with a FD4 standard curve.

2.7 | Determination of Plasma Biochemical Parameters

Plasma LPS levels were quantitatively measured using an endotoxin detection system (Toxin Sensor, Genscript, New Jersey, USA) based on a Limulus Amebocyte Lysate (LAL) colorimetric assay. The manufacturer's protocol was followed in all cases. Commercial ELISA kits were used to quantify the plasma levels of intestinal fatty acid-binding protein (iFABP) (KifeSpan Biosciences, Seattle, Washington, DC, USA) and interleukin-6 (IL-6) (Thermo Scientific, Spain).

2.8 | Gene Expression Analysis

Total RNA was extracted from 50 mg of duodenum and both proximal and distal colon samples using Trizol (AMbion, USA) following the instructions as described previously by Gil-Cardoso et al. [35]. cDNA was obtained using the high capacity cDNA Reverse Transcription kit (Applied Biosystems, Madrid, Spain) following the manufacturer's instructions.

We performed quantitative real-time polymerase chain reaction (qPCR) amplification and detection in a qPCR system (CFX96 Touch-Real Time PCR, Bio-Rad, Madrid, Spain). All samples were run in duplicate in 96-well reaction plates. Gene expression of claudin-2 (*Cldn2*) (Rn02063575_s1), claudin-3 (*Cldn3*) (Rn00581751_s1), tight junction protein zonula occludens-1 (*Tjp1*) (Rn01420322_g1), and occludin-1 (*Ocel1*) (Rn01420322_g1) were performed using TaqMan Universal PCR Master Mix and Taqman probes (Applied Biosystems, Madrid, Spain). All the results were normalized with respect to cyclophilin E (*Ppia*) (Rn00690933_m1). Reactions with the TaqMan Universal PCR Master Mix were performed as previously described by Gil-Cardoso et al. [21].

Gene expression of the myosin light-chain kinase (*Mlck*), mucin-2 (*Muc2*), and cyclin-dependent kinase inhibitor p21 (*p21*) were carried out using Universal SYBR Green Supermix (Bio-Rad, Spain). We also normalized mRNA expression levels with respect to *Ppia* as an endogenous control gene. Primer sequences for the targeted SYBR rat genes are summarized in Table S1. Reactions with the iTaq Universal SYBR Green Supermix were performed using the following thermal profile: 30 s at 95°C, 40 cycles of 5 s at 95°C, and 30 s at 55–61°C (depending on specific annealing temperatures). The relative mRNA expression levels were calculated following the $2^{-\Delta\Delta Ct}$ method.

2.9 | Histological Assessment of Intestinal Samples

Proximal colon samples were added to a 4% formaldehyde solution for 24 h and transferred to a 70% ethanol solution until further paraffin inclusion. After that, 4-µm-thick tissue sections were prepared with a microtome. Alcian-blue/nuclear-fast red staining was performed using standard procedures. All tissue sections were analyzed under a light microscope to detect changes in mucus production and goblet cells architecture. ImageJ software (Java 1.8.0_172, NIH, Maryland, USA) was used to quantify the area of goblet cells as well as the crypt width and height. The goblet cell area (µm^2) was normalized to the total area occupied by the mucosal layer (µm^2). Twenty-five fields were measured on each sample.

2.10 | Quantification of Myeloperoxidase Activity

Tissue samples were homogenized with a Tissue Lyser LT system (Qiagen) in 50 mM potassium phosphate buffer (Panreac). The homogenate was centrifuged at 15 000 × *g* for 15 min at 4°C, and the resulting supernatant was discarded. The pellet was then homogenized with hexadecyltrimethylammonium bromide (Sigma-Aldrich) and 50-mM-potassium phosphate buffer. The homogenate was sonicated (20 s), subjected to three freeze-thaw cycles and centrifuged at 15 000 × *g* for 10 min at 4°C. The supernatant was mixed into a solution of phosphate buffer, 0.22% guaiacol (Sigma-Aldrich), and 0.3% H_2O_2 (Sigma-Aldrich), and absorbance was read at 470 nm. Enzyme activity was defined as the amount of MPO needed to degrade 1 µmol of H_2O_2 in 1 min (U = µmol/min) and was normalized to milligram of total protein content, which was measured using the Bradford method.

2.11 | Statistical Analysis

The data are represented as the mean ± standard error of the mean (SEM). Non-parametric statistical comparisons between groups were assessed by applying Kruskal–Wallis and Mann–Whitney U tests. Analyses were performed with XLSTAT 2021.03.1 (Addinsoft, Barcelona, Spain). p values < 0.05 were considered statistically significant.

3 | Results

3.1 | GSPE Produced Long-Term Changes on Colonic Microbiome From Aged Rats

We explored whether the metabolic effects of GSPE treatment could be linked to changes in the gut microbiota. Alpha diversity (within-sample diversity) relates both richness and evenness of the taxa analyzed in each rat. An initial analysis of colonic microbial alpha diversity at the phylum level revealed that there were some significant changes in their diversity (Shannon entropy, Kruskal–Wallis statistic: 5.26, p value: 0.072; Simpson's index, Kruskal–Wallis statistic: 7.85, p value: 0.020; Observed index, Kruskal–Wallis statistic: 2.20, p value: 0.335) (Figure 2A). These analyses show a decreasing tendency in the aged group whereas it is increased back in the GSPE group. Beta diversity

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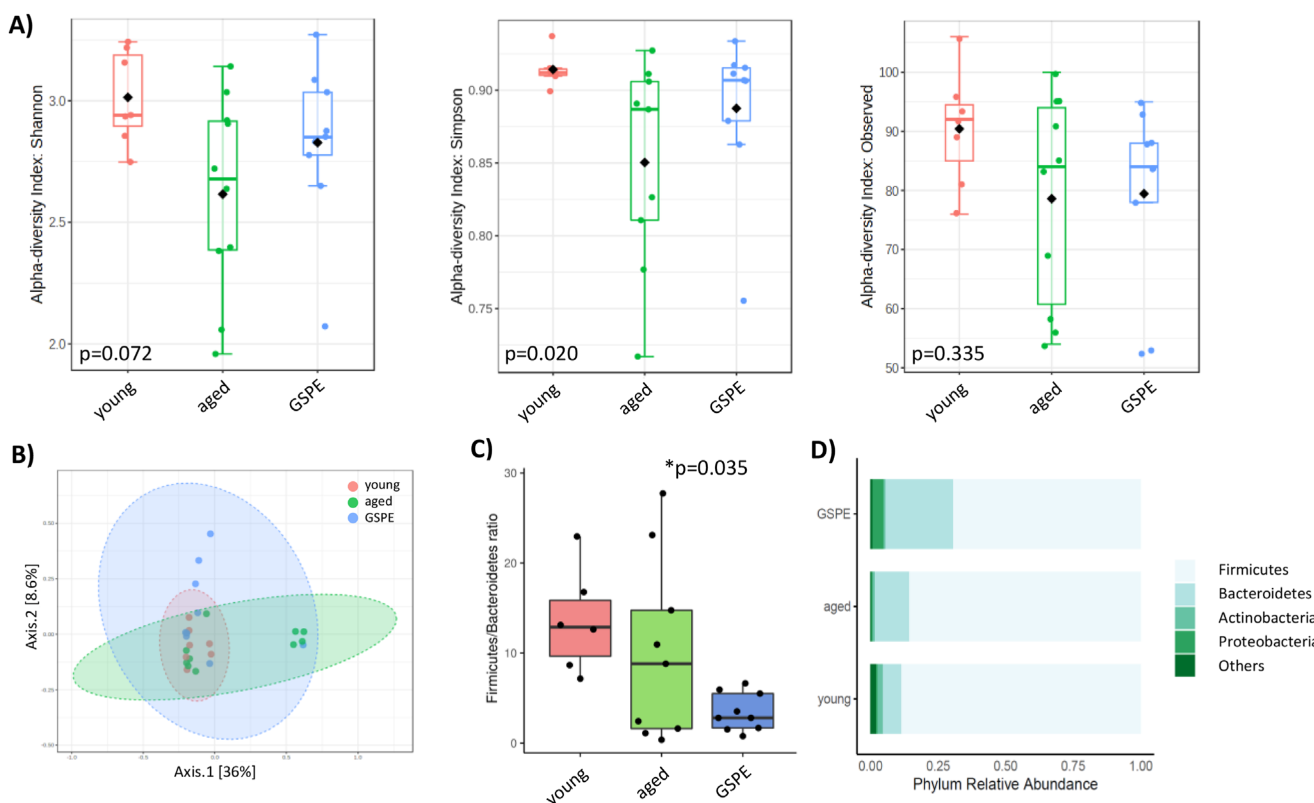

FIGURE 2 | Ageing and GSPE effect on gut microbiota composition. (A) Microbial alpha diversity (Featured level) across the different groups. Kruskal–Wallis *p* values are in the bottom-left corner. (B) Beta diversity PCoA plot based on the Bray–Curtis distance matrix of all groups. (C) The relative proportion of *Firmicutes* to *Bacteroidetes* ratio. (D) Composition of gut microbiota at the phylum level.

index, which refers to the change in taxa diversity between groups, showed that there were significant differences between their distribution (PCoA, Jaccard Index, [PERMDISP] *F*-value: 4.097; *p* value: 0.031) (Figure 2B). As shown in Figure 2D, all the groups were characterized by the dominance of the major phylum *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria*. However, the relative abundance (RA) of some major phyla differed between groups. *Firmicutes/Bacteroidetes* ratio was decreased in the GSPE-treated rats (Figure 2C), mainly due to an increase in *Bacteroidetes* (young = 6.9%; aged = 12.8%; GSPE = 25.1%) rather than a significant change in *Firmicutes* (young = 88.7%; aged = 85.8%; GSPE = 69.5%). *Actinobacteria* was decreased in both aged groups compared to young rats (young = 2%; aged = 0.8%; GSPE = 0.7%) and *Proteobacteria* was increased only in the GSPE group (young = 0.5%; aged = 0.4%; GSPE = 4.0%).

A deeper analysis at the genus level revealed significant differences in the microbiomes of the groups. Figure 3A shows that the most differentially abundant bacterial taxa in aged rats belonged to the genus *Bilophila*, an unclassified genus from the family Rikenellaceae, and another from the family Ruminococcaceae. Interestingly, the relative abundance (RA) of these unclassified genera was reduced in GSPE-treated rats.

On the other hand, *Roseburia*, *Coprococcus*, *Ruminococcus*, *Clostridium*, *Lachnospira*, *Collinsella*, and *Odoribacter* were among the genera most markedly decreased in aged rats compared to the young group. Indeed, the genus *Clostridium* was enriched in the GSPE group, along with *Bacteroides*, *Sutterella*, and *Bifidobacterium*. Heat tree analysis confirmed the community differences between the groups (Figure 3B). Notably, *Desulfovibrionaceae* were more abundant in aged rats than in the young and GSPE-treated groups. In contrast, *Butyricimonas* were less abundant in aged rats than in GSPE-treated animals.

Finally, we analyzed the core microbiome of each group to better understand the global changes occurring (Figure S1). In addition to the previously mentioned genera, we found that *Roseburia* had a higher prevalence in the GSPE group compared to aged rats and that *Prevotella* and *Akkermansia* were present in the core microbiome of GSPE-treated rats but absent in the aged rats.

To investigate the co-occurrence of genera that specifically define the GSPE effects, we performed a Spearman correlation network, which also depicted the relative abundances of genera in the different groups (Figure S2A). The genus *Bacteroides*, which was differentially increased in the GSPE group, showed a negative correlation with *Dehalobacterium* and a strong positive correlation with *Sutterella* from the phylum Proteobacteria. Roseburia, Coprococcus, and Ruminococcus from the phylum Firmicutes, which were decreased in aged rats, were positively correlated with each other. Lastly, Akkermansia, a genus present only in the core microbiome of GSPE-treated rats, was negatively associated with Faecalibacterium, Dorea, and Gemmiger from the phylum Firmicutes (Figure S2B).

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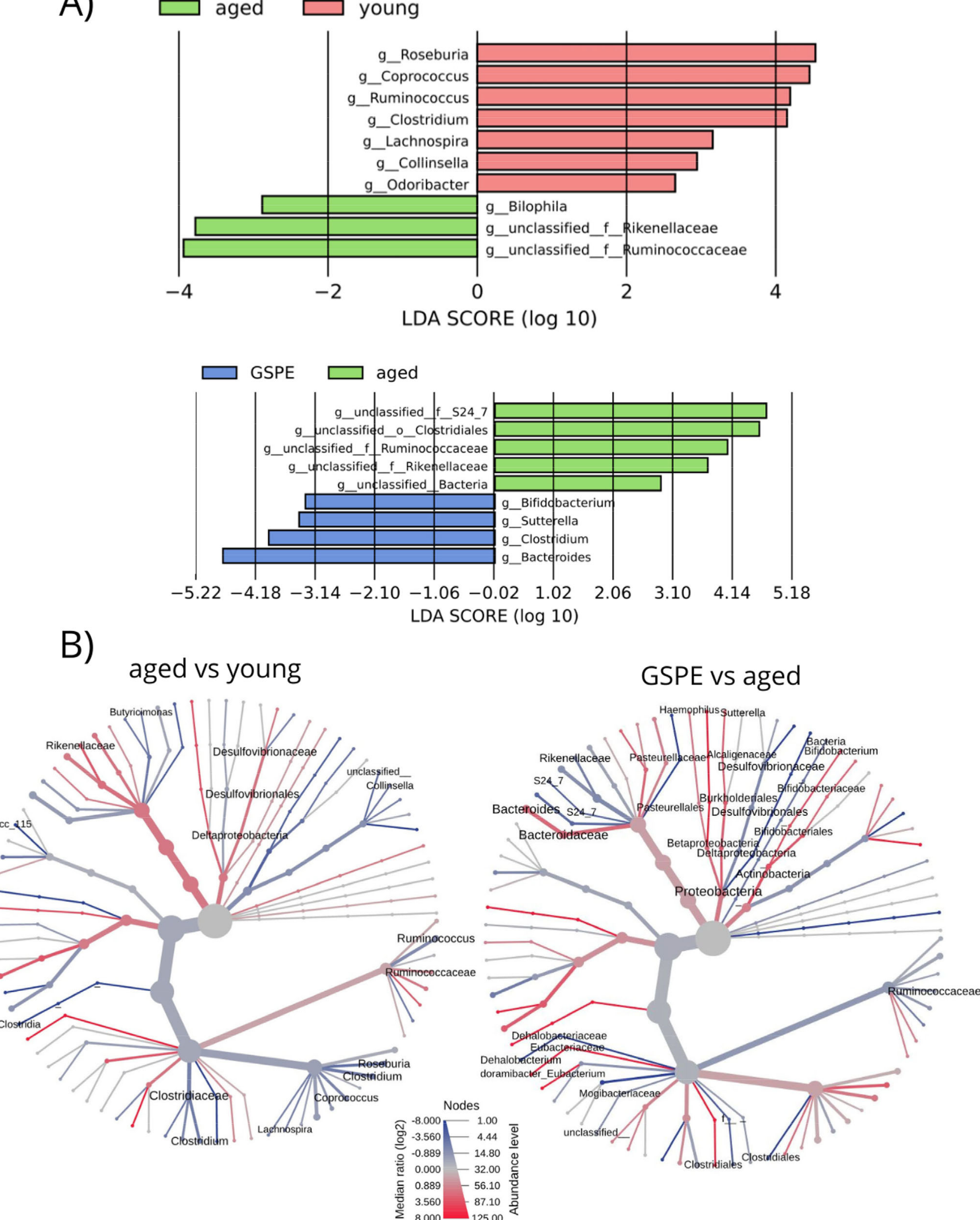

FIGURE 3 | Microbiota analysis at the genus level in the different groups analyzed. (A) The bar graph of LDA score calculated by LEfSe ranked according to the effect size and associated with the genus with the highest median value. This analysis was calculated in LEfSe using the default settings (alpha value of 0.05). (B) Heat tree visualization of taxonomic differences between the two selected groups, aged versus young (left panel) and GSPE versus aged (right panel). The color gradient and the size of node, edge, and label are based on the log2 ratio of median abundance. Blue and red indicate that corresponding taxa are lower and higher, respectively.

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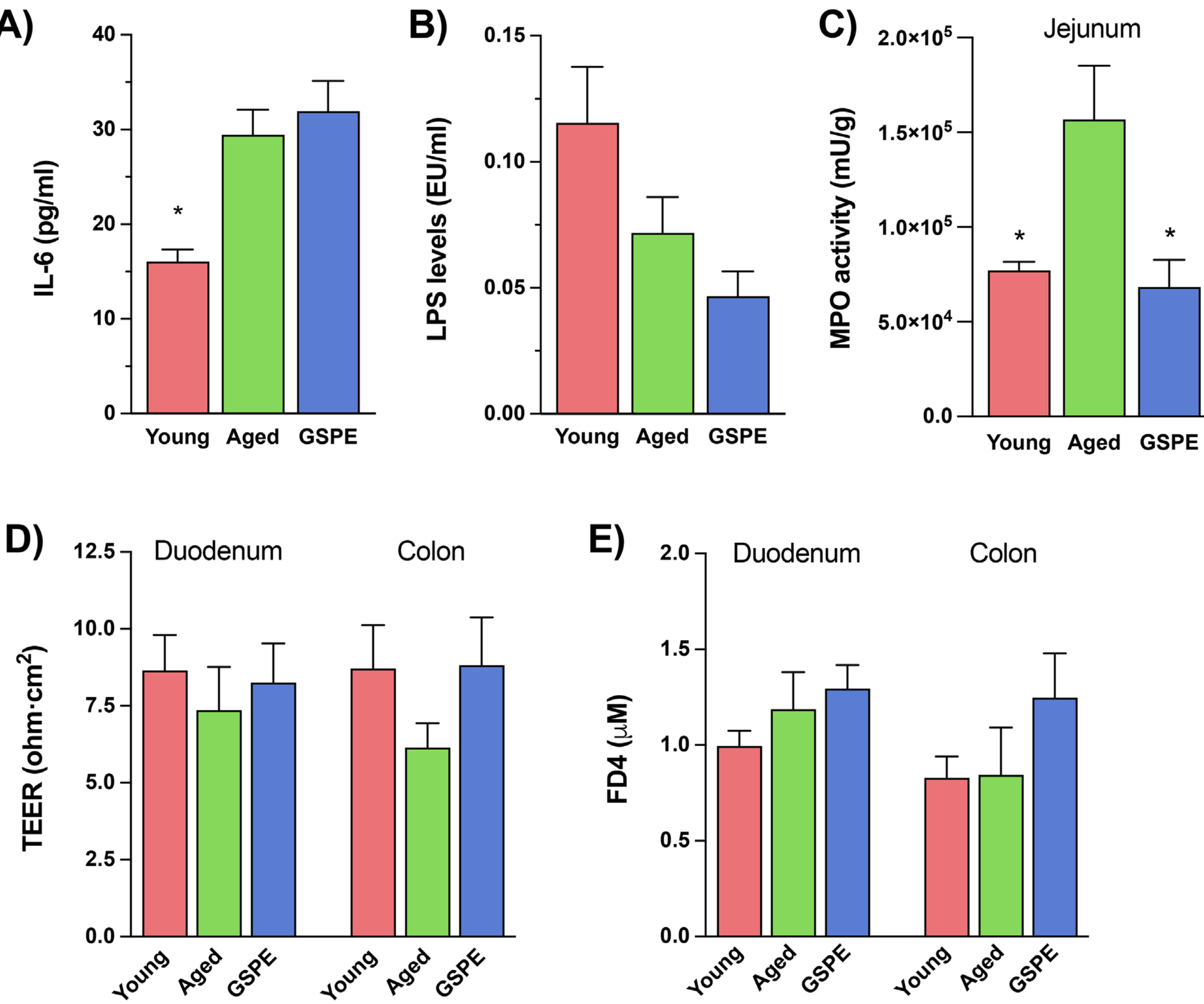

FIGURE 4 | Systemic inflammation & intestinal permeability assessment. (A-B) IL-6 and endotoxin levels in plasma. (C) Myeloperoxidase (MPO) activity from jejunum used as neutrophile infiltration marker. (D) Evaluation of TEER values and (E) quantification of FD4 transport across the intestinal barrier in duodenal and colonic mucosal preparations mounted in an Ussing chamber system. Values are represented as the means ± SEM. $^{*}p < 0.05$ compared to the aged group.

3.2 | GSPE Preventive Treatment Ameliorates Some Aspects of the Aging Phenotype

As previously reported [26], aged rats presented increased visceral adiposity and altered glucose and lipid homeostasis (Table S2). Fasting insulin levels and HOMA indexes were increased in this group. Also, triglyceride and cholesterol levels were higher in aged rats, altogether showing a typical human aging phenotype. GSPE preventive treatment ameliorated some of these aging traits. It reduced insulin levels and the HOMA-B index indicating a better management of glucose homeostasis and consequently, reduced insulin resistance. We found that associated to the ameliorated systemic insulin sensitivity, visceral adiposity was significantly reduced in GSPE-treated rats (Table S2). Finally, IL-6 concentration in the plasma was determined as a biomarker of systemic inflammation. Aged animals exhibited significantly higher IL-6 levels compared to young animals (Figure 4A), indicating a certain degree of inflammaging. However, GSPE had no effect on IL-6 levels.

3.3 | Aging Does Not Alter Gut Epithelial Integrity, Morphology, or Senescence Markers, but Modifies Neutrophil Infiltration in the Jejunum

The levels of circulating LPS were measured to assess endotoxin intestinal translocation. We did not observe any significant change among groups; however, LPS levels were generally lower in aged rats (Figure 4B). Interestingly, GSPE tended to reduce LPS levels. MPO activity, measured as an indicator of neutrophil infiltration, was increased in the jejunum of aged rats and reduced in the GSPE group (Figure 4C). No changes were found in the colon of these animals (data not shown).

Segments of the duodenum and colon were harvested for ex vivo assessments. Intestinal integrity was evaluated through measurements of electrical resistance (TEER) and the translocation of macromolecules (FD4) into the basolateral compartment using Ussing chamber equipment. Surprisingly, no loss of integrity associated with aging was observed when comparing young and

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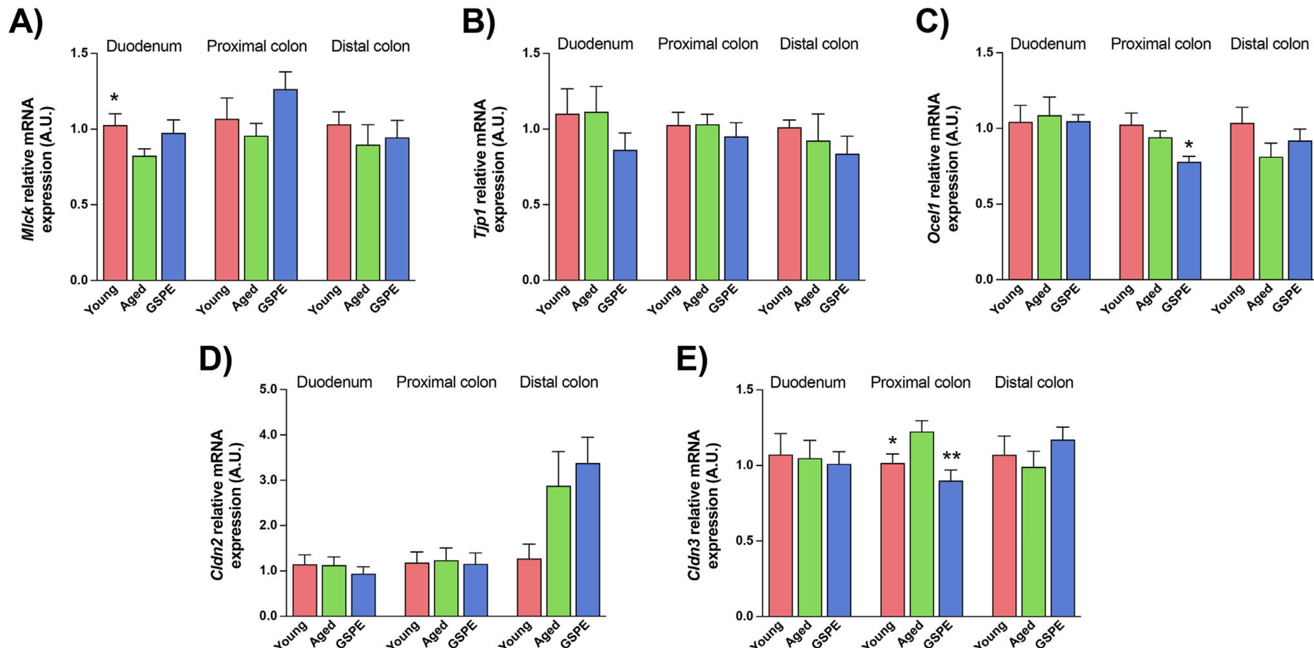

FIGURE 5 | Gene expression of *Mlck* and TJ-related genes in the duodenum, proximal and distal colon. The expression of target genes was normalized to Cyclophilin A gene expression (*Ppia*). Values are represented as the means ± SEM. *$p < 0.05$ compared to the aged group; **$p < 0.005$ compared to the aged group.

aged rats. No changes in the measured parameters were observed with any treatment of GSPE (Figure 4D,E).

Tight-junctions, consisting in claudins, zoonulins, occludins, and JAM proteins, are related to the translocation of macromolecules through the intestinal paracellular pathway. TJ opening is mainly controlled by myosin light-chain kinase (MLCK) [36]. In this study, we assessed the effects of aging and GSPE supplementation on *TJ* and *Mlck* gene expression in the duodenum, proximal colon, and distal colon. Aging induced a significant decrease in *Mlck* gene expression in the duodenum (Figure 5A), and GSPE, tended to reverse this condition (Figure 5A). In general, *TJ* gene expression was not affected by either age or GSPE treatment, with the exception of *Ocel1*, whose expression was reduced in the proximal colon of the GSPE-treated group, and *Cldn3*, which showed significantly higher expression in the proximal colon of animals in the Aged group (Figure 5B–E).

The mucus layer is also crucial in maintaining the intestinal barrier homeostasis and protecting against gut permeability. Goblet cells of intestinal mucosa secrete gel-forming proteins, mostly Muc2. In the ileum GSPE treatment significantly increased the expression of *Muc2* (Figure 6A). Conversely, in the distal colon, age significantly increased *Muc2* gene expression while GSPE treatment showed no effects (Figure 6A).

We also performed a histological analysis of intestinal samples from distal colon. First, we quantified the area of goblet cells per area of tissue. In line with the results of *Muc2* gene expression, the number of goblet cells was not changed by age nor the treatment with GSPE (Figure 6B). As shown in the histological pictures (Figure 6C), we did not find morphological differences neither in the density of goblet cells among the different experimental groups.

The cyclin-dependent kinase inhibitor *p21* is a gene expressed in senescent cells that causes growth arrest. We measured *p21* gene expression in the duodenum, proximal colon, and distal colon. No changes were observed in the duodenum and proximal colon (Figure 6D).

Intestinal fatty acid-binding protein (iFABP) is a widely used biomarker for assessing intestinal mucosal damage. In this study, we evaluated the iFABP concentration in the plasma. Although the difference was not significant ($p = 0.102$), the iFABP concentration was clearly higher in the aged group compared with the young group (Figure 6E).

4 | Discussion

Aging is characterized by the gradual deterioration of the body's ability to maintain homeostasis, resulting in a decline in physiological functions and an increased vulnerability to mortality. This process is associated with various age-related conditions, including infectious, neoplastic, metabolic, and degenerative diseases, along with frailty and cognitive decline. In our research, we have focused on the decline of gut function, emphasizing the gut barrier and microbiota profiling as critical components of homeostasis maintenance throughout aging. For this purpose, we have used an experimental animal model that successfully replicates some metabolic disruptions observed in humans during the aging process [37]. In aged rats under a standard chow diet, we observed insulin resistance, dyslipidemia, and inflammaging [26].

The gut microbiome composition also changes as the host ages. It acts as a mediator of environmental signals and has an impact on the risk of developing diseases at all stages of life.

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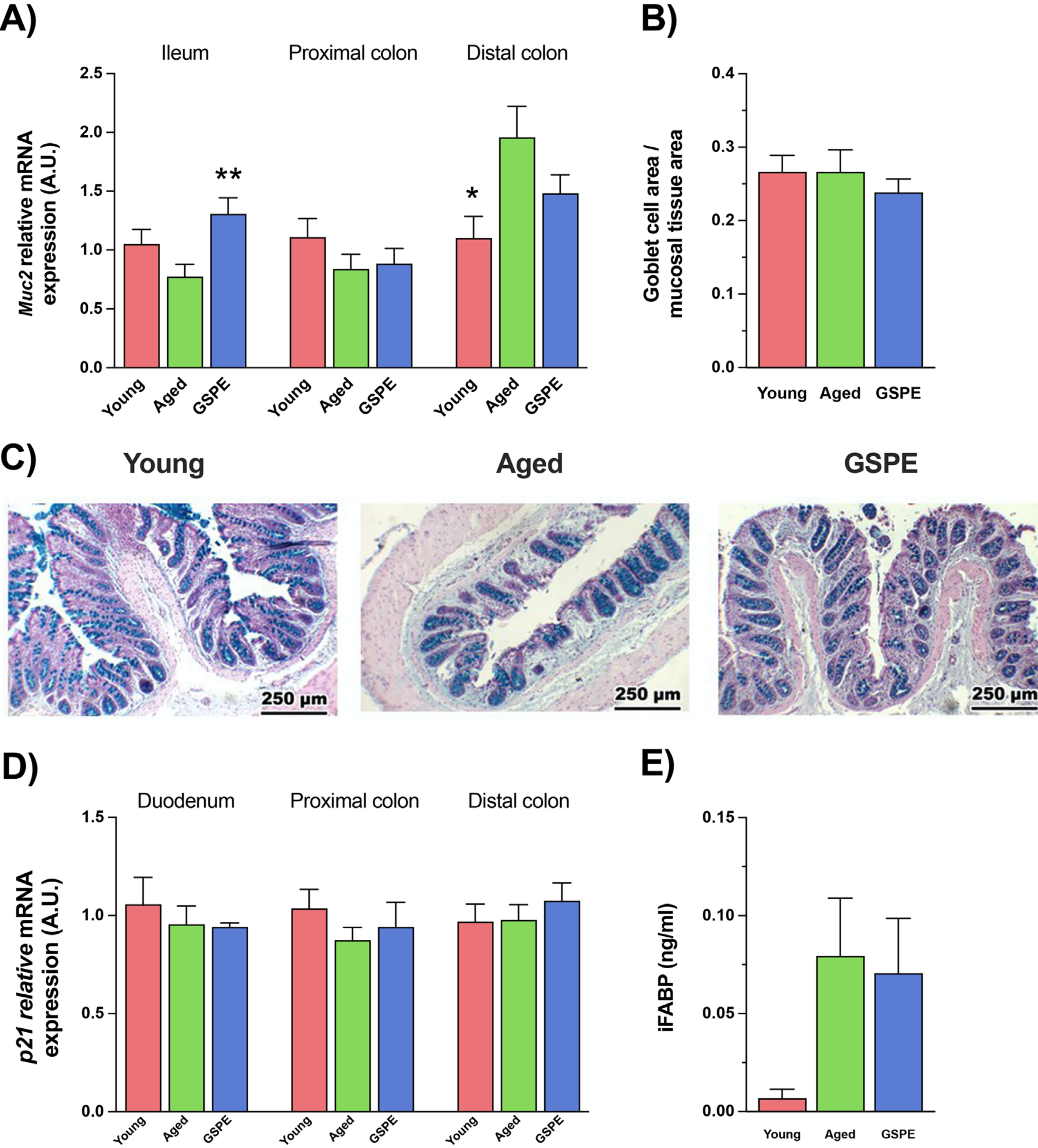

FIGURE 6 | Mucus production, senescence and damage of the intestinal mucosa. (A) Gene expression of *Muc2* in ileum, proximal, and distal colon. The expression of the target gene was normalized to cyclophilin A gene expression (*Ppia*). (B) Goblet cell quantification in distal colon. (C) Alcian Blue-Nuclear Fast red staining of representative histological sections of distal colon. Scale bar = 250 μm in all pictures. GSPE, grape seed proanthocyanidin extract; *Muc2*, mucin-2. (D) Gene expression of p21 as a biomarker for intestinal cellular senescence in duodenum, proximal and distal colon. (E) Plasma iFABP concentration. Values are represented as the means ± SEM. *$p < 0.05$ compared to aged group.

These changes in the microbiome are highly individualized and influenced by both personal and external environmental factors. It should be noted that there is no single, universally accepted configuration of a healthy microbiome. However, studies have shown that there are notable differences in the gut microbiota between frail and healthy older adults [38, 39]. Ghosh et al. [39] recently reported that frail elderly microbiomes are primarily characterized by loss of commensals that are reduced with aging in general (i.e., *Faecalibacterium*, *Roseburia*, *Coprococcus, Eubacterium rectale, Bifidobacterium*, and *Prevotella*) and a gain of pathobionts (such as *Eggerthella*, *Desulfovibrio*, *Enterobacteriaceae* family members, disease-associated *Clostridium* species,

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and *Ruminococcus torques*). By contrast, *Akkermansia*, *Odoribacter*, *Butyricimonas*, *Butyrivibrio*, *Oscillospira*, *Christensenellaceae*, and *Barnesiellaceae* increase in abundance in a healthy aging trajectory but are lost when there is a shift to a state of physiological decline. Our experimental model showed an increase in the genera *Rikenellaceae*, *Bilophila*, and *Desulfovibrio* in aged rats, while genera as *Butyricimonas*, *Roseburia*, *Ruminococcus, Odoribacter*, or *Coprococcus* were decreased. Globally, these changes in the gut microbiome of the aged rats closely resemble those observed in humans during the aging process [39]. It is important to highlight that we raised healthy, conventional animals in open cages for nearly 4 months under controlled, health-monitored conditions, allowing for natural antigenic exposures and indigenous gut flora, mimicking a microbiota closer to adult humans [40].

After defining a solid experimental model of human aging, we aimed to investigate a potential anti-aging treatment. Current treatments include nutritional and lifestyle interventions and some drugs. While senolytic drugs, which target senescent cells, have been shown to improve age-related functional decline and reduce inflammation [37], the most effective anti-aging treatment remains caloric restriction [41]. This has been demonstrated to extend lifespan and delay the onset of age-related diseases in various model organisms. Additionally, metformin, a drug commonly prescribed for type 2 diabetes, has anti-aging effects by its inhibition of signaling through the mTOR pathway and reducing oxidative stress [42, 43]. It is worth noting that these anti-aging treatments are generally applied after a disease or condition has already developed. Our approach to anti-aging treatment takes a more comprehensive healthcare strategy by utilizing a preventive treatment prior to the onset of metabolic decline in the old adults. For patients, a preventive treatment reduces the chance of developing a condition, avoiding pain, discomfort, and expense associated with corrective treatments. For healthcare, it leads to cost savings, reduces hospitalization and improves overall population health, and reduces the burden of chronic diseases.

The effects of proanthocyanidins on gut microbiota and metabolic health in young models suggest their potential to mitigate age-related metabolic disorders, such as dyslipidemia, insulin resistance, and fat accumulation [44–50]. However, few studies focus specifically on aging populations. By examining gut barrier function and microbiome composition, we gained a comprehensive view of GSPE's impact on gut health and aging. In this study, 21-month-old female rats (equivalent to 50–55 years in humans [51, 52]) were treated with GSPE. Previous findings showed that after 4 months, GSPE improved insulin sensitivity and reduced visceral fat [26], and this study aims to further explore these long-term effects.

Aged rats treated with GSPE showed significant improvements in gut microbiota, with a reduction in bacteria associated with unhealthy aging, such as *Desulfovibrionaceae* and Rikenellaceae, and an increase in beneficial bacteria like Bacteroides, Bifidobacterium, and *Butyricimonas*, which are linked to a healthier state [39]. Furthermore, we found that GSPE changed microbiota composition toward the young profile, even restoring lost strains in aged rats as *Prevotella* [39]. Ghosh et al. [39] reviewed the dynamic relationship between the gut microbiome and aging in humans, highlighting how microbial composition shifts significantly with age, having important implications for age-related diseases. They emphasize the reciprocal influence between the host and microbiota as follows: while aging alters the microbiome, these microbial changes can accelerate physical and cognitive decline. In line with our findings, Ghosh et al. described a decreased abundance of commensal bacteria such as Roseburia, *Faecalibacterium*, and Coprococcus in older populations, all of which are typically associated with healthy gut function and the production of short-chain fatty acids (SCFAs). These SCFAs possess anti-inflammatory properties and are crucial for maintaining gut barrier integrity, which is essential for overall health during aging [39].

The study by Sheng et al. [53] further expands on these findings, demonstrating that GSPE not only alters the microbiota but also improves oxidative stress and inflammation through the gut–brain and gut–liver axes [53] . Consistent with our results showing a reduced neutrophile infiltration in the jejunum, their mouse model of D-galactose-induced aging revealed that GSPE significantly increased the abundance of beneficial bacteria like Bifidobacterium and Akkermansia, which are known to promote gut barrier integrity and reduce inflammation, thus preventing systemic metabolic disturbances such as dyslipidemia and insulin resistance. These microbial changes were associated with decreased pro-inflammatory markers and oxidative stress in both the liver and brain, suggesting a broader systemic effect of GSPE in mitigating age-related metabolic diseases [53].

Rubio et al. [54] add further support by demonstrating that aged Wistar rats exhibit increased fat deposition and gut barrier deterioration, both linked to changes in gut microbiota, including an increase in mucin-degrading bacteria that weaken gut integrity [54]. GSPE treatment, by restoring beneficial bacteria, may help reinforce the gut barrier and reduce endotoxemia, thereby alleviating insulin resistance and metabolic dysregulation.

Previous studies from our group in young obese rats demonstrated that a cafeteria diet led to increased gut permeability, contributing to metabolic endotoxemia and intestinal inflammation [21, 22, 27]. Similar findings have been reported in other animal models of diet-induced obesity, where gut permeability is also elevated [55]. However, the impact of aging on gut permeability remains poorly understood, and the current literature presents conflicting evidence. In our animal models, aging did not result in significant adverse effects on either small or large intestinal barrier function. This aligns with findings by Wilms et al., who reported no significant differences in gut permeability between healthy young adults and the elderly, whether examining the small intestine, colon, or whole gut [56, 57]. Similarly, Valentini et al. [58] observed that the small intestinal mucosal barrier in humans does not deteriorate with aging per se, but rather, is compromised by low-grade inflammation in combination with conditions such as type 2 diabetes. In contrast, other studies suggest an age-related decline in barrier function, with old non-human primates exhibiting higher gut permeability compared to younger ones [59]. Comparable results were observed in aged rats and mice, where intestinal dysfunction was noted [60–62]. However, these studies utilized specific pathogen-free (SPF) animals, which are typically raised under germ-free conditions and, more importantly, kept in pathogen-free environments [63,

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64]. Under such conditions, rodents are not exposed to a lifetime of pathogens and antigens, lack a typical microbiota and may experience dysbiosis, potentially influencing their barrier function [65]. In fact, Dobson et al. [40] discussed the pros and cons of maintaining the specific pathogen-free (SPF) environment for animals in biomedical research and presented individual cases where altering the gut microbiome has dramatically changed the animal's basic physiology, immune/inflammatory functions, and susceptibility to infection and disease [63]. Taken together, these findings underscore the importance of determining whether the observed changes in barrier function are solely a consequence of the normal aging process or instead represent features of unhealthy aging, marked by low-grade inflammation and dysbiosis.

Although this study was conducted in an animal model, the findings may have relevant implications for the prevention of intestinal dysfunctions associated with aging in humans. The dose used (500 mg/kg body weight) was selected based on previous studies from our group showing its effectiveness in improving intestinal and metabolic parameters in young rats [23, 66]. Applying the body surface area normalization method proposed by [67], this corresponds to a human equivalent dose (HED) of approximately 5 g/day for a 60-kg adult. While this dose is relatively high, it is noteworthy that commercial formulations of grape seed extract containing 500 mg per capsule are currently available, which may facilitate its use in a clinical setting. Moreover, as recommended for translational studies, the HED can initially be reduced by a safety factor of 10 to determine a conservative starting dose in humans [67]. Based on this, an initial dose of 500 mg/day would be both feasible and safe, allowing for a gradual escalation depending on tolerability and efficacy outcomes. In support of this, a clinical study reported that oral intake of up to 2500 mg/day of GSPE for 4 weeks was generally safe and well tolerated in healthy humans [68]. In addition to this approach, our previous work in young and aged obese rats [24, 69] demonstrated that short-term, intermittent administration of GSPE produced long-lasting metabolic and intestinal benefits. Therefore, an intermittent treatment strategy may also be considered in humans to reduce the need for continuous high-dose supplementation, while preserving the protective effects. Taken together, these considerations support the translational potential of GSPE as a dietary or nutraceutical intervention aimed at preserving intestinal health during aging.

This study has some limitations. The findings of this study were obtained from female rats. It is possible that male rats may exhibit different outcomes, considering the reported sex differences in gut microbiota observed in animal studies [70]. Although we have previously evaluated the composition of the GSPE [71], working with a procyanidin extract instead of pure molecules has limitations such as variable bioavailability, and difficulty in determining precise dosing and mechanism of action. Finally, further research should prioritize the investigation of the metabolites derived from the degradation of proanthocyanidins, its mode of action, and the potential divergent outcomes between genders. In conclusion, our findings underscore the potential of GSPE preventive anti-aging therapy with long-lasting benefits that address the altered gut microbiome in elderly individuals. By modulating the gut microbiota, GSPE offers a promising approach to restoring metabolic balance and promoting healthier aging. These insights contribute to the growing body of evidence that targeting the gut microbiome is a viable strategy for preventing or reversing metabolic decline in aging populations. Further studies are warranted to more clearly link GSPE-induced changes in microbiota with the metabolic improvements observed.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon request.

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