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Original Article

Aging in Male Wistar Rats Associates With Changes in Intestinal Microbiota, Gut Structure, and Cholecystokinin-Mediated Gut–Brain Axis Function

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Abstract

Aging in mammals is characterized by failure of the homeostatic mechanisms that regulate energy balance. Several mechanisms have been proposed such as the presence of a low-grade chronic inflammation in different tissues, as well as leptin and insulin resistance, but the primary alteration is not fully elucidated. The gut microbiota has recently emerged as a key player in a variety of metabolic and neurological disorders. A main concept in this context is the gut–brain axis that refers to alterations in the gut that mediate effects in the central nervous system, including those related with the control of energy balance. Using 16S rRNA analysis, we demonstrate that aged male Wistar rats have increased presence of mucin-degrading and lipopolysaccharide (LPS)-producing bacteria. In addition, old animals exhibit a lower number of neutral mucin secreting goblet cells, and a decrease of tight junctions and adherens junctions marker proteins, zonula occludens protein-1 (ZO-1) and β-catenin, respectively. These data are compatible with a thinner mucus layer and a weaker gut barrier in older animals that likely facilitate LPS leakage. Our data also show that cholecystokinin (CCK) satiating effect is impaired in aged rats, one of the expected effects of increased LPS leakage. In contrast, no overt signs of gut or systemic inflammation are observed. Changes in microbiota in old male Wistar rats present features of situations of increased adiposity, but different from those of obese animals. These could partly explain the increased adiposity and fat deposition in liver and heart as observed here.

Keywords: Adiposity, Gut bacteria, Mucin, Satiety

During the last decade, growing evidence has demonstrated a relevant association of intestinal microbiota with obesity, type 2 diabetes, and the metabolic syndrome. Thus, conventionalizing of germ-free mice results in an increase of fat mass despite lower food intake (1). Moreover, fecal transplant from lean donors improves insulin sensitivity in patients with metabolic syndrome (2) and transplant of microbiota from discordant twins for obesity to germ-free mice induces a differential increase of their respective body weights in agreement with the obese phenotype of the donor (3).

Changes in intestinal microbiota composition could be responsible for several effects such as impairment of the gut barrier integrity, endotoxemia, and presence of inflammation in the gut and other peripheral tissues that, in turn, might lead to development of insulin resistance and lipid deposition in different organs (4). The emerging concept of the microbiota–gut–brain axis postulates that alterations in the microbial populations of the intestine also influence neurophysiological-governed behaviors including those related with appetite control and energy balance (5).

Aging in rodents and humans is characterized by insulin resistance, fat accretion, and increased body weight (6). Studies in rats have clearly shown an alteration of homeostatic energy balance mechanisms including the impaired anorexigenic hypothalamic action of leptin and insulin (7–9). Although many studies in different models of obesity and insulin resistance have explored alterations in gut microbiota and structure, little information is currently available from aging models that partially share metabolic characteristics such as increased adiposity and lower insulin sensitivity. Moreover, there are scarce evidences about changes on gut–brain axis mechanisms involved on energy homeostasis in aged rats.

In this work, we explore, in a previously well-characterized model of aging in rats, changes in the fecal microbiota composition using 16S rRNA analysis. In addition, we study the alterations in the gut structure, inflammation, and barrier integrity. Our data suggest a possible influence of altered microbiota and gut barrier integrity on the satiety effects of cholecystokinin (CCK) mediated by lipopolysaccharide (LPS) leakage (10). Therefore, we also analyzed CCK satiating effect in aged male Wistar rats to gain insight on possible alterations of the gut–brain axis.

Materials and Methods

Animals

Four- and 24-month-old male Wistar rats (from our in-house colony, Centre of Molecular Biology Severo Ochoa, Madrid, Spain) were used. Animals were fed standard diet (Harlan 2014 2.9 kcal/g; 4% fat; 14.3% protein) throughout their life span. Handling of animals was performed according to European Union laws and the guidelines of the National Institutes of Health (United States). The institutional committee of research ethics and regional authorities approved the experimental protocols (PROEX Number 051/14).

Microbiome Analysis

Fecal content was freshly collected from individual animals, weighted, and frozen at −80°C until analysis. Genomic DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. Homogenization (10 seconds at a speed setting of 5.5) was performed using a FastPrep Instrument (MP Biomedicals). DNA concentration was measured using the NanoVue plus system (GE Healthcare Life Science) followed by dilution of all samples to a final concentration of 100 ng/µl. 16SV3V4-Fw: ACACTGACGACATGG TTCTACACCTACGGGNGGCWGCAG and 16SV3V4-Rv: TAC GGTAGCAGAGACTTGGTCTGACTACHVGGGTATCTAATCC primers were used for PCR amplification of the V3-V4 hypervariable regions of 16S rRNA genes (11). Amplicons were mixed in a single tube and after removing the amplification primers, amplicons were sequenced using the Illumina-Myseq system. An average of 160.000 readings per sample were obtained. Paired reads were joined using PandaSeq Assembler. Only sequences ranging from 300 to 460 bp were considered. Operational taxonomic units (OTUs) were determined using the Quantitative Insights Into Microbial Ecology (QIIME) program and Green Genes reference sequences database. α-Diversity was calculated by the Shannon index. β-Diversity was analyzed using the Unweighted UniFrac method and visualized by Principal Coordinates Analysis (PCoA).

The Miseq sequences derived from the 16S profiling were deposited in the European Nucleotide Archive and are available under the following accession number: PRJEB13754.

Colon Mucosal Integrity Evaluation by Light Microscopy

Samples of distal colon were paraffin embedded and dehydrated with ethanol and *n*-butanol for 48 hours at 4°C. Five-micron-thick sections were obtained using a standard microtome (Microm) and mounted on silanized glass slides.

Colonic expression of neutral and acid mucins was determined following the PAB (PAS-Blue Alcian) histochemical technique (see "PAB Histochemical Technique" section in Supplementary Material). The neutral mucins were stained in magenta, while the acid mucins were stained in blue. Overall neutral and acid mucin scores were obtained using the Fiji version of ImageJ software (http://fiji.sc/Fiji). The results correspond to the optical density of the surface from magenta and blue colors. The wavelengths of the 2 colors were discriminated before their quantification. Data are expressed in arbitrary units (A.U.).

Determination of ZO-1 by Immunofluorescence Staining

Immunofluorescence staining of ZO-1 was performed on deparaffinized and rehydrated colonic sections as previously described (12). Rabbit anti-ZO-1 (1:50 dilution) was used as primary antibody (Invitrogen, ThermoFisher Scientific Inc.). After washing, the slides were probed with a 1:100 dilution of Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody and a 1:1000 dilution of 10 mg/ml of DAPI solution (both from Invitrogen, ThermoFisher Scientific, Inc., Waltham, MA). Images for quantification were taken with a confocal microscope LSM 710 (Zeiss, Oberkochen, Germany). The intensity of the area stained was measured using the Fiji version of Image J software. Two different colon sections of 4 young and 4 old rats were evaluated.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism v5. Unpaired 2-tailed Student's *t* test and Mann–Whitney *U* test were used to do pairwise comparisons in samples with normal or nonnormal distribution of data, respectively. Shapiro–Wilk test was run to determine if the samples showed a parametric distribution. Two-way ANOVA with repeated measures, with post hoc Bonferroni test, was employed to compare the satiating effect of

CCK in young and old animals. The data are presented as the mean ± SEM. Differences were expressed by reporting p values. A threshold value of $p < .05$ is considered statistically significant. Nevertheless, following recent suggestions on statistical significance (13), we also discuss differences with a p value slightly higher than .05.

Results

Changes in Adiposity and Body Composition With Aging

Aged rats exhibit 1.9-fold increase in body weight (Supplementary Table 1) reflecting total fat accretion (4-fold), but also a 1.4-fold increment in lean body mass (LBM) (Supplementary Figure 1A and B). Fat increase is also observed in subcutaneous and visceral depots both when expressed as total fat or relative to body weight, whereas the proportion of LBM decreases with aging (Supplementary Figure 1C). Thus, fat increase is higher than that of LBM as indicated by the fat mass/LBM ratio (Supplementary Figure 1D). Analysis of ectopic lipid deposition shows an increase of triglyceride (6.3-fold) and cholesterol (2.6-fold) content in the liver. Likewise, in cardiac muscle, an increment of 1.4-fold in triglyceride and cholesterol is observed with aging (Supplementary Table 1).

Analysis of Fecal Microbiota in Young and Old Rats

16S rRNA analysis indicates that the number of OTUs, the richness, and evenness determined by the Shannon index and the β-diversity of microbial communities are not different between young and old animals (Supplementary Figure 2A–C). Nevertheless, fecal profiling reveals significant changes in microbiota composition at different taxonomic levels (Supplementary Figure 2D and Table 1). As expected, taxa from the phyla Firmicutes and Bacteroidetes represent around 95% of all species. Taxa from phylum Verrucomicrobia increase significantly with aging, whereas those from Bacteroidetes are less abundant in older rats. In addition, the less abundant phyla Proteobacteria and TM7 decrease significantly in aged animals (Supplementary Figure 2D and Table 1).

Some genera of gram-negative bacteria that are recognized for their capacity to produce LPS (*Bacteroides*, *Prevotella*, and *Odoribacter*) increase in aged rats. In addition, anti-inflammatory bacteria from the genus *Lactobacillus* decrease in old animals, whereas bacteria from the families Turicibacteriaceae and Ruminococcaceae, usually associated with gut inflammation and bowel disease (14), are increased (Table 1).

Our analysis also identifies a lower presence of genera *Clostridium* (family Clostridiaceae) and *Coprococcus* (family Lachnospiraceae) with aging, although the differences show a p value slightly over the significance threshold ($p = .056$ and $p = .055$, respectively). Both genera include beneficial butyrate-producing bacteria characterized by their contribution to the maintenance of gut epithelial barrier integrity. Concerning mucin-degrading bacteria, in addition to the increase of phylum Verrumicrobia, a higher presence of family Ruminococcaceae is observed with aging (Table 1).

Effect of Aging in Markers of Colonic Epithelial Integrity and Inflammation

Colonic epithelial integrity depends mainly on the presence of tight junctions and adherens junctions proteins (15), as well as the thickness of the mucus layer of mucin secreted by goblet cells. Immunofluorescence (Figure 1) and immunohistochemistry (Supplementary Figure 3A and B) of distal colon samples show that ZO-1 and β-catenin, markers of tight junctions and adherens junctions, respectively, decrease in old rats. Measurements of the 2-dimensional planar sections of the colon showed a significant reduction with aging in the colonic crypt width, without changes in the crypt depth (Supplementary Figure 3C).

Figure 2A shows the presence of goblet cells expressing neutral (magenta) and acid (blue) mucins in distal colon from young and old rats. The study of the colonic sections at higher magnification (20×) revealed that in the aged group, the number of goblet cells per gland decreases when compared with the young group (Figure 2A and B). The study of the intensity of mucins using ImageJ program reveals a reduction of neutral mucins without changes in acidic mucins with aging (Figure 3).

Concerning inflammation, we do not observe an increase in leukocyte infiltration in the mucosa of aged rats with respect to young animals (Figure 3 and Supplementary Figure 3). Moreover, serum levels of several markers of inflammation such as C-reactive protein (CRP), IL-1α, IL-1β, IL-6, IL-10, MCP-1, TNF-α, and AF-1 are similar in young and old rats (Supplementary Table 2). All these observations

Table 1. Changes in Fecal Microbiota From Wistar Rats at Different Taxonomic Levels During Aging

Phylum	Class	Order	Family	Genus
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae↑ *	
			Peptococcaceae↓ *	*Rc4-4*↓**
			Lachnospiraceae	*Blautia*↑*
	Bacilli	Lactobacillales	Lactobacillaceae↓* *	*Lactobacillus*↓ *
		Turicibacterales	Turicibacteriaceae↑ *	*Turicibacter* ↑*
Bacteroidetes↓**	Bacteroidia	Bacteroidales	Bacteroidaceae↑**	*Bacteroides* ↑ **
			Paraprevotellaceae↓***	*Paraprevotella*↓***
			Prevotellaceae↑**	*Prevotella* ↑**
			Odoribacteraceae	*Odoribacter*↑*
Proteobacteria↓*	Betaproteobacteria	Burkholderiales	Alcaligenaceae↓*	*Sutterella*↓*
	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae↓**	
Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae↓ *	*Anaeroplasma*↓*
TM7↓***				
Verrucomicrobia↑ *	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae↑ *	*Akkermansia*↑*

Notes: Comparisons between 4- and 24-month-old rats. ↑ indicates increase and ↓ indicates decrease with aging. Data correspond to 10 young and 16 old animals.

*$p < .05$. **$p < .01$. ***$p < .001$, significantly different between 4- and 24-month-old rats.

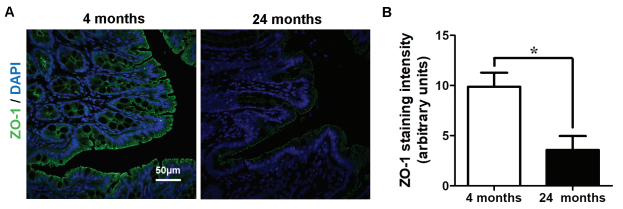

Figure 1. Immunofluorescence staining of ZO-1 in colon sections from 4- and 24-month-old rats. Presence of ZO-1 was determined by immunofluorescence staining as indicated in "Materials and Methods" and analyzed by confocal microscopy. (**A**) Representative distal colon sections from young and old rats. (**B**) Stained areas from 2 sections per rat were quantified using Image J software. Data are expressed in arbitrary units and correspond to the mean ± SEM of the staining density observed in 4 animals per age. *$p < .05$. Full color version is available within the online issue.

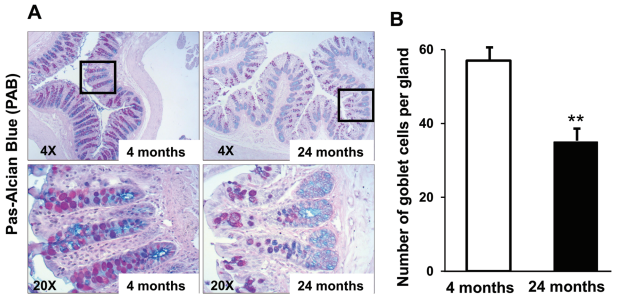

Figure 2. Differential mucin staining of colonic mucosal crypts from 4- and 24-month-old rats. (**A**) Pas-Alcian Blue (PAB) staining of colonic crypts observed by light microscopy at 4× and 20× magnifications. The image of 20× correspond to the rectangle indicated in the 4× magnification. Acidic (blue in online version, black in print version) and neutral mucins (magenta in online version, gray in print version) expressed in colonic crypts are shown. (**B**) The chart illustrates the number of goblet cells per colonic gland. Two independent pathologists blinded to the origin of the sample performed the counting of the number of goblet cells in both conditions. Five sections of each sample were evaluated. Values are mean ± SEM of 5 different animals per age. **$p < .01$. Full color version is available within the online issue.

suggest that the colonic mucosal barrier of aged rats is weakened, but there are no overt signs of gut or systemic inflammation.

CCK Satiating Effect

A weaker colonic mucosal barrier together with a higher presence of LPS-producing microorganisms suggests an increased leakage of LPS through the intestinal wall in aged rats. Since LPS has been reported to induce leptin resistance in vagal afferents and, subsequently, inhibit the satiating effect of CCK (10), we analyzed the effect of an i.p. injection of CCK on food intake in overnight fasted young and old rats. As shown in Figure 4A, in young rats, injection of CCK induces a marked decrease of food intake with respect to saline injected animals up to 90 minutes after finishing the overnight fast. Old rats injected with saline exhibit 50% lower food intake in the short term than young animals. In contrast to that observed in young rats, in old animals, the injection of CCK does not result in a significant decrease of food intake, suggesting the presence of CCK resistance in rats with aging ($p > .2$ at all 3 times tested).

Interestingly, determination of serum CCK concentrations during fasting and after 90 minutes of feeding indicates a poor CCK secretory response of enteroendocrine cells in aged rats that could be partly due to the lower short-term food intake observed (Figure 4B).

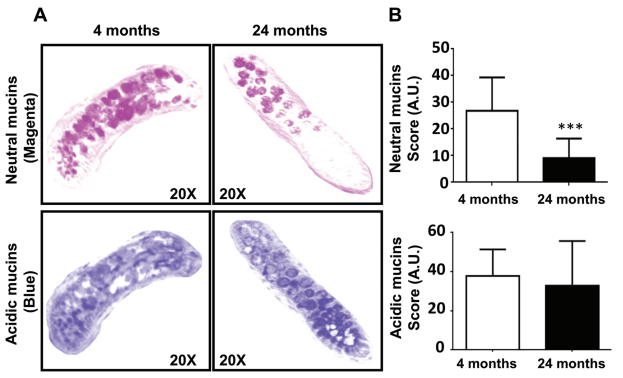

Figure 3. Estimation of neutral (magenta) and acidic (blue) mucins of colonic mucosal crypts from 4- and 24-month-old rats. (**A**) Spectral separation of magenta and blue colors of Pas-Alcian Blue (PAB) staining of colonic crypts from young (4 months) and old (24 months) rats observed by light microscopy at 20× magnification. (**B**) The charts illustrate the magenta and blue scores in the glands of the colon. The score was obtained using the Fiji version of ImageJ software as described in "Materials and Methods." Values are mean ± SEM of 5 different animals per age. ***$p < .001$; $p > .2$ for acidic mucins score. A.U. = arbitrary units. Full color version is available within the online issue.

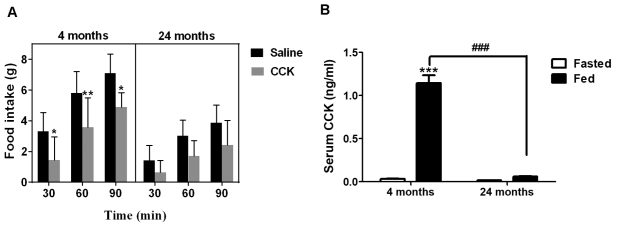

Figure 4. Short-term satiety effect of CCK and CCK serum concentration under fasting and fed conditions in 4- and 24-month-old rats. (**A**) After overnight fasting, rats were injected i.p. with saline (black bars) or CCK (gray bars) as indicated in "Materials and Methods," and food intake was determined after 30, 60, and 90 min. Two-way ANOVA with repeated measures, with post hoc Bonferroni test was employed to compare the satiating effect of CCK in young and old animals. Data are expressed as mean ± SEM of 8–10 different animals per age and treatment, *$p < .05$ and **$p < .01$ CCK vs saline; for old animals, $p > .2$ CCK vs saline at all 3 times tested. (**B**) Blood was taken from the tail vein of 4- and 24-month-old rats under overnight fasting (white bars) or after 90 min feeding ad libitum (black bars). Serum CCK was determined by specific ELISA kit as indicated in "Materials and Methods." Two-way ANOVA with Tukey post hoc test was used for statistical analysis. Data are expressed as mean ± SEM of 5 animals per experimental condition. ***$p <$ .001 fed vs fasted; ###$p < .001$ young vs old.

Discussion

In this work, we studied changes in the microbial community and gut structure of male Wistar rats with aging, and its possible relationship with the well-known alterations in adiposity and energy homeostasis regulation observed in aged rats (16).

Bacterial richness (OTUs number), and α- and β-diversity are not different between 4- and 24-month-old rats. These data are similar to that reported in Fischer rats (17), but in contrast with the increase in the first 2 parameters and the marked separation in β-diversity observed with aging in the more obese aged Sprague-Dawley rats (18). In humans, overlapping microbial communities have been reported for healthy young and old people, while a separation is observed with elderly under long-stay residential care (19). A recent study in rhesus macaques also reports no differences in α- and β-diversity thorough aging (20).

The data in this work show a higher presence of LPS-producing bacteria from genera *Bacteroides*, *Prevotella*, and *Odoribacter* in aged animals, whereas the anti-inflammatory genus *Lactobacillus*, that inhibits LPS production (17), decreases in old rats. We also show an aging-dependent decrease of tight junctions and adherens junctions markers ZO-1 and β-catenin, respectively, in the distal colon, structures needed for the maintenance of the mucosal barrier integrity (15) and reported to be altered in aged humans and old mice (21). In addition, aged rats seem to have a slightly lower abundance of genera *Clostridium* and *Coprococcus* which produce butyrate that protects intestine against inflammation and maintains gut mucosa integrity by enhancing the expression of intestinal mucosal tight junction proteins such as occludin, claudin 3, and ZO-1 (22,23). We also detect a lower presence of neutral mucins producing goblet cells that would contribute to a weaker mucus layer with lower buffering capacity in aged rats despite normal viscosity (24). All these data point to the presence of a weaker mucosal barrier that likely allows LPS leakage in old rats. Interestingly, in aged F344 rats and in old baboons, an increased concentration of fecal and plasmatic LPS together with a reduction in colonic tight junction protein expression (17,25) has been already reported.

Alterations in mucin composition have been associated with colonic inflammation in rats (26). A weak gut barrier in old rats might favor the invasion of internal medium by bacteria or bacteria-released substances, as previously described in older adults (27) and Fisher rats (17). In fact, some of the bacteria more abundant in old animals can reach the immune system. Thus, *Prevotella* and *Akkermansia* are known to be present in the lymph node samples (28). Nevertheless, our data do not identify signs of colon inflammation. In addition, circulating markers of inflammation remain unchanged in old rats. Previous data from our group showed unchanged levels of IL-6, but higher serum levels of IL-1β in old rats (29). However, in this work, this last difference does not reach statistical significance. Moreover, we observed a relevant proinflammatory macrophage infiltration in hypothalamus, liver, and adipose tissue and elevated expression of MCP-1 and TNF-α in adipose tissue and IL-1β and TNF-α in liver of aged animals (29). Of note, in the present study, we observed a significant decrease of phylum *TM7* in aged animals. This phylum has been reported to have potential immune suppression ability by inhibiting TNF-α production in macrophages (30) and its decrease might promote inflammation in aged animals. Whether organ inflammation in old rats derives from changes in gut microbiota and barrier permeability remains to be elucidated and cannot be ruled out.

We find in aged male Wistar rats a significant increase of phylum Verrucomicrobia whose sole representative specie is the mucin-degrading bacteria *Akkermansia muciniphila*. This contrasts with the decrease of this species in aged Sprague-Dawley rats (18). *Akkermansia muciniphila* is elevated in lean animals and humans relative to those with obesity, as well as during prolonged fasting or in mice treated with metformin (23). Moreover, administration of *A muciniphila* decreases circulating LPS, increases insulin sensitivity, and improves glucose profile in diet-induced obese mice (31). Moreover, feeding old mice with high-fat diet with heat-killed *Lactobacillus paracasei* improves gut barrier integrity promoting mucus synthesis by goblet cells and an increased presence of *A muciniphila* (32). However, *A muciniphila* has no effect on fat mass, fasting glucose, and mucus thickness in control mice (33). The Sprague-Dawley model of aging is characterized by a marked obesity, whereas Wistar rats, despite an increase in adiposity, are clearly different from obese rats (6). This fact might explain the differences between both rat strains with respect to the changes in α- and β-diversity indicated above, and allows speculating that the increased presence of *A muciniphila* in aged Wistar rats is rather related to the aging process itself and not to the associated fat accretion. Of note, a higher presence of Verrucomicrobia has been observed in healthy older adults (19) and aged Fisher rats (17).

The thickness of the mucus layer results from the balance between the action of mucin-degrading bacteria and host mucin-producing goblet cells. Here, we report a lower presence of goblet cells producing neutral mucins in aged rats together with a higher presence of mucin-degrading bacteria such as *A muciniphila* and family Ruminococcaceae. Thus, aging seems to associate with a misbalance between mucin-degrading bacteria and mucin-producing goblet cells likely leading to mucus layer thinness and the subsequent facilitation of LPS leakage. A thinner mucus layer has been related with elevated *A muciniphila* in a mouse model of colitis (34) and its introduction in a germ-free mouse model was sufficient to promote inflammatory colitis (35). An increase of bacterial family Ruminococcaceae has also been shown in human patients of Chron's disease (36).

The variations in the composition of the gut microbiota reported herein suggest that levels of LPS increase likely in aged rats, as reported for old mice (17) and older adults (37). LPS is known to interact with intestinal vagal afferents inhibiting the sensitizing action of leptin on CCK satiating effect without interfering with its hypothalamic function (10). Interestingly, our data herein reveal the presence of CCK resistance in aged rats as expected from the alterations observed in gut microbiota. Aged Wistar rats, despite a lower short-time food intake, exhibit slightly higher total daily food intake (38) and increased fat depots in liver, heart, and adipose tissues together with a higher LBM. Of note, fully defective CCK satiating action in Otsuka Long Evans Tokushima Fatty (OLEFT) rats has been shown to associate with obesity and hyperphagia (39). Thus, it can be postulated that the defective CCK satiating effect in aged rats could partly contribute to the observed body weight gain. It should also be noted that enteroendocrine CCK-secreting cells seem to have a poor response to food intake in aged animals something that could also contribute to the increase in body weight. More experimental work would be necessary to establish whether this defective response is due to altered microbiota.

We also observe changes in the gut microbiota of aged rats usually associated to situations of fat accretion and adiposity. Thus, several bacterial genera (*Lactobacillus*, *Rc4-4*), family Desulfovibrionaceae and phylum TM7, that decrease in response to a diet enriched in saturated fatty acids, have a lower presence in aged rats, whereas genus *Bacteroides* and family Turicibacteriaceae are more abundant in both mice under saturated fatty acid-enriched diet (40) and aged rats. Altogether, these data show the presence of a gut microbiota profile in aged rats usually associated with fat accretion and adiposity.

Summarizing, the data in this work identify a misbalance between mucin-producing and degrading mechanisms in aged rats, with lower presence of goblet cells that secrete neutral mucins and higher abundance of mucin-degrading bacteria. We also observe a decrease in tight junctions and adherens junctions markers during aging. All these likely result in a thinner mucus layer and more permeable gut barrier in old animals. In addition, gut bacterial profile in aged animals is compatible with a higher LPS production that likely leaks through the intestinal barrier. The lower CCK satiating effect observed in aged rats is an expected consequence of LPS leakage. In contrast, no signs of gut inflammation are observed. Microbiota changes with aging are similar to situations of increased adiposity but not identical to those of obese animals. Whether these changes in microbiota are a primary modification during the aging process that lead to fat accretion, insulin resistance, and gut–brain axis dysfunction, or a consequence of some of these changes, remains to be elucidated.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

None declared.

Author Contributions

C.R. has performed the major part of the experiments, participated in the processing and discussion of data, and is responsible for the final draft of the manuscript. D.A.-C., M.F.-L., and P.P.-P. contributed to perform, process, and discuss experiments on microbiota analysis and CCK satiating effect. P.S.-S., M.V.T.-L., and L.G.G. contributed to perform, process, and discuss experiments on gut structure and inflammatory markers. E.L., C.A., and F.E. contributed to perform, process, and discuss RMN experiments and fat determination in heart and liver. A.M.V. and J.M.C. designed the study, discussed all the data, and are responsible for the final version of the manuscript. All authors critically reviewed the work and approved the final version.

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