Intrinsic aerobic capacity governs the associations between gut microbiota composition and fat metabolism age-dependently in rat siblings

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ABSTRACT

Host genetic factors affecting the gut microbiome play an important role in obesity. Yet limited attention has been paid on the host genetic factors linked to physical fitness in modifying the microbiome. This study determined whether sibling-matched pairs of rats selectively bred for high (HCR) and low (LCR) aerobic capacity differ in their microbiome age-dependently and which taxa associate with differential in metabolism. Several taxa in young adult rats (hereafter young) linked to inherited aerobic capacity while in older adult (hereafter old) rats most of the differences between the lines associated with body weight. Despite the absence of weight differential between LCR and HCR when young, the LCR microbiome contained more *Actinobacteria*, *Veillonellaceae*, *Coriobacteriaceae*, *Phascolarctobacterium* and *Ruminococcus;* taxa previously linked to obesity. This raises the question whether the microbiome contributes to the later development of obesity in LCR. Age-related differences were detected in almost all taxa in both rat lines. The young HCR measured higher for serum glycerol and free fatty-acids and lower for cholesterol, HDL, LDL and triglycerides than LCR. The old HCR differed from the old LCR by lower LDL. Several metabolites including LDL associated age- and genetic background-specifically with the microbiome that might explain the metabolic differences between the lines. While old lines did not differ in visceral adipose tissue gene expression, the young HCR expressed more inflammatory genes than LCR, and several taxa including *Proteobacteria* associated with these genes. In conclusion, intrinsic aerobic capacity governs the microbiome that may influence body weight, metabolism and gene expression.

NEW AND NOTEWORTHY

Several microbial taxa were linked to inherited aerobic capacity. Despite the lack of weight difference between the younger rat lines, LCR had more taxa previously linked to obesity that may contribute to the later development of obesity in LCR. Several metabolites and visceral adipose tissue genes associated age- and genetic background-specifically with the gut microbiota and might explain the metabolic and gene expression differences between the rat lines.

INTRODUCTION

During the last decade an increasing number of studies show that the gut microbiota plays an important role in the development of obesity and metabolic disorders. Given that these gut-inhabiting microbial cells count up to 100-fold more genes than our genome, it is not surprising how important the impact of gut microbiota is on metabolic and immunologic health (38). For example, gut microbiota regulates host lipid storage and metabolism (1). The long co-evolution of the human host and intestine-colonizing microbiota has resulted in several positive and negative health consequences of the symbiotic relationship (4). Nevertheless, a number of factors that affect the equilibrium between the harms and benefits caused on the host depend mostly on the balance of the gut microbiota composition as a whole. Further, the balance of gut microbiota is readily influenced by environment, for instance diet and nutrition (38).

In addition to diet as a lifestyle factor, several studies have suggested that a link between physical activity and gut microbiota composition exists. However, the link is currently not well understood. A number of studies in mice have shown that exercise results in a variety of modulations in microbial communities (7). Yet, it seems that the effects of exercise may depend on the metabolic state of the host as the gut microbiota of diabetic vs. healthy animals respond differently to exercise (22). In addition to animal experiments, cross-sectional studies in humans have shown that the gut microbiota of professional athletes is more diverse than that of sedentary controls (9), and that cardiorespiratory fitness associates with the microbial diversity (13).

Besides lifestyle and environment, the genetic influence governs the gut microbiota composition in the host, and thus the microbiota that the offspring inherit from their parents could predispose to a certain risk phenotype (25, 42, 43). Studies in twins have suggested that the genetic influence determines the gut microbiota composition of an individual, and that the siblings' microbiota resemble more each other than that of unrelated individuals (25, 42, 43). Several recent reports have shown that the host genetic factors importantly affect the gut microbiome and its functions. It has been reported that in laboratory animals within a controlled environment, the genetic background explains a substantial fraction of the abundance of most common microbiota (33) and its relationship with the development of obesity and metabolic disorders (44). A study in human twins demonstrated that metabolic syndrome-related gut microbial taxa were heritable, and their abundances were associated with a single nucleotide polymorphism that is a genetic risk factor for the disease (26). Another study comprising 93 unrelated individuals showed that the host genetic

variation in immunity-related pathways, especially those enriched in host genes that have been previously associated with microbiome-derived complex inflammatory and obesity-related disorders were significantly driving the associations with gut microbiota (5).

Surprisingly little attention has been paid on the role of genetic factors in physical activity and fitness and their association with the gut microbiota composition. The Human Gene Map for Performance and Health-related Fitness Phenotypes has listed several genes that are suggested to be linked to exercise performance and response to exercise training (6). However, even identical twins have been shown to be discordant for physical activity (24) and therefore, other factors linked to and governed by the genetic differences must contribute to the physical fitness. It was recently reported that exercise induced changes in the abundances of *Firmicutes*, *Proteobacteria* and *Cyanobacteria* phyla in ovariectomized female rats selectively bred for high (HCR) and low (LCR) aerobic capacity (27). Moreover, several taxa responded differently to exercise between the LCR and HCR rat lines and differences were also detected between the lines in the sedentary condition. This polygenic HCR/LCR rat model system has been developed by selective breeding over 30+ generations based on the endurance running capacity (19, 35). As a consequence of strong selection pressure, two lines with distinct intrinsic aerobic capacity and risk factors for metabolic disorders have been generated. Compared with each other, LCR rats gain more weight and have higher adiposity while HCR rats have higher oxidative metabolism capacity and are protected from diet-induced insulin resistance (19, 48).

In this study we aimed to find out whether also male rats with dissimilar intrinsic aerobic capacity differ on their gut microbiota composition. The genetic differences in the rat lines were expected to affect the host genome-gut microbiota associations and further contribute to the metabolic differences between the lines. We also studied age-dependent differences by examining young rats versus their older siblings (42). Sedentary rats were chosen to exclude acute effects of exercise and to specifically concentrate on the genetic influence of the intrinsic aerobic capacity on the gut microbiota.

MATERIALS AND METHODS

Animals

The rats in this study were born at the University of Jyväskylä, Jyväskylä, Finland and approved for use by the national ethics committee of animal experimentation in Finland. Their parents were of the 35th generation of selection and phenotyped at the University of Michigan, USA and measured over a 10-fold difference in endurance exercise capacity on a treadmill running test (48). The best running distance of HCR males in the test averaged 1993 ± 202 meters and of LCR males 191 ± 40 meters. The best running distance of HCR females averaged 2444 ± 204 meters and of LCR mothers 213 ± 90 meters. All animals were kept sedentary without access to a running wheel. In young adult (hereafter young) rats the microbiota of ten HCR (mean age 7.90 ± 0.27 weeks, mean weight 177.8 ± 25.4 grams) and ten LCR (mean age 7.77 ± 0.29 weeks, mean weight 183.4 ± 23.4 grams) were analyzed. After sequencing one HCR was excluded from the analyses due to different clustering i.e. grouping of the reads compared to other HCR rats. In old adult (here after old) siblings 12 HCR (mean age 39.98 ± 0.33 weeks, mean weight 376.8 ± 48.0 grams) and ten LCR (mean age 39.81 ± 0.34 weeks, mean weight 477.3 ± 43.2 grams) were analyzed. These ages were chosen to avoid spontaneous effects of aging in old rats.

Fecal sample processing and 16S rRNA gene sequencing

After the animals were euthanized, the abdominal cavity was opened and the colon content was collected into sterile tubes and immediately frozen in liquid nitrogen. For DNA extraction the samples were thawed gently on ice. To enhance the cell lysis ~60-80 mg of feces in 1.4 mm Ceramic Bead Tubes (MO BIO Laboratories, Inc., Carlsbad, CA, USA) were vortexed for 10 min and afterwards the DNA was extracted with semi-automated GXT Stool Extraction Kit VER 2.0 (Hain Lifescience GmbH, Nehren, Germany).

The microbial 16S rDNA profiles were analyzed with Illumina MiSeq 16S rRNA gene sequencing targeting the V4-V5 regions of the bacterial 16S rRNA gene as previously described (36). The quality of the raw sequence data was checked with FastQC quality control tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and the datasets were analyzed with QIIME 1.9 pipeline utilizing GreenGenes 13.08 database. Sequence reads were filtered with a

quality score acceptance rate of 20 or better, and the generated OTU table was filtered by dropping out OTUs that represent less than 0.05% of the total sequence count. To minimize the effect of inter-sample variation in the sequencing efficiency, samples were subsampled (rarefied) by random sampling without replacement to the lowest common sequencing depth.

Blood analyses

Rats were euthanized with carbon dioxide anesthesia followed by cardiac puncture. Blood glucose was measured with HemoCue Glucose (201^{+}, HemoCue AB, Ängelholm, Sweden). Serum total cholesterol, LDL, HDL, free fatty acids (FFA), triglycerides and glycerol were analysed using the KONELAB 20XTi analyser (Diagnostic Products Corporation, Los Angeles, CA, USA).

Real-time mRNA analyses from visceral fat

Total RNA from ~100 mg of pulverized visceral adipose tissue was extracted using Tri reagent (Ambion, Thermo Fischer Scientific, Waltham, MA, USA) and by homogenizing using TissueLyser (Qiagen, Hilden, Germany) according to the supplier's protocol. One microgram of total RNA was reverse transcribed according to the manufacturer's instructions using High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR analysis was performed using in-house designed primers (iQ SYBR Supermix and CFX96™ Real-time PCR Detection System (Bio-Rad Laboratories, Richmond, CA, USA).

Each sample was analyzed in duplicate and PCR cycle parameters were as follows: +95ºC for 10 min, 40 cycles at +95ºC for 10 s, at +53-61ºC (depending on gene, Table 1) for 30 s and at +72ºC for 30 s, followed finally by 5 s at +65ºC . Relative expression levels were calculated using the CFX Manager Software (Bio-Rad Laboratories) and normalized to the expression of β-actin (*ACTB*). Amplification efficiencies for each gene were 100±2%.

Statistical analyses

All data was checked for normality with Shapiro Wilks test using IBM SPSS Statistics 22 (Armonk, NY, USA). As most of the data was not normally distributed non-parametric tests were chosen for group comparisons and correlation analyses. Statistical analyses of the 16S rRNA gene sequence

data were performed together with QIIME statistical tools (21) and SPSS Statistics 22. All analyses were made from the randomly subsampled OTU table with rarefaction level matching the sample with the lowest total OTU count. The bacterial diversity of the samples (α-diversity metrics) and statistically significant differences in the OTU abundances were computed with QIIME. Differences between the groups of the taxonomic levels Phylum, Family and Genus were studied using Kruskal Wallis test with FDR correction in QIIME. A False Discovery Rate (FDR) $P < 0.05$ was considered as statistically significant. Outliers in the microbial taxa were analyzed with GraphPad Prism (San Diego, CA, USA). If significant outliers were found in the microbial taxa, they were removed from the data and the differences between the groups were further analyzed using Mann-Whitney U test with IBM SPSS Statistics 22. In SPSS $P < 0.05$ was considered as statistically significant. OTUs existing in less than 25% of the samples were filtered away before testing.

The group differences in gene expression, body weight and fat metabolism-related blood variables were analyzed by non-parametric tests using SPSS Statistics 22. Specifically, the differences between the groups were identified and statistical significance determined using Mann-Whitney U test. The associations between the gut microbiota and other variables were determined using Spearman's rank correlation coefficient in IBM SPSS Statistics 22.

RESULTS

LCR and HCR rats differ from each other on the gut microbiota composition age-dependently

Among the young rats' microbial community, a total of 9 phyla, 13 families and 17 genera, and in old rats 8 phyla, 19 families and 26 genera were identified (Figures 1-3). In both young and old rats, the most abundant phylum was *Bacteroidetes* (54.8% and 65.8%, respectively; Figure 1) and the

most abundant family *S24-7* of the order *Bacteroidales* (29.3% and 26.5%, respectively; Figure 2). In young rats the most common genus was an unknown genus of *S24-7* family that represented 29.3% of the sequences. In old rats the genus *CF231* of the *Paraprevotellaceae* family was the most common representing 26.5% of the sequences (Figure 3).

No differences in alpha diversity measures were detected between the rat lines, either young or old siblings (data not shown). However, the young HCR rats differed from LCR rats by harboring less phylum *Actinobacteria* (p=0.041, Figure 1), less families *Veillonellaceae* (p=0.007) and *Coriobacteriaceae* (p=0.041) (Figure 2), less genera *Phascolarctobacterium* (p=0.011) and *Ruminococcus* (p=0.019), and more genus *Lactobacillus* (p=0.043) (Figure 3).

The old HCR rats, in turn, differed from LCR rats by harboring less phylum *Bacteroidetes* (p=0.023), more phyla *Spirochaetes* (p=0.003) and *Deferribacteres* (p=0.019) (Figure 1), more families *Spirochaetaceae* (p=0.003) and *Deferribacteraceae* (p=0.019) (Figure 2), more genera *Prevotella* of *Paraprevotellaceae* family (p=0.002), *Mucispirillum* (p=0.017), and *Treponema* (p=0.002), less genera *Phascolarctobacterium* (p=0.031) and unknown genera of *Erysipelotrichaceae* (p=0.031) (Figure 3).

Though siblings under controlled laboratory conditions are expected to resemble each other in their gut microbiota composition, in both rat lines the abundance in the majority of taxa differed between young and old siblings. The significantly differing taxa are presented in supporting figure 1 for HCR and in supporting figure 2 for LCR of the supporting material.

The differences in the lipid metabolism-related variables and weight and their association with gut microbiota

The young HCR rats measured higher for serum glycerol (p=0.043) and free fatty acids (FFA, p=0.0001), and lower for total cholesterol (p=0.008), HDL (p=0.002), LDL (p=0.0001) and triglycerides (p=0.017) than the young LCR rats (Table 2). No differences were found in body weight or serum glucose between the younger lines. The old HCR rats differed from old LCR by lower serum LDL values (p=0.017) and lower body weight (p=0.0001) (Table 2).

Several lipid metabolism variables and body weight were found to associate with different microbial taxa depending on intrinsic aerobic capacity and age. The significant associations are shown in Tables 3 and 4.

Gene expression in the visceral adipose tissue and its association with gut microbiota composition

The young HCR rats expressed more *IL1B* and *CD45* but less *AdipoQ* and *TLR5* than LCR ($p < 0.01$ for all, Figure 6A). In young rats, *IL1B* with *Rikenellaceae* (R= 0.714, p=0.047) in HCR, and *Deferribacteres* (R= -0.667, p=0.05) and CF231 (R= 0.85, p=0.004) in LCR, *AdipoQ* with *Cyanobacteria* (R=-0.667, p=0.05) and *Ruminococcus* (R= -0.717, p=0.03) in HCR, and with *Bacteroidetes* (R= -0.782, p=0.008), *Deferribacteres* (R= 0.855, p=0.002), *Elusimicrobia* (R= 0.681, p=0.03), *Ruminococcaceae* (R= 0.648, p=0.043) and unidentified genus of *Lachnospiracheae* (R=0.721, p=0.019) in LCR. *TLR 4* expression associated with the abundance of *Proteobacteria* (R= -0.733, p=0.025) in HCR, *Cd45* with *Rikenellaceae* (R= 0.738, p=0.037) in HCR, and with *Proteobacteria* (R= -0.733, p=0.025) and *Veillonellaceae* (R= 0.733, p=0.025) in LCR, and *TLR5* with *Tenericutes* (R= 0.673, p=0.033) in LCR rats.

In old rats no differences were found in the expression levels of *AdipoQ*, *IL1B*, *TLR4*, *CD45* or *TLR5* (Figure 6B). However, also in old HCR *IL1B* associated with *Deferribacteres* phyla, *Deferribacteraceae* family, *Mucisprillum* genus (R= -0.599, p=0.018 for all) as well as unknown family and genus of *Bacteroidales* (R= -0.782, p=0.038 for both). In old LCR *IL1B* correlated with *Bacteroidetes* (R= -0.893, p=0.007), *AdipoQ* associated in old HCR with the unknown family and genus of *Clostridiales* (R= -0.857, p=0.014 for both) and *Clostridiaceae* family (R= -0.893, p=0.007), and in LCR with *Ruminococcaceae* family (R= 0.762, p=0.028) and an unidentified genus of the family (R= 0.857, p=0.007). In HCR *TLR4* correlated with *Proteobacteria* (R= -0.811, p= 0.027) and *Clostridiaceae* family (R= -0.929, p=0.003). No correlation were found for *Cd45* in HCR, while in LCR it correlated with the abundance of *Firmicutes* (R= 0.714, p=0.047), *Ruminococcacaeae* (R= 0.786, p=0.021), *Prevotella* of the *Paraprevotellaceae* family (R= 0.805, p= 0.016), unidentified genus of *Ruminococcaceae* (R= 0.833, p=0.01) and *Corpococcus* (R= 0.843, p=0.009). *TLR5* associated with *Deferribacteres*, *Deferribacteriaceae*, *Mucisprillum* (R= 0.786, p=0.036 for all), unknown family and genus of *Bacteroidales* (R= -0.810, p=0.015) and *Phascolarctobacterium* (R= -0.805, p=0.029) in old HCR and in old LCR with *Elusimicrobia* (R= -0.782, p=0.038), unknown genus of *Elusimicrobiaceae* (R= -0.831, p=0.011) and unknown family and genus of *Clostrdiales* (R= -0.810, p=0.015).

DISCUSSION

The long co-evolution of the gut microbiota with the host has resulted in a mutualistic relationship, in which the microbiota importantly impacts the host metabolism and the host genetic factors affect the gut microbial composition. Recently, several genes belonging to immunity-related pathways in the host have been shown to correlate with the microbiome (5). Despite the considerable impact on health, little attention has been paid on the effects of genetic predisposition to low aerobic capacity on the gut microbiome. As the gut microbiota composition is suggested to be a polygenic trait (3), the polygenic HCR/LCR model was the most adequate to study the influence of inherited aerobic capacity on the microbial taxa.

The gut microbiota composition is highly associated with body weight (42). The young HCR and LCR rats did not differ in bodyweight. Yet, an abundance of gut microbiome taxa did differ between the young representatives of the two rat lines. Thus, we suggest these differences are specifically linked to the intrinsic aerobic capacity. These taxa were the phylum *Actinobacteria*, the families *Veillonellaceae* and *Coriobacteriaceae*, and the genera *Ruminococcus* and *Lactobacillus.* Previous studies using sedentary ovariectomized and sham-operated female rats have also found *Ruminococcus* to be more abundant in LCR rats (10, 27), while *Actinobacteria*, *Veillonellaceae*, *Coriobacteriaceae* or *Lactobacillus* did not differ between the adult female rat lines when sedentary. Comparable sex-specific associations of gut microbiota with health despite similar dietary background, matched age and BMI, have been previously described in humans (16). In addition, a study in 89 inbred mice lines showed that several taxa exhibited significant gender-specific differences in the microbiota composition (32). However, contrary to our findings in rats, a study in humans did not report associations between these bacterial taxa and cardiorespiratory fitness (13).

The gut microbiota composition of young rats differed significantly from old rats. Age-related changes in gut microbiota are rather well documented. Elderly humans have higher proportions of *Bacteroidetes* (28), which was also seen in this study between young and old LCR rats but not in HCR. The changes in the microbiome during aging can importantly affect the health of the host. For

297 instance, an age-associated decrease in *Lactobacillus* and an increase in *Ruminococcus* - detected
298 also in this study - have been reported to associate with high frailty in elderly (45). Some elements
299 of frailty that the gut microbiota are suggested to modulate are aging-related changes in innate
300 immunity, sarcopenia and cognitive function (31).

301 The old HCR and LCR rats differed significantly from each other in weight. Therefore the weight
302 difference may have influenced the gut microbiota (or *vice versa*) as all taxa, except *Bacteroidetes*,
303 *Spirochaetes,* the family *Spirochaetaceae* and further, the genus *Treponema* associated with weight.
304 To our knowledge, no studies before have reported associations of these taxa with inherited
305 difference in aerobic capacity, except that female HCR have been described to have lower
306 *Bacteroidetes* than LCR (10). Studies in mice have reported that exercise increases the abundance
307 of *Bacteroidetes*. This phylum may convey some metabolic advantage to the host as in addition,
308 high-fat sedentary mice had very low levels of *S24-7* family of *Bacteroidales* order that were
309 elevated by exercise (14). An interesting taxon is *Treponema* belonging to *Spirochates* phylum and
310 *Spirochaetaceae* family that is absent in western human populations but very abundant in hunter
311 communities of Amazon and Tanzania with very nature-bound way of life (30, 37). *Treponema* uses
312 xylane, xylose, and carboxymethylcellulose to produce high levels of butyrate that has anti-
313 inflammatory effects. However, whether *Treponema* plays a role in physical fitness and healthy
314 metabolism remains to be determined.

315 Interestingly, despite the lack of body weight difference between the young rat lines, young LCR
316 rats had more *Actinobacteria*, *Veillonellaceae*, *Coriobacteriaceae*, *Phascolarctobacterium* and
317 *Ruminococcus* that all have been previously linked to obesity (17, 23, 41, 42). This raises a question
318 whether the gut microbiota contributes to the later development of obesity in LCR rats. Indeed,
319 animal studies have demonstrated that an altered gut microbiota composition is a cause and not a
320 consequence of obesity. In the study of Turnbaugh et al. transplantation of the caecal microbiota
321 from obese but not lean mice into the gut of germ-free mice increased fat gain and insulin resistance
322 (40, 43). The authors suggested that compared with “lean”, an “obese” microbiome more efficiently
323 harvests energy from the diet (2). *Phascolarctobacterium, Veillonellaceae*, *Actinobacteria* and
324 *Ruminococcus* produce high amounts of butyrate, acetate and propionate from non-digestible
325 carbohydrates (8, 12, 46). While for instance butyrate is beneficial, acetate is suggested to be
326 obesogenic (8). Therefore, through the metabolites that they produce these bacteria may induce
327 obesity in LCR rats.

Several taxa were rat line and age-specifically associated with fat metabolism variables. Generally, *Ruminococcaceae* and *Actinobacteria* associated negatively while *Clostridiales* positively with serum glycerol and LDL cholesterol. Our findings are in line with those showing that low abundance of *Ruminococcaceae* and *Actinobacteria* and higher abundance of *Clostridiales* are associated with negative traits in host metabolism (11, 29, 34, 42). Thus, these taxa might, at least partly, explain the metabolic differences between HCR and LCR as the associations differed between the rat lines. *Ruminococcaceae* correlated negatively with FFA in young HCR that had higher FFA but not in LCR. *Prevotellaceae* and the unknown genus of *Ruminococcaceae* correlated negatively with LDL in old LCR rats that had higher LDL but not in HCR. In agreement, an increase in *Prevotellaceae* has been shown to associate with improved lipid metabolism (15), and a decrease in *Ruminococcaceae* with disturbed lipid metabolism (11). In addition, in young HCR that had lower total cholesterol and LDL than LCR rats, *Allobaculum* that belongs to the *Erysipelotrichaceae* family correlated negatively with cholesterol and LDL. Supporting our findings, *Erysipelotrichaceae* has been negatively associated with cholesterol synthesis (29).

The gut microbiota has been associated with visceral adipose tissue (VAT) inflammation and metabolic disturbances as blood and lymphatic vessels draining the gut localize in VAT that is therefore exposed to bacteria-derived components of the gut (20). For that reason, we analyzed associations between gut microbiota and VAT gene expression and found that the associations differed between the two rat lines and, thus, may explain some of the differences in the gene expression levels. Specifically, adiponectin expression levels were higher in young LCR than HCR rats and were associated in LCR positively with several gram-positive bacteria while negatively with gram-negative *Bacteroidetes* in LCR and *Cyanobacteria* in HCR. Gram-negative bacteria have been shown to decrease and gram-positive increase the secretion of adiponectin from VAT (39). Surprisingly, the young HCR rats expressed higher levels of *IL1B* and *CD45* of inflammatory leukocytes that were both associated with the abundance of *Rikenellaceae*. Previous studies have also associated *Rikenelleceae* family with inflammation (18). *Proteobacteria*, in turn, have been linked to improved inflammatory profile, which is line with its negative association with *TLR4* in old HCR and *CD45* in young HCR (47).

In conclusion, several microbial taxa in young rats were linked to inherited aerobic capacity while in older rats most of the differences between the lines may be influenced by body weight. Despite the lack of weight difference between the younger rat lines, LCR had more *Actinobacteria*, *Veillonellaceae*, *Coriobacteriaceae*, *Phascolarctobacterium* and *Ruminococcus*, which have been previously linked to obesity. Thus, the gut microbiota may contribute to the later development of

obesity in LCR. Aging-related differences were detected in the abundances of almost all taxa between young and old HCR as well as young and old LCR. Several metabolites and visceral adipose tissue genes associated age- and genetic background-specifically with the gut microbiota and might explain the metabolic and gene expression differences between the lines. The main findings are summarized in Figure 5.

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DISCLOSURE STATEMENT

No conflicts of interest, financial or otherwise, are declared by the authors.

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524 FIGURE LEGENDS

525

526 **Figure 1. Phylum level abundances of gut microbiota in young and old HCR and LCR rats.**

527 Nine young HCR, 10 young LCR, 12 old HCR and 10 old LCR were included in the 16S rDNA
528 sequencing. All data are presented as mean ± SD. The statistical significance was set to $p<0.05$ and
529 the significant differences are presented with lines and * between the groups. The young HCR rats
530 differed from LCR rats by harboring less *Actinobacteria*. The old HCR rats differed from LCR rats
531 by harboring less *Bacteroidetes* and more *Spirochaetes* and *Deferribacteres*.

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Figure 2. Family level abundances of gut microbiota in young and old HCR and LCR rats.

Nine young HCR, 10 young LCR, 12 old HCR and 10 old LCR were included in the 16S rDNA sequencing. All data are presented as mean ± SD. The statistical significance was set to $p<0.05$ and the significant differences are presented with lines and * between the groups. The young HCR had less *Veillonellaceae* and *Coriobacteriaceae* than young LCR. The old HCR had more families *Spirochaetaceae* and *Deferribacteraceae* than the old LCR.

Figure 3. Genus level abundances of gut microbiota in young and old HCR and LCR rats.

Nine young HCR, 10 young LCR, 12 old HCR and 10 old LCR were included in the 16S rDNA sequencing. All data are presented as mean ± SD. The statistical significance was set to $p<0.05$ and the significant differences are presented with lines and * between the groups. The young HCR harbored less *Phascolarctobacterium* and *Ruminococcus,* and more genus *Lactobacillus* than the young LCR. The old HCR had more genera *Prevotella* of *Paraprevotellaceae* family, *Mucispirillum* and *Treponema*, and less genera *Phascolarctobacterium* and unknown genera of *Erysipelotrichaceae* than the old LCR.

Figure 4. Visceral adipose tissue inflammatory gene expression in young and old HCR and LCR rats.

Nine young HCR, 10 young LCR, 12 old HCR and 10 old LCR were included in the 16S rDNA sequencing. All data are presented as mean ± SD. The statistical significance was set to $p<0.05$ and the significant differences are presented with lines and * between the groups. The young HCR rats expressed more *IL1B* and *CD45* but less *AdipoQ* and *TLR5* than LCR. In old rats no mRNA expression differences between the lines were found.

Figure 5. Main findings of the study.

Several microbial taxa in young rats were linked to inherited aerobic capacity while in old adult rats most of the differences between the lines may be influenced by body weight. Despite the lack of weight difference between the younger rat lines, LCR had more *Actinobacteria*, *Veillonellaceae*, *Coriobacteriaceae*, *Phascolarctobacterium* and *Ruminococcus*, which have been previously linked

to obesity. Young HCR expressed more inflammatory genes than LCR in the visceral adipose tissue. The young HCR measured higher for serum glycerol and free fatty-acids (FFA) and lower for cholesterol (chol), HDL, LDL and triglycerides (trigly) than LCR. The old adult HCR differed from the old adult LCR by lower LDL.

TABLES

Table 1. The differences in the lipid metabolism-related variables and weight between the young and old rat lines. The differences between the groups were studied in SPSS Statistics 22 using non-parametric Mann Whitney U-test. $P < 0.05$ was considered statistically significant.

Variable	HCR young mean ± SD n=9	LCR young mean ± SD n=10	p	HCR old mean ± SD n=12	LCR old mean ± SD n=10	p
Chol (mmol/L)	2.13 ± 0.47	2.75 ± 0.43	**0.008**	2.63 ± 0.68	3.30 ± 1.05	0.080
HDL (mmol/L)	1.69 ± 0.38	2.28 ± 0.29	**0.002**	2.26 ± 0.69	2.87 ± 0.77	0.059
LDL (mmol/L)	0.28 ± 0.06	0.60 ± 0.16	**< 0.001**	0.46 ± 0.25	0.81 ± 0.51	**0.017**
Trigly (mmol/L)	1.05 ± 0.36	0.70 ± 0.12	**0.017**	1.38 ± 0.43	1.13 ± 0.33	0.107
FFA (µmol/L)	556 ± 382	145 ± 40	**< 0.001**	403 ± 298	438 ± 292	0.456
Glycerol (µmol/L)	160± 15	139 ± 29	**0.043**	242 ± 46	210 ± 41	0.123
Glucose (mmol/L)	7.96 ± 0.86	8.63 ± 1.22	0.400	7.12 ± 0.68	7.27 ± 0.51	0.557
Weight (g)	177.8 ± 25.3	183.4 ± 23.4	0.780	376.8 ± 48.0	477.3 ± 43.2	**< 0.001**

Chol, serum total cholesterol; Trigly, triglycerides; FFA, free fatty acids

TABLE 2. Primer sequences and annealing temperatures used in quantitative PCR.

Primer name	Annealing temp.	Primer sequence
TLR5 fwd	61°C	CCGAGGTTGTAACCTTACCCAG
TLR5 rev	61°C	GTCAAGCGAGCATACTGGGTC
ACTB fwd	60°C	GGCACCACACTTTCTACAAT
ACTB rev	60°C	AGGTCTCAAACATGATCTGG
AdipoQ fwd	60°C	AATCCTGCCCAGTCATGAAG
AdipoQ rev	60°C	CATCTCCTGGGTCACCCTTA
CD45 fwd	60°C	CCGTTGTACACCAGAGATGA
CD45 rev	60°C	TCCCAAAATCAGTCTGCAC
IL1B fwd	53°C	CAACAAAAATGCCTCGTGC
IL1B rev	53°C	TGCTGATGTACCAGTTGGG
TLR4 fwd	60°C	GAGACCAGGAAGCTTGAATCCCTGC
TLR4 rev	60°C	TGTCTCCACAGCCACCAGATTCTG

Table 3. Significant associations of microbial taxa with body weight and lipid metabolism variables in young HCR and LCR. The correlations were studied in SPSS Statistics 22 using non-parametric Spearman's rank correlation coefficient. $P < 0.05$ was considered statistically significant.

		Weight	Chol	HDL	LDL	Glyc	FFA	Trigly	Glc
YOUNG HCR (n=9)									
Cyanobacteria	***P***	NS	NS	NS	NS	0.024	NS	NS	0.012
	R					-0.734			0.784
Deferribacteres	***P***	NS	0.030	0.040	NS	NS	NS	NS	NS
	R		-0.716	-0.675					
Tenericutes	***P***	NS	0.033	NS	NS	NS	NS	NS	NS
	R		-0.748						
Actinobacteria	***P***	NS	NS	NS	0.030	NS	NS	NS	NS
	R				-0.717				
Coriobacteriaceae	***P***	NS	NS	NS	0.030	NS	NS	NS	NS
	R				-0.717				
Bacteroidetes	***P***	NS	0.044	NS	NS	NS	NS	NS	NS
	R		0.679						
Bacteroidaceae	***P***	NS	NS	NS	NS	0.037	NS	0.046	NS
	R					-0.696		0.676	
Bacteroidales S24-7	***P***	0.038	NS	NS	NS	NS	NS	NS	NS

	R	- 0.693							
***S24-7* unknown genus**	***P***	NS	NS	NS	NS	NS	NS	NS	NS
	R								
Bacteroides	***P***	NS	NS	NS	NS	0.037	NS	NS	NS
	R					-0.696			
Porphyromonadaceae	***P***	NS	0.018	NS	NS	NS	0.023	NS	NS
	R		0.759				0.738		
Parabacteroides	***P***	NS	0.018	NS	NS	NS	0.023	NS	NS
	R		0.759				0.738		
Lachnospiracheae	***P***	NS	NS	NS	0.048	NS	NS	NS	NS
	R				-0.672				
***Lachnospiracheae* unknown genus**	***P***	NS	NS	NS	0.032	NS	NS	NS	NS
	R				-0.709				
Ruminococcaeae	***P***	NS	NS	NS	NS	NS	0.023	NS	0.015
	R						-0.773		0.773
Allobaculum	***P***	NS	0.046	NS	0.029	NS	NS	NS	NS
	R		-0.675		-0.721				
***Veillonellaceae* uknown genus**	***P***	0.015	NS	NS	NS	NS	NS	NS	NS
	R	-0.769							
Phascolarctobacterium	***P***	0.038	NS	NS	NS	NS	NS	NS	NS
	R	0.695							
YOUNG LCR (n=10)									
Spirochaetes	***P***	0.048	NS	NS	NS	0.010	NS	NS	NS
	R	0.637				-0.766			
Treponema	***P***	0.048	NS	NS	NS	0.010	NS	NS	NS
	R	0.637				-0.766			
Actinobacteria	***P***	NS	NS	NS	NS	NS	NS	0.043	NS
	R							-0.647	
Coriobacteriaceae	***P***	NS	NS	NS	NS	NS	NS	0.043	NS
	R							-0.647	
Bacteroidetes	***P***	NS	NS	NS	NS	0.004	NS	0.020	NS
	R					0.814		0.717	
Bacteroidales S24-7	***P***	0.034	NS	NS	NS	NS	NS	NS	0.009
	R	- 0.669							0.771
***S24-7* unknown genus**	***P***	0.034	NS	NS	NS	NS	NS	NS	0.009
	R	- 0.669							0.771
Prevotellaceae	***P***	NS	NS	NS	NS	NS	0.046	NS	NS
	R						-0.640		
Paraprevotellaceae CF231	***P***	NS	NS	NS	NS	0.038	NS	NS	NS
	R					-0.753			
***Lachnospiracheae* unknown genus**	***P***	0.042	NS	NS	NS	0.012	NS	NS	NS
	R	0.650				-0.753			
Ruminococcaeae	***P***	NS	NS	NS	NS	0.026	NS	0.043	NS
	R					-0.694		-0.647	
Ruminococcus	***P***	NS	NS	NS	NS	NS	NS	NS	NS
	R								
Lactobacillaceae	***P***	NS	NS	NS	NS	NS	NS	NS	0.008
	R								0.778
Lactobacillus	***P***	NS	NS	NS	NS	NS	NS	0.017	NS
	R							-0.761	

Chol, total cholesterol; glyc, glycerol; FFA, free fatty acids; trigly, triglycerides; glc, glucose, NS, not significant

609 **Table 4. Significant associations of microbial taxa with body weight and lipid metabolism**
610 **variables in old HCR and LCR.** The correlations were studied in SPSS Statistics 22 using non-
611 parametric Spearman's rank correlation coefficient. $P < 0.05$ was considered statistically significant.

612

		Weight	Chol	HDL	LDL	Glyc	FFA	Trigly	Glc
OLD HCR (n=12)									
Cyanobacteria	***P***	0.049	NS	NS	NS	NS	NS	NS	NS
	R	0.579							
Cyanobacteria YS2F	***P***	0.049	NS	NS	NS	NS	NS	NS	NS
	R	0.579							
Cyanobacteria YS2F **unknown genus**	***P***	0.049	NS	NS	NS	NS	NS	NS	NS
	R	0.579							
Deferribacteres	***P***	0.025	NS	NS	NS	NS	NS	NS	NS
	R	-0.639							
Deferribacteriaceae	***P***	0.025	NS	NS	NS	NS	NS	NS	NS
	R	-0.639							
Mucispirillum	***P***	0.025	NS	NS	NS	NS	NS	NS	NS
	R	-0.639							
Bacteroidetes	***P***	NS	NS	NS	NS	NS	NS	NS	NS
	R								
Bacteroidaceae	***P***	NS	NS	NS	0.014	NS	NS	NS	NS
	R				0.683				
Bacteroidales S24-7	***P***	NS	NS	NS	NS	NS	NS	0.050	NS
	R							-0.567	
S24-7 **unknown genus**	***P***	NS	NS	NS	NS	NS	NS	0.050	NS
	R							-0.567	
Prevotellaceae	***P***	NS	NS	NS	0.048	NS	NS	NS	NS
	R				-0.580				
Prevotella	***P***	NS	NS	NS	0.048	NS	NS	NS	NS
	R				-0.580				
Paraprevotellaceae	***P***	NS	0.020	0.004	0.016	NS	0.035	NS	0.049
prevotella	***R***		0.658	0.763	0.677		0.611		-0.640
Bacteroides	***P***	NS	NS	NS	0.014	NS	0.038	NS	NS
	R				0.683		0.602		
Clostridiales	***P***	NS	0.027	NS	0.003	NS	0.022	NS	0.038
unknown family	***R***		0.633		0.776		0.650		-0.630
Clostridiales	***P***	NS	0.027	0.012	0.003	NS	0.022	NS	0.038
unknown genus	***R***		0.633	0.763	0.776		0.650		-0.630
Veillonellaceae	***P***	NS	NS	NS	0.014	NS	NS	NS	0.014
	R				0.684				-0.741
Rikenellaceae	***P***	NS	NS	NS	NS	NS	NS	NS	NS
unknown genus	***R***								
Erysipelotrichaceae	***P***	NS	NS	NS	NS	0.013	NS	NS	NS
unknown genus	***R***					-0.720			
Oscillospira	***P***	0.001	NS	NS	NS	NS	NS	NS	0.043
	R	-0.811							-0.616
Phascolarctobacterium	***P***	0.050	NS	NS	NS	NS	NS	NS	NS
	R	0.576							
OLD LCR (n=10)									
Proteobacteria	***P***	NS	NS	NS	NS	NS	NS	0.009	0.039
	R							0.770	-0.657
Elusimicrobia	***P***	NS	NS	NS	NS	NS	NS	NS	0.036
	R								0.698

***Elusimicrobiaceae* unknown genus**	***P***	0.049	NS	NS	NS	NS	NS	NS	NS
	R	-0.634							
Firmicutes	***P***	NS	NS	NS	NS	NS	0.016	NS	NS
	R						0.733		
***Clostridiales* unknown family**	***P***	NS	NS	NS	NS	NS	NS	NS	0.033
	R								0.673
***Clostridiales* unknown genus**	***P***	NS	NS	NS	NS	NS	NS	NS	0.033
	R								0.673
Ruminococcus	***P***	NS	NS	NS	NS	NS	0.007	NS	0.002
	R						0.817		-0.881
***Ruminococcaceae* unknown genus**	***P***	NS	NS	NS	0.035	NS	NS	NS	NS
	R				-0.699				
Clostridiaceae	***P***	NS	NS	NS	NS	NS	0.033	NS	NS
	R						0.673		
Lactobacillaceae	***P***	NS	NS	NS	NS	NS	0.038	NS	NS
	R						0.661		
Lactobacillus	***P***	NS	NS	NS	NS	NS	0.038	NS	NS
	R						0.661		
Erysipleotrichaceae	***P***	NS	NS	NS	NS	NS	NS	0.012	NS
	R							-0.753	
***Erysipleotrichaceae* unknown genus**	***P***	NS	NS	NS	NS	NS	NS	NS	NS
	R								
***Veillonellaceae* unknown genus**	***P***	NS	NS	NS	NS	NS	0.010	NS	NS
	R						0.768		
Coprococcus	***P***	0.019	NS	NS	NS	NS	NS	NS	NS
	R	0.720							
Bacteroidetes	***P***	NS	NS	NS	NS	NS	0.050	NS	NS
	R						-0.667		
Bacteroidales S24-7	***P***	NS	NS	NS	NS	0.038	0.038	NS	0.021
	R					-0.661	-0.661		-0.710
***S24-7* unknown genus**	***P***	NS	NS	NS	NS	0.038	0.038	NS	0.021
	R					-0.661	-0.661		-0.710
Prevotellaceae	***P***	NS	NS	NS	NS	0.016	NS	NS	NS
	R					0.733			
Prevotella	***P***	NS	NS	NS	NS	0.016	NS	NS	NS
	R					0.733			
Paraprevotella	***P***	NS	NS	NS	NS	NS	NS	NS	0.043
	R								-0.648

613 Chol, total cholesterol; glyc, glycerol; FFA, free fatty acids; trigly, triglycerides; glc, glucose, NS,
614 not significant