The role of Ames dwarfism and calorie restriction on gut microbiota

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Abstract:

The gut microbiome (GM) represents a large and very complex ecosystem of different microorganisms. There is an extensive interest in the potential role of the GM in different diseases including cancer, diabetes, cardiovascular diseases, and aging. The GM changes over the lifespan and is strongly associated with various age-related diseases. Ames dwarf (df/df) mice are characterized by an extended life- and healthspan, and although these mice are protected from many age-related diseases, their microbiome has not been studied. To determine the role of microbiota on longevity animal models, we investigated the changes in the GM of df/df and Normal control (N) mice, by comparing parents before mating and littermate mice at three distinct time points during early life. Furthermore, we studied the effects of a 6-month calorie restriction (CR), the most powerful intervention extending the lifespan. Our data revealed significant changes of the GM composition during early life development, and we detected differences in the abundance of some bacteria between df/df and N mice, already in early life. Overall, the variability of the microbiota by genotype, time-point and breeding pair showed significant differences. In addition, CR caused significant changes in microbiome according to gastrointestinal (GI) location (distal colon, ileum and cecum), genotype and diet. However, the overall impact of the genotype was more prominent than that of the CR. In conclusion, our findings suggest that the gut microbiota plays an important role during post-natal development in long-living df/df mice and CR dietary regimen can significantly modulate the GM.

Introduction:

Long-living Ames dwarf mice (df/df) are characterized by a mutation of the *Prop1* (df) gene which leads to deficiencies of the growth hormone (GH), prolactin (PRL) and thyrotropin (TSH) [1]. Several independent studies showed that these dwarf mice live significantly longer and healthier in comparison to their normal (N) littermates [2]. Due to an increase between 40-60% in lifespan, the df/df mouse model represents an excellent model for aging studies [3]. In addition to an extended lifespan, df/df mice are also protected from age-related decline in metabolic function, development of insulin resistance and diabetes [2, 4], and they are less prone to developing cancer [5]. The gut microbiome represents a community of microorganisms including bacteria, archaea, viruses and eukaryotic microbes that inhabits the gastrointestinal (GI) tract. The GI tract can host up to 1000 bacterial species with many of them encoding protein functions absent in the human genome [6]. Thereby, the gut microbiome has, for instance, the ability to impact metabolic functions, protect against pathogens and modulate the host immune system [7]. Moreover, it is believed that the microbiome can modulate the aging processes and a “healthy” microbiota can have a protective effect on the development of age-related diseases. Several studies have shown that aging causes a decrease in microbiome variety and an increase of its fragility, which has been associated with age-related constipation and low-level chronic inflammation [8]. Environmental factors such as diet are recognized as the main determinants in shaping microbiome composition, suggesting that modulating and maintaining a healthy diet may help to stimulate a microbiome that improves health and extends lifespan [9]. Importantly, changes in the microbiome and bacterial metabolites during aging have been associated with higher risk of Alzheimer’s [10] and Parkinson’s disease [11, 12], which might be related to alterations in mucosal barrier function [13].

Calorie Restriction (CR) is a dietary intervention that has been shown to extend lifespan in several different animal models. It has been shown that CR regimen in mice can extend lifespan by up to 40%, while in non-mammalian models, the effects of CR was even more pronounced [14, 15]. This dietary intervention reduces body weight and plasma glucose and insulin levels, while improving insulin sensitivity and overall health in laboratory animals [16]. Ames dwarf mice share several of the characteristics of CR mice, however the mechanism of action is not identical since previous studies have shown that CR further extends longevity and enhances insulin sensitivity in these long-living animals [17]. Moreover, it is well established that CR also reduces blood pressure and cholesterol levels [18]. CR studies in humans have demonstrated several health benefits including reducing obesity with decreased body weight [19], reduced atherosclerosis [18] and an improvement of cardiac function [20]. Furthermore, CR decreases the risk of age-related diseases such as type 2 diabetes and cancer [21]. Other studies have also shown that CR strongly inhibits microglial activation and as well neurodegeneration after an injury [22]. Since the Ames dwarfism mutation and CR intervention both extend the lifespan and healthspan in mice, we studied the impact of this mutation and dietary regimen on the composition of gut microbiome. Our goal was to determine whether the genetic mutation that extends lifespan in mice will shift microbiome composition in comparison to phenotypically normal heterozygous (N) littermates coming from the same parents and maintained under the same environmental conditions. Additionally, we investigated whether dietary interventions with CR will introduce similar shifts in GM when comparing df/df and N mice.

Material and Methods

Ames dwarf mice experimental design

Ames dwarf (df/df) mice were bred and maintained in the Burnett School of Biomedical Sciences, University of Central Florida under light- and temperature-controlled conditions, with a 12-hour light and 12-hour dark cycle and a constant temperature of 20-23°C. The Vivarium at UCF is AAALAC accredited and a specific pathogen-free barrier facility. All personnel entering animal facility must always wear PPE, which include shoe covers, one-time use lab coat, face cover, hair cover and gloves. To enter the facility each person passes through air-shower barrier. Before and after entering air-shower as well as before entering the room with animals the shoe cover and gloves are sprayed with chlorine dioxide for disinfection. Mice used in the study were kept in Tecniplast filtered cages with automated watering in laminar flow racks. All caging equipment was autoclaved before use. Food and water were irradiated to eliminate any possible contaminations. Beside high standard of handling animals with extensive usage of PPE, for any animal manipulations the cage was placed in laminar flow workstation and before opening, the whole cage was sprayed with chlorine dioxide. Additionally, every six months PCR evaluation indicated that mice were negative for: *Aspicularis tetraptera*, *Corynebacterium bovis*, *Helicobacter bilis*, *Helicobacter ganmani*, *Helicobacter hepaticus*, *Helicobacter mastomyrinus*, *Helicobacter rodentium*, *Helicobacter* spp., *Helicobacter typhlonius*, murine norovirus, *Myocoptes*, *Pasteurella pneumotropica* biotype Heyl, *Pasteurella pneumotropica* biotype Jawetz, *Radfordia/Myobia*, *Syphacia muris* and *Syphacia obvelata*.

Phenotypically normal heterozygous (N) females were mated with homozygous Ames dwarf (df/df) males to produce df/df and N offspring. Both male and female offspring

were used in the study. All experiments were approved by UCF Laboratory Animal Care Committee.

Fecal pellets were collected at 4 individual time point:

At Collection 1 (C1) fecal pellets were collected from non-pregnant female (N) and male mice (df/df) before mating. (C1; males n=4 and females n=4).

At Collection 2 (C2) fecal pellets were collected from individual N and df/df pups on day 22 (C2; one day after weaning of N mice; N males n=7 and N females n=6).

At C2 only N pups were weaned at day 21, while df/df pups, due to slower growth and development, had to stay until day 41 with their mothers following breeding protocol, when collection was performed (C3).

At Collection 4 (C4) fecal pellets were collected from individual N and df/df pups on day 70 (C4; N males n=7, N females n=6, df/df males n=4 and df/df females n=6).

On the last collection at day 70, mice were anesthetized with isoflurane, bled by cardiac puncture and sacrificed by cervical dislocation and fecal samples were also collected from distal colon, terminal ileum and cecum. All samples were snap frozen in liquid nitrogen and stored at -80°C for further analysis.

Calorie restriction experimental design

Ames dwarf mice and their N littermate controls were housed in single cages at controlled temperature (20-24°C) with a 12-hour light and dark cycle. Mice were fed ad libitum (AL) diet (Rodent Laboratory Chow 5001; not autoclaved; 23.4% protein, 4.5% fat, 5.8% crude fiber; LabDiet PMI Feeds, Inc., St. Louis, MO). At the age of three months, df/df and N male mice were divided into 4 groups (n=10 mice/group): N mice with fed ad libitum (N-AL), N mice subjected to 30% CR (N-CR), df/df mice fed ad libitum (df/df-AL) and df/df mice subjected to 30% CR (df/df-CR). Mice selected for dietary intervention were subjected to gradual CR by reducing food intake to 90%

during the first week, 80% during the second week and 70% from the third week through the end of the study. The AL mice food consumption was measured weekly to adjust food dosage to CR groups. Ames dwarf and N mice on CR were fed every day. After approximately 6 months of CR (9 months old mice), mice were anesthetized with isoflurane, bled by cardiac puncture and sacrificed by cervical dislocation. Fecal pellets from the distal colon, terminal ileum and cecum were collected and snap frozen in liquid nitrogen and stored at -80°C for further analysis.

16S rDNA Amplification, Sequencing and Microbiome Analysis

For microbiome identification, the amplification of the V4 region (F515/R806) of the 16S rRNA gene was performed as described in previous protocols [23]. For DNA-based amplicon sequencing 25ng of DNA was used for each 30 μL PCR reaction. The PCR consisted of initial denaturation for 30 s at 98°C , followed by 25 cycles of 10 s at 98°C , 20 s at 55°C and 20 s at 72°C . Every sample was amplified in triplicate and pooled. Subsequently, PCR amplicons were sequenced on the Illumina MiSeq platform (PE250).

The reads were assembled, quality controlled and clustered using the Usearch 8.1 software package (<http://www.drive5.com/usearch/>). Briefly, the reads were merged using `-fastq_mergepairs` with `-fastq_maxdiffs 30` and quality filtering was done with `fastq_filter` (`-fastq_maxee 1`) and a minimum read length of 200 bp. Using the UPARSE algorithm [24] the OTU clusters and representative sequences were determined, ensued by taxonomy assignment using the Silva database v128 [25] and the RDP Classifier [26] with bootstrap confidence cut off of 80%. The OTU absolute abundance table and mapping file were used for statistical analyses and data visualization in the R statistical programming environment [27] using the phyloseq package [28].

Results:

GH deficient df/df mice have an altered microbiota composition

To assess whether GH deficiency influences the intestinal microbiota of df/df mice, a longitudinal littermate control study was performed according to Stappenbeck & Virgin 2016 [29] (see Fig 1A). This experimental design was necessary as familial transmission and environmental factors have been demonstrated to strongly influence microbiota composition in laboratory mice [30]. Therefore, df/df GH deficient male mice were crossed to female heterozygous (N) mice and microbiota composition was analyzed in offspring (df/df and N) mice by 16S rRNA gene sequencing after 22 days (feces), 42 days (feces) and 70 days (content distal colon). Analysis of alpha diversity revealed a similar number of detected species (Observed species) (Figure 1B) and a comparable evenness (Shannon index) (Figure 1C) between df/df and N littermate control mice at both time points. Strikingly, analysis of beta diversity using Bray–Curtis dissimilarity and non-metric multidimensional scaling (NMDS) readily revealed differences between df/df and N mice at both time points (Figure 1D). Permutational multivariate ANOVA (Adonis) statistical testing was performed to assess the contribution of genotype, breeding pair, and gender to variability within the microbiota (Figure 1D). This analysis demonstrated that after weaning both breeding pair ($R^2=0.195$, $p=0.06$) and genotype ($R^2=0.130$, $p=0.013^*$) contributed to the observed variability. The effects of the genotype were even stronger ($R^2=0.314$, $p=0.001^{***}$) after 70 days (C4) (Figure 1D). In addition to Bray–Curtis dissimilarity, weighted Unifrac distances were used to assess differences in microbiota composition between mice. After 70 days, weighted Unifrac distances between df/df mice were small compared to the distances between df/df and N mice, corroborating the development of genotype-dependent alterations in the microbiota composition (Figure 1E). In order to further characterize these differences, we quantified the ratio between Bacteroidetes

and Firmicutes (B/F ratio). Notably, while the B/F ratio was not different at weaning, the B/F ratio at 10 weeks was significantly different (0.25 vs 0.5, $p < 0.001$) between the df/df and N mice (Figure 1F). The beta-diversity analyses of microbiota composition on the family level demonstrated the presence of prevalent commensal bacteria including Muribaculaceae, Lachnospiraceae and Lactobacillaceae in both groups (Figure 1G). To identify which bacterial taxa displayed significantly altered relative abundances between the two genotypes at 10 weeks of age, LEfSe analysis was performed utilizing standard parameters (linear discriminant analysis [LDA] score > 3.0) [31]. In df/df mice, LEfSe identified an enrichment of the families of Muribaculaceae (BacteroidalesS24_7 group), Enterococcaceae, Enterobacteriaceae, ClostridialesvadinBB60group, and Porphyromonadaceae, and a reduction of Lactobacillaceae, Rikenellaceae, and Planococcaceae compared to littermate control mice (Figure 1H). In addition to LEfSe, we also applied DESeq2 [32], which utilizes distinct statistical approaches and models for identification of significant differences on the levels of individual OTUs. In df/df mice, we observed an enrichment of OTUs belonging to the Muribaculaceae (=Muribaculum S24-7 group), Enterococcus spp., and Klebsiella spp., and a reduction of an OTU belonging to Lactobacillus spp., compared to N mice (Figure 1H). Together, this data provides evidence that GH deficiency in df/df mice affects microbiota composition during post-natal development.

Alterations in intestinal microbiota are not limited to the colonic microbiome

To assess whether df/df mice also have altered microbiota composition in the small intestine and cecum, a detailed analysis of microbial communities in these organs was performed. In line with the data from the distal colon, analysis of beta diversity in the ileum and in the cecum demonstrated differences between mice from the two genotypes for both organs (Figure 2A and B). Permutational multivariate ANOVA demonstrated

that both breeding pair ($R^2 = 0.341$, $p = 0.004$ **, Ileum) ($R^2 = 0.288$, $p = 0.001$ ***, Cecum) and genotype ($R^2 = 0.417$, $p = 0.001$ ***, Ileum) ($R^2 = 0.172$, $p = 0.001$ ***, Cecum) contributed to the observed variability. In line with previous findings in other mouse lines [33], the microbiome in the small intestine of df/df and N mice is largely dominated by Lactobacillaceae (Figure 2C). LEfSe (LDA score > 3.0) identified the families Lactobacillaceae, Enterobacteriaceae and Enterococcaceae as being overrepresented in the ileum of df/df, while the relative abundance of the families Muribaculaceae, Lachnospiraceae and Ruminococcaceae were reduced (Figure 2D). Notably, only one OTU was identified, *Lactobacillus spp.*, with increased abundance, while several other low abundance OTUs were decreased (Figure 2E). Similar to the microbiota in the distal colon, the cecal microbiota of df/df and N mice is largely dominated by Muribaculaceae, Lachnospiraceae and Lactobacillaceae in both groups (Figure 2F). LEfSe identified the family of Enterobacteriaceae as being increased in the cecum of df/df mice, which correlates with the increased relative abundance of this family in the distal colon (Figure 2G). However, other bacterial families increased in the distal colon were not altered in the cecum, e.g. Muribaculaceae. Of note, the same OTU (OTU_2) from the family Muribaculaceae was identified in both the cecum and the distal colon of df/df mice suggesting the presence of shared alterations in both cecum and distal colon (Figure 2H). In contrast to the distal colon, the relative abundance of Lactobacillaceae was not as strongly altered in the cecum suggesting the presence of site-specific genotype-dependent changes in the microbiome. Hence, in summary, GH deficiency in df/df mice distinctively affects intestinal communities in cecum and colon.

Vertical inheritance of microbiota in df/df mice

Having revealed an effect of GH deficiency on the GI tract microbiota, we next assessed next whether the alterations observed in df/df offspring mice are characteristic across generations by comparing the microbiota between the parents (F0, genotype: mother: N/df; father: df/df) and offspring (F1, genotypes: N and df/df). Analysis of beta diversity demonstrated distinct pattern influenced both by genotype (Adonis, $R^2=0.212$, $p=0.001$ ***) and generation ($R^2=0.131$, $p=0.022$ *) (Figure 3A). To further assess the influence of the genotype, we performed a comparison of microbial communities by calculating weighted Unifrac distances between individual mice of the different groups. If genotype-dependent effects are preserved over generations, we would expect smaller weighted Unifrac distances between mice of the same genotype compared to mice of the same generation but another genotype. In accordance with that hypothesis, the comparison of distances between the F1 mice revealed significant differences dependent of the genotype (Figure 3B and C). Notably, the closest related group, i.e. next shortest distance, for the F1 df/df mice were the F0 males, which shared the same genotype (Figure 3B). For the F1 N/df mice the closest related group were the F0 mothers, which similarly shared the genotype (Figure 3C). Together, this provides further evidence of distinct genotype-dependent alterations in GH deficient df/df mice compared to N mice.

Calorie restriction affects the microbiota in df/df and N mice

Next, we investigated whether CR induces similar or distinct alterations in the microbiota of df/df and N mice. Therefore, df/df and N mice were subjected to 30% CR starting at 3 months of age, and microbiome analysis was performed after 6 months of dietary intervention (Figure 4A). We initially assessed alpha diversity in the ileum, cecum and distal colon of df/df-AL, df/df-CR, N-AL and N-CR mice, but aside from a higher number of observed species in the cecum of N-AL compared to N-CR mice, no

significant differences were observed between mice of different genotype or diet (Figure 4B - D). In contrast, analysis of beta diversity using Bray–Curtis dissimilarity and non-metric multidimensional scaling (NMDS) revealed differences among groups for the separate sample sites (Figure 4E - G). As with sampling time points in previous experiments, alterations were detected between df/df-AL and N-AL mice (data not shown) demonstrating that there are genotype-dependent changes in the microbiota at different time points. In addition to the effect of genotype, overall assessment of variability identified a large cage-driven effect and minor contributions from the variable diet (Figure 4E-G). The comparison of microbiota composition between N-AL and N-CR mice using LEfSe at genus level revealed that CR induced significant changes in the intestinal microbiota at all investigated sites (Figure 5A). There was an overabundance of different members of Lachnospiraceae (OTU_184 and OTU_21) and Ruminococcaceae (*Acutalibacter* OTU_71) in the ileum (Figure 5B). Strikingly, in N-AL mice, there was a significant enrichment of the Erysipelotrichaceae (*Turicibacter* OTU_6) family in the cecum and colon suggesting that this group of bacteria responds to CR (Figure 5C and D). In contrast, LEfSe identified only minor CR induced changes in the microbiota of df/df mice (Figure 5E-G). This observation suggests that there are different CR-induced changes between the two genotypes since the strong CR-induced reduction in the family Erysipelotrichaceae in N mice was not observed in df/df mice. Together, this data establishes that CR alters the microbiota composition differently in N mice than in df/df mice.

Discussion:

Since the gut represents one of the major entrances into the body, it is well equipped with an intricate intrinsic immune system. Moreover, it represents one of the major

organs regulating food processing and nutrient intake. Maintaining a healthy gut microbiome is one of the most important requirements for healthy aging and extended lifespan. Ames dwarf mice are known to live longer and healthier when compared to their N littermate controls, however, the gut microbiota in these long-lived df/df mice has not yet been examined. In our study, we analyzed the alterations of the gut microbiome in GH deficient df/df mice compared to their N littermate mice. This analysis has been performed in both parents and offspring, taking into account gender and genotype. Furthermore, we evaluated the changes of the gut microbiome in CR-treated compared to AL-fed N and df/df mice in the three different GI locations (Ileum, Cecum and Distal Colon).

In our study, significant changes in the gut microbiota composition in long-living df/df mice compared to N mice were demonstrated. Considering, common parents, identical diet, and a pathogen-free environment as previously described, this suggests that GH deficiency is involved in the regulation of bacterial composition in the gut of those mice, and intestinal communities in the cecum and colon are differentially affected. Moreover, our results showed a strong change in the microbiota composition between df/df mice and their N controls at 70 days of age, and differences in the microbiota composition were small between df/df mice in comparison to the overall difference between df/df and N mice. These results confirm the important role of genotype-dependent alterations in the microbiota composition.

In the present study, we also quantified the ratio between Bacteroidetes and Firmicutes (B/F ratio), which is a well-known measurement for a healthy microbiota [34, 35]. These two above-mentioned main bacterial phyla, Bacteroidetes and Firmicutes are the most dominant bacteria (more than 99%) in the gut [36]. Studies with humans have shown that the Bacteroidetes/Firmicutes ratio correlates with the bodyweight. It was

shown that the gut microbiota in obese subjects had elevated proportion of Firmicutes and a decreased population of Bacteroidetes when compared to lean subjects and that the proportion of Bacteroidetes increases with weight loss on a low-calorie diet [34]. Additionally, the B/F ratio is also altered in ob/ob mice [37]. Importantly, our findings show that df/df mice are characterized by a significant higher B/F ratio in comparison to the N littermate controls. Studies have also linked changes in the B/F ratio with aging in human subjects [38] and the B/F ratio is higher in younger than older mice [39]. Therefore, this specific gut microbiota composition in df/df mice could be considered beneficial for these long-lived df/df mice.

One should emphasize that Muribaculaceae (previously known as the Bacteroidales S24-7) belongs to the phylum Bacteroidetes; and, in turn, the Lachnospiraceae, Ruminococcaceae, Lactobacillaceae and Erysipelotrichaceae belong to the phylum Firmicutes [40]. We have identified the Muribaculaceae, Lachnospiraceae and Lactobacillaceae bacteria in both genotypes. In detail, df/df mice had an increase of Muribaculaceae, Enterococcaeae, Enterobacteriaceae, Clostridialesvadin BB60 group, and Porphyromonadaceae, and a reduction of Lactobacillaceae, Rikenellaceae, and Planococcaceae compared to their N littermate controls. It is known that the diversity of Bacteroidales increases during childhood in humans [41, 42] and reaches adult levels at about 17 years of age [43]. The amount of Bacteroides stays almost stable during life but decreases during aging [38]. In our study, we demonstrated in df/df mice an enrichment of the bacterial family of Muribaculaceae, which belongs to the order Bacteroidales. Previously, it has also been shown that the Rikenellaceae family has been found in the microbiome from elderly people [9]. Interestingly, the amount of this bacteria family is decreased in long-lived df/df mice and increased in N mice. This may suggest an important role for the Rikenellaceae family in lifespan regulation.

Our study indicated that GH deficiency affects the gut microbiota early during postnatal development. Many factors like nutrition or environmental conditions may affect the microbiota composition. Interestingly, in our study, we identified one OTU *Lactobacillus* with increased abundance in df/df mice. It has been previously reported that increased levels of *Lactobacilli* and *Enterococci* were found in the elderly compared to young adults [44]. However, others have shown that *Lactobacilli* were lower in old individuals than in young adults, and no differences in *Bacteroides* and *Eubacterium* levels were found [45]. Furthermore, it was reported that the bacteria composition in the gut in elderly individuals is very variable [9, 46]. Consistently, recent studies reported lower levels of *Bacteroides* and *Eubacterium* in older individuals compared to younger [47, 48].

Overall, our data indicate significant microbiota changes during early development. There is strong evidence in the literature that during advanced aging the changes in microbiome are even greater in both humans and animals [49]. In our study, we did not follow animals throughout the complete aging process, however, we clearly demonstrate how a different microbiome is developed during early life in models of extended longevity. We believe that studying the microbiota during early development might be likewise important and informative concerning the impact of the microbiome on aging especially in its intersection with GH signaling. In recent studies we could already show that treating GH-deficient df/df mice with GH for as short as six weeks during early development pre-program the lifespan of these mice by shortening their longevity [50]. This finding indicates that GH-dependent physiological alterations during early development might represent important factor pre-programming the process of aging. Overall, this could suggest that the differences in gut microbiota in GH-deficient df/df mice during early development might represent important shifts in

bacteria populations promoting healthy lifespan throughout aging. However, more follow-up studies to determine longitudinal changes in gut microbiome in these long living dwarf mice are needed.

Follow-up with translational studies to provide better relevance of these findings to human aging are needed as well. As we discussed above, there are some important similarities, i.e. low abundance of Rikenellaceae in long-living df/df mice already at young age when comparing with N mice, while in humans these bacteria are known to be increased during aging. This might suggest that df/df mice are protected from overpopulations of these bacteria from early life on. Yet, this can be only speculated until further longitudinal studies are available. More importantly, it would be of great value to follow-up with microbiota studies in humans with IGHD type 1B, caused by a mutation of the GHRH receptor gene or individuals affected by Laron dwarfism [51], to obtain a clearer relevance for our findings and their translational value.

It has been previously reported that the genus *Lactobacillus* was significantly increased in CR [52]. The increase of *Lactobacillus* relative abundance pairs well to lower cholesterol and triglycerides levels as an effect of CR [52], although it is not clear which changes occur first. Calorie restriction is a well-known experimental method which can lead to decreased bodyweight, decreased plasma insulin and glucose levels, and improved insulin sensitivity, health span and lifespan [16]. Knowledge about the effects of CR on the gut microbiota is limited; however it was previously shown that diet is an important factor affecting the gut microbiota during aging [53]. The gut microbiota plays an important role in regulating the effects of a diet intervention including weight loss, maintenance of a high basal metabolic rate, and a decrease in blood glucose and serum cholesterol level [54]. Furthermore, it was demonstrated that antibiotic treatment eliminates the metabolic-regulatory functions of the bacteria in the gut and leads to loss

of the health benefit of the gut microbiota [54]. In our study, we aimed to investigate how the CR treatment in df/df and control mice may affect bacteria composition in three different GI locations. CR had a more pronounced impact on the microbiome in N than df/df mice. Moreover, we observed in N-AL mice, an overabundance of different members of the order Bacteroidales in ileum and a significant enhancement of the family Erysipelotrichaceae in cecum und colon. However, Erysipelotrichaceae was not altered in CR. Erysipelotrichaceae plays a central role in the development of metabolic disorders [55]. Furthermore, larger amounts of this bacteria family were observed in obese individuals [56]. A recent study showed that depletion of gut microbiota rendered mice resistant to CR-induced loss of body weight, resulting in increased fat mass [54], suggesting that the microbiota might be the central mediator of CR effects. In fact, df/df mice benefit from the effects of CR [57]. However, changes in body weight are not so pronounced as in N mice [58], and some effects on insulin signaling are abolished by GH replacement [59]. This evidence is in line with our findings that df/df mice microbiota diversity is less affected by CR than it is in N mice. Based on this and previous findings, not all mice models benefit from CR effects on longevity. Therefore, we believe that it would be important to study in detail the microbiota in humans exposed to long-term caloric restriction, which would provide important knowledge towards future functional studies, which could be followed with ongoing human CR studies or with help from volunteers involved in CRON-diet (Calorie Restriction with Optimal Nutrition).

In summary, our study underlines the different gut microbiota in GH-deficient df/df mice compared with N mice. There were significant differences in the B/F ratio and genotype-dependent alterations in the microbiota composition during postnatal development. We also showed that changes of microbiota from df/df mice were

associated with parental vertical transmission. Furthermore, we observed that changes of the gut microbiota after a dietary treatment were more evident in N than df/df mice, reflecting the divergent effects of CR on the different starting microbial communities. Further studies will be required for a better understanding of the role of GH deficiency and CR in the regulation of the gut microbiome. Nevertheless, one could hypothesize that gut microbiome composition likely contributes to the phenotypic differences and health benefits that we see in long-lived df/df mice and could be considered a new potential hallmark of age-related and metabolic alterations.

Conflict of Interest

None

Acknowledgments

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Individual contributions to the present work was as follows: conceptualization:

D.W., K.S., A.S. and MM; methodology: E. G., L. S., B. V., T. S. and M. M.; software: E. G. and T. S.; validation: D.W., K. A., A. A. and M. M.; formal analysis: E. G., T. S., K. S., D. W., and M. M.; experimental investigation: D.W., K.S., A.S., L. S., B. A., K. A., A.G. and M. M.; resources: K.S., T. S. and M. M.; manuscript writing, reviewing, and editing: D.W., E. G., L.S., B. V., B.A., A.S., A.G., K. A., T.S., K.S. and M. M.; supervision: K.S. and M. M.; funding acquisition: M. M..

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Figure1. Gut microbiota variation in N and df/df littermates is explained by genetics and breeding pair

(A) Experimental design for microbiome normalization using littermates Normal (N) and Ames dwarf mice (df/df). Fecal samples for DNA isolation were taken from parents (P-N, P-df/df) and offspring C2 (2nd week) and C4 (10th -week) after weaning. Luminal samples were collected from indicated locations (GI locations: Ileum, Caecum and Distal Colon) at C4.

(B) Alpha-diversity estimation of offspring fecal microbiome at C2 and C4 using observed-richness and (C) Shannon index. Ratio of Bacteroidetes /Firmicutes (calculated as the relative abundance of Bacteroidetes /sum(Firmicutes + Bacteroidetes)) at timepoint C2 and C4. Mann-Whitney U test of Bacteroidetes /Firmicutes ratio at C4.

(D) Beta-diversity analysis. Variance effect size using ADONIS test and NMDS ordination analysis of microbiota composition using Bray-Curtis distances at timepoint C2 and C4.

(E) Weighted UniFrac distances of samples by genotype at timepoint C4. In blue, comparisons between the same category, in red, inter-group comparison.

(F-G) Taxonomic bar plot of dominant bacterial families per sample (top 14 Families) sorted by Bacteroidetes /Firmicutes ratio.

(H) Differential abundance analysis (DA) between df/df and N using LEfSe and DESeq2. Upper chart displays LEfSe comparison at Family level ($\log_{10}(\text{LDA score}) > 3.5$). Bottom, MA plots displays DA OTUs (Up: red; down: blue; P values < 0.05 after correction for multiple tests). For ADONIS test a significant effect was attributed when

P-value is < 0.05 and R^2 is > 0.10 (equivalent to 10% of explained variance);
*** $P < 0.001$ ** $P < 0.01$, * $P < 0.05$.

Figure 2. Distinct microbial composition in Ileum and Cecum in df/df mice

Beta-diversity analysis of intestinal microbiota in distinct anatomical sites at timepoint C4. Variance effect size using ADONIS test and NMDS ordination analysis using Bray-Curtis distances from luminal samples in (A) Ileum and (B) Cecum. Taxonomic bar plot of dominant bacterial families per sample (top 14 Families) sorted by Bacteroidetes /Firmicutes ratio (C-D).

(E-H) Differential abundance analysis (DA) between df/df and N using LEfSe and DESeq2. Upper chart displays LEfSe comparison at Family level ($\log_{10}(\text{LDA score}) > 3.5$). Bottom, MA plots displays DA OTUs (Up: red; down: blue; P values < 0.05 after correction for multiple tests). A significant effect was attributed when P-value is < 0.05 and R^2 is > 0.10 (equivalent to 10% of explained variance); *** $P < 0.001$ ** $P < 0.01$, * $P < 0.05$.

Figure 3. df/df mice offspring recapitulate genotype-associated microbiome composition

(A) Beta-diversity analysis of offspring vs parents (homozygotes df/df and heterozygotes N). Variance effect size of “Genotype”, “Gender” and the interaction between “Generation and genetics” using ADONIS test and NMDS ordination analysis using Bray-Curtis distances of colonic samples.

(B-C) Comparison of Weighted UniFrac distances of homozygotes df/df and heterozygotes N offspring vs parents. In blue, distances between the same category, in red, inter-group comparisons.

Figure 4. Gut microbiota composition associated with calorie restriction in N and df/df mice.

(A) Experimental design for microbiome normalization using littermates Normal (N) and Ames dwarf mice (df/df). Fecal samples for DNA isolation were taken from parents (N-AL, N-CR, df/df-AL, df/df-CR). Luminal samples were collected from indicated locations (GI locations: Ileum, Caecum and Distal Colon) after the mice were sacrificed.

(B-D) Alpha-diversity estimation using observed-richness, Shannon index and Firmicutes/Bacteroidetes ratio.

(E-G) Beta-diversity analysis of intestinal microbiota in distinct anatomical sites. Variance effect size using ADONIS test and NMDS ordination analysis using Bray-Curtis distances from luminal samples.

Figure 5. The Effect of Calorie Restriction in N and df/df mice

(A) Taxonomic bar plot of dominant bacterial families per sample (top 16 Families) sorted by Bacteroidetes /Firmicutes ratio.

(B-D) Differential abundance analysis (DA) between df/df and N using LEfSe comparison at Genus level ($\log_{10}(\text{LDA score}) > 3.5$).

(E) Taxonomic bar plot of dominant bacterial families per sample (top 16 Families) sorted by Bacteroidetes /Firmicutes ratio.

(F-G) Differential abundance analysis (DA) between df/df and N using LEfSe comparison at Genus level ($\log_{10}(\text{LDA score}) > 3.5$).

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5