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The Effect of Lifespan Extending Mutations on Healthspan in Ames Dwarf Mice

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List of Abbreviations

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ANOVA	Analysis of variance
AL	Ad Libitum
Ba(OH)2	Barium hydroxide
B/F	Bacteroidetes and Firmicutes
bp	base pair
C1	Collection 1
C2	Collection 2
C3	Collection 3
C4	Collection 4
CR	Calorie Restriction
CRON	Calorie Restriction with Optimal Nutrition
DA	Differential abundance
df/df	Ames dwarf
2-DG	2-Deoxyglucose
dL	deciliter
DNA	Desoxyribonucleic Acid
endoRa	Rate of endogenous glucose appearance
etc.	et cetera
F0	Parents (genotype mother: N/df; father: df/df)
F1	Offspring (genotypes: N and df/df)
Fig.	Figure
g	gram
GH	Growth Hormone
GHR	Growth Hormone Receptor
GHRH	Growth Hormone Releasing Hormone
GHRKO	Growth Hormone Receptor Knockout
GI	Gastrointestinal
GM	Gut Microbiome
GTT	Glucose Tolerance Test
h	Hour
HPLC	High-performance liquid chromatography
i.e.	id est
IGF-1	Insulin-like growth factor 1

IGHD	Isolated growth hormone deficiency type 1B
IR	Insulin receptor
IRS1	Insulin receptor substrate-1
ITT	Insulin Tolerance Test
KOH	Potassium hydroxide
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis Effect Size
mg	milligram
min.	Minute
ml	Milliliter
mTOR	mammalian target of rapamycin gene
N	Normal
n	Number
ng	Nanogram
NIA	National Institute on Aging
nm	Nanometer
NMDS	Non-metric multidimensional scaling
ob/ob	obese
OTU	Operational taxonomic unit
P	Parents
p-value	probability value
PCA	Perchloric acid
PCR	Polymerase Chain Reaction
PRL	Prolactin
Prop1$^{df/df}$	Prophet of pituitary factor 1 gene
Pit-1	Pituitary-specific transcription factor 1
PPE	Personal protective equipment
Ra	Rate of glucose appearance
Rd	Rate of glucose disappearance
RDP	Ribosomal Database Project
Rg	Glucose metabolic index
rRNA	ribosomal ribonucleic acid
s	seconds
spp.	several species
SEM	Standard Error of the Mean
t	time

TSH	Thyroid-stimulating hormone
UCF	University of Central Florida
vs.	versus
ZnSO4	Zinc sulfate
µCi	Microcurie
µl	Microliter
%	Percent
°C	Degree Celsius

Abstract

Ames dwarf (df/df) mice, exhibit a significant increase in longevity, and carry a homozygous and spontaneous mutation of the *prophet of pituitary factor 1* gene (*Prop1*$^{df/df}$), which inhibits the development of the anterior pituitary cells: somatotrophs, lactotrophs and thyrotropes. These mice, are therefore characterized by a growth hormone, prolactin and thyrotropin deficiency. Ames dwarf mice, are widely used as a valuable aging research model because they have a significantly longer (40-60%) and healthier lifespan compared to their normal (N) littermate controls. In addition to this extraordinary longevity, df/df mutants carry significant protection from the majority of age-related diseases including insulin resistance, metabolic syndrome, diabetes, cancer or neurodegeneration. Ames dwarf mice exhibit decreased levels of fasting insulin and glucose as well as an improved glucose clearance measured by glucose tolerance test combined with a high sensitivity to injected insulin. These mutants have also shown to have enhanced insulin signaling in diverse insulin target organs. Importantly, insulin tolerance test (ITT), a measure of insulin sensitivity, showed that there is a positive correlation between insulin sensitivity and longevity. However, the basic ITT does not reveal insulin sensitivity in different insulin-responsive organs including skeletal muscle, adipose tissue as well as glucose regulation in the liver. To investigate this tissue-specific response, for the first time, the hyperinsulinemic-euglycemic clamp study was performed *in vivo* in long-living df/df mice. These findings showed that in df/df mice the glucose infusion rate needed to be ~2-fold higher than in normal (N) control mice to maintain euglycemia at a rate of approximately 160 mg·dL^{-1}. This study presented that df/df mice had significantly greater uptake of glucose in the gastrocnemius and vastus muscles along with adipose tissue. More importantly, there was a pronounced hepatic response in df/df mice indicated by complete suppression of endogenous glucose production, while in N mice only 60% suppression was achieved during the clamp. In addition to the hyperinsulinemic-euglycemic clamp study, the effects of Ames dwarfism mutation on gut microbiota development in df/df and N mice was also investigated. The gut microbiome was never studied before in these long-living dwarf mice, while there is an overall increasing interest in understanding gut microbiome in diverse diseases including diabetes, cardiovascular diseases, cancer, aging and many others. There is also strong evidence showing that the gut microbiome changes over the lifespan of an individual and might be related to development or protection from diverse age-related diseases. In this study, the changes in the gut microbiome of df/df and N mice were examined through a comparison of parents before mating and littermate mice at three different time points during early life development. Furthermore, as calorie restriction is shown to be an intervention that significantly enhances longevity in animal models, it was studied the effects of a 6-month calorie restricted (CR) diet on the microbiota. These results show that the gut microbiota

composition changes significantly with the aging process and it also demonstrates divergences in the abundance of several bacteria when comparing df/df mice with N littermates already during early life development. Overall, the gut microbiota showed significant differences in genotype, time-point and in the animal breeding pair when comparing df/df and N mice. Additionally, it was also demonstrated that CR causes significant changes in the gut microbiome when comparing different GI location (Distal Colon, Ileum and Cecum), genotypes and the diet. Overall, the effect of the genotype was more evident than the one of the diet.

In conclusion, the results of the hyperinsulinemic-euglycemic clamp study suggests that improved insulin sensitivity in various insulin responsive organs and enhancement in overall metabolic condition might promote prolonged life-span of Ames dwarf mice. In addition, these findings of the gut microbiota study indicated that the microbiota have significant impact during postnatal development in promoting longevity in df/df mice and that CR could also modulate longevity by altering gut microbiota. In summary, it can be said that both studies have shown potential novel longevity markers in Ames dwarf mice.

Zusammenfassung

Ames dwarf (df/df) Mäuse weisen eine signifikant höhere Lebenserwartung auf und tragen eine homozygote und spontane Mutation des Gens, welches als *prophet of pituitary factor 1* gene (*Prop1*$^{df/df}$), bezeichnet wird. Aufgrund dessen gibt es keine Entwicklung der vorderen Somatotropin, Lactotropin und Thyreotropin produzierenden Hypophysenzellen. Diese Mäuse sind daher durch einen Mangel an Wachstumshormon, Prolaktin und Thyreotropin gekennzeichnet. Ames dwarf Mäuse werden häufig als wertvolles Forschungsmodell für das Altern verwendet, da sie im Vergleich zu ihren normalen (N) Wurfgeschwister-Kontrollen eine signifikant längere (40-60%) und gesündere Lebensdauer haben. Zusätzlich zu der verlängerten Lebensdauer sind die df/df-Mutanten auch vor den meisten altersbedingten Krankheiten wie Insulinresistenz, Metabolisches Syndrom, Diabetes, Krebs oder neurodegenerativen Erkrankungen geschützt. Ames dwarf Mäuse weisen einen verringerten Nüchterninsulin- und Glukosespiegel sowie eine verbesserte Glukose Freigabe auf. Letztere wurde durch einen Glukosetoleranztest und durch eine hohe Empfindlichkeit gegenüber injiziertem Insulin gemessen. Zusätzlich konnte gezeigt werden, dass diese Mutanten eine verstärkte Insulinsignalisierung in den verschiedenen Insulin-Zielorganen aufweisen. Der Insulintoleranztest (ITT), welcher ein Maß für die Insulinsensitivität ist, zeigte, dass eine positive Korrelation zwischen Insulinsensitivität und Langlebigkeit besteht. Allerdings zeigt der grundlegende ITT keine Insulinsensitivität in den verschiedenen auf Insulin ansprechenden Organen an. Betroffen sind hier der Skelettmuskel, das Fettgewebe sowie die Glukoseregulierung in der Leber. Um diese gewebespezifische Reaktion zu untersuchen, wurde zum ersten Mal die hyperinsulinämisch-euglykämische Clamp-Studie *in vivo* an langlebigen df/df-Mäusen durchgeführt. Diese Ergebnisse haben gezeigt, dass bei df/df-Mäusen die Glukoseinfusionsrate ~ 2-fach höher sein musste als bei den normalen (N) Kontrollmäusen, um die Euglykämie bei gleicher Insulininfusion bei etwa 160 mg·dL^{-1} zu halten. Ebenfalls hat diese Studie gezeigt, dass df/df-Mäuse eine signifikant höhere Aufnahme von Glucose im Gastrocnemius- und Vastus-Muskel sowie im Fettgewebe aufwiesen. Noch wichtiger ist, dass bei df/df-Mäusen eine ausgeprägte Leberreaktion auftrat, die durch eine vollständige Unterdrückung der endogenen Glukose Produktion angezeigt wurde, während bei N-Mäusen nur eine Unterdrückung von 60% erreicht wurde. Zusätzlich zur hyperinsulinämisch-euglykämischen Clamp-Studie wurden auch die Auswirkungen der Ames dwarf Mutation auf die Entwicklung der Darmmikrobiota bei df/df- und N-Mäusen erforscht. Das Darmmikrobiom wurde noch nie zuvor bei diesen langlebigen Zwergmäusen untersucht, während das Interesse am Verständnis des Darmmikrobioms bei verschiedenen Krankheiten wie Diabetes, Herz-Kreislauf-Erkrankungen, Krebs, Altern und vielen anderen insgesamt zunimmt. Es gibt auch starke Hinweise darauf, dass sich das Darmmikrobiom im Laufe der

Lebensdauer eines Individuums verändert und möglicherweise mit der Entwicklung oder dem Schutz vor verschiedenen altersbedingten Krankheiten zusammenhängt. In dieser Studie wurden die Veränderungen im Darmmikrobiom von df/df- und N-Mäusen durch einen Vergleich der Eltern vor der Paarung und der Wurfgeschwister zu drei verschiedenen Zeitpunkten während der frühen Lebensentwicklung untersucht. Da sich gezeigt hat, dass Kalorienreduzierung eine Intervention ist, die die Langlebigkeit in Tiermodellen signifikant verlängert, wurden die Auswirkungen einer 6-monatigen kalorienreduzierten (CR) Diät auf die Mikrobiota untersucht. Die Ergebnisse haben gezeigt, dass sich die Zusammensetzung der Darmmikrobiota mit dem Alterungsprozess signifikant verändert. Zusätzlich wurden Unterschiede in der Häufigkeit mehrerer Bakterienstämme zwischen df/df- und N-Mäusen bereits während der frühen Lebensentwicklung gezeigt. Insgesamt zeigte das Darmmikrobiom beim Vergleich von df/df- und N-Mäusen signifikante Unterschiede im Genotyp, Zeitpunkt und Brutpaar. Zusätzlich wurde gezeigt, dass CR signifikante Veränderungen im Darmmikrobiom verursacht, wenn die verschiedenen Bereiche innerhalb des Gastrointestinaltrakts (Distal Colon, Ileum und Cecum), die Genotypen und auch die kalorienreduzierte Diät verglichen werden. Insgesamt war die Auswirkung innerhalb des Genotyps jedoch deutlicher zu sehen als die der Diät.

Zusammenfassend legen die Ergebnisse der hyperinsulinämisch-euglykämischen Clamp-Studie nahe, dass eine verbesserte Insulinsensitivität in den verschiedenen auf Insulin ansprechenden Organen sowie eine Verbesserung des gesamten Stoffwechsels, die verlängerte Lebensdauer von den Ames dwarf Mäusen fördern könnte. Darüber hinaus haben die Ergebnisse der Darmmikrobiota-Studie gezeigt, dass die Mikrobiota während der postnatalen Entwicklung eine wichtige Rolle bei der Förderung der Langlebigkeit bei df/df-Mäusen spielt und dass CR auch die Lebensdauer durch Veränderung der Darmmikrobiota modulieren könnte. Zusammenfassend kann gesagt werden, dass die beiden untersuchten Studien neue potenzielle Langlebigkeitsmarker bei Ames dwarf Mäusen gezeigt haben.

1. Introduction

1.1 Aging

Aging is an organism's progressive, biological and irreversible process, ending with death. This process is linked with a time-dependent progressive rise in disease vulnerability. Nearly all recognized organism’s age. Despite the fact, that the maximum duration of life varies from one organism to another, the curve shape, which is also indicative of the organism's health, is extremely consistent throughout species (MITCHELL et al., 2015). Until the end of the 21st century, the proportion of human population over the age of 65 is expected to rise from about 7% to more than 20% worldwide (http://esa.un.org/wpp/). In response to this aging phenomenon, the elderly as well as the general population, have become extremely unhealthy, independent of a slight increase in life span of a small increase in life expectancy over the last decades (FREID et al., 2012). Because of this incoming unhealthy lifestyle and the rising elderly population, it is becoming increasingly more important to investigate factors that lead to a healthy aging. In recent decades, work into the root causes of aging has contributed to impressive developments, not just in comprehension of the mechanisms of aging but also in interventions that may improve life expectancy and, most significantly, healthspan (BARTKE, 2016). Model organisms, including *C. Elegance, Drosophila,* laboratory studied rodents and non-human primates have actually been at the frontline of this research and have provided riches of knowledge, helping us to decipher pathways that might regulate human aging. Mammalian target of rapamycin (mTOR) is one of the well-established modulators of aging, and its inhibition has been shown to extend life expectancy in flies, yeast as well as nematodes (HARRISON et al., 2009; JIA et al., 2004; KAEBERLEIN et al., 2005; KAPAHI et al., 2004; LAMMING et al., 2012). Model organisms are critical for aging studies, as ethical concerns, long natural life period, ecological impacts, genetic heterogeneity, and numerous other restricting elements complicate the use of human subjects in aging research. Excellent progress has been made after McCay's crucial reports on explaining the lifespan expansion of calorie restriction (CR) in rats (MCCAY et al., 1975). The maximum lifespan of an individual is driven by aging. There are many mutant or knockout animals which are characterized by an extended lifespan, providing valuable tools for studying the molecular mechanisms of aging. Based on previous research studies, growth hormone (GH) and insulin-like growth factor 1 (IGF-1) are important hormones, which are involved in the regulation of aging and longevity (MASTERNAK, BARTKE, 2012). Throughout aging individuals are more vulnerable to age-related diseases including diabetes, atherosclerosis, cancer, Alzheimer disease, osteoporosis, etc. along with a drastic decline in the immune system function and chronic conditions and triggering inflammatory processes (FRANCESCHI et al., 2000). Due to a global increase in the

population of the elderly over 65 years old, there is a rising interest in studying the mechanisms of aging with a focus on determining molecular and cellular processes that regulate aging, healthspan and lifespan. Several hypotheses have been developed during the decades of aging studies attempting to explain the aging phenomena, and two major theories were classified. The first theory is based on programmed aging and the second theory is called the wear and tear theory (JIN, 2010).

1.2 Models of Aging

Many aging models have been established which demonstrate that genes play an important role in extending life span. Due to their delayed aging, the Ames dwarf, Snell dwarf, and growth hormone (GH) knockout receptor (GHRKO) mice are the typical mouse models used for aging studies. Such strains demonstrate extraordinary longevity by alterations in the GH-pathway which leads to low-circulating IGF-1 (FLURKEY et al., 2001; HSIEH et al., 2002). Both Ames and Snell dwarf mice have a mutation due to a loss of function of the *Prop-1* and *Pit-1* genes which leads to a lifespan extension due to the resulting deficiencies in circulating levels of thyrotropin, prolactin, and GH (BARTKE, BROWN-BORG, 2004). Ames dwarf mice show a remarkably 40-60% increase of their longevity (BARTKE, BROWN-BORG, 2004). The Snell dwarf mice live up to 50% longer compared to their wild-type littermates (BARTKE, BROWN-BORG, 2004; FLURKEY et al., 2001). These mice reveal some of the features of CR, consisting of a lower body temperature (HUNTER et al., 1999), enhanced insulin sensitivity (HSIEH et al., 2002), improved antioxidant defenses (ROMANICK et al., 2004), and also a postponed onset of neoplasia (BARTKE et al., 2007; IKENO et al., 2003). These factors might play a major role for their enhanced lifespan. The GHRKO mouse was developed, by the targeted disruption of the GH receptor as well as GH-binding protein (FLURKEY et al., 2001). The amino acid sequence of the growth hormone-binding protein (GHBP) is the same as the extracellular constituent of the GH receptor component (GHR) (BAUMANN, 2002). Growth hormone (GH) signaling pathway disruption enhances the insulin sensitivity and is related to delayed aging and a prolonged longevity (GESING et al., 2017). GH receptor gene-disrupted knockouts (GHRKO) mice are actually defined through a reduced GH axis along with a significant reduction of body size as well as lowered plasma insulin-like growth factor-1 (IGF-1) and also insulin levels (GESING et al., 2017). These mice are long-living and have a decrease in glucose, insulin, thyroid hormones, as well as in body temperature (FANG et al., 2020). All of these characteristics are similar to that observed in the df/df mice (HAUCK et al., 2001). The GHRKO mice reveal a comparable rise in life expectancy across males and females of 23% and 25%, respectively (FLURKEY et al., 2001). Decreased levels of glucose,

insulin and thyroid hormones, as well as reduced body temperature might be very important to the fundamental mechanisms of delayed aging in these animals. Surprisingly, GHRKO mice are obese, yet are sensitive to insulin (FLURKEY et al., 2001), which is paradoxically contrary to what was observed in CR. A recent study that analyzed the role of visceral fat in adiposity and insulin sensitivity showed that removal of visceral fat caused an increase in insulin sensitivity in N mice, whereas it made the GHRKO mice more resistant to insulin (MASTERNAK et al., 2012). Interestingly, there is no lifespan expansion when GHRKO mice are subjected to CR (BONKOWSKI et al., 2009; BONKOWSKI et al., 2006), possibly because CR decreases adiposity, which may not be advantageous for GHRKO mice (MASTERNAK et al., 2012). The GHRKO mice attain lifespan extension through a mechanism that overlaps the effects of CR, considering that CR cannot further increase longevity in these mutants. In the current study, long-living df/df mice were used to investigate detailed regulation of insulin sensitivity in different insulin signaling organs and the impact of Ames dwarfism mutation on health and gut microbiota. The gut microbiota showed variations in long-living df/df mice during early life development which could represent major changes in bacteria populations that can promote a healthy lifespan during aging through differential stimulation of immune responses and overall lower inflammatory grade.

1.3 Ames dwarf mice

Ames dwarf mice (df/df) were first described in 1961 by Schaible and Gowen (SCHAIBLE R, 1961). They discovered that long-living df/df mice possess a homozygous, recessive mutation of the *prophet of pituitary factor* 1 gene (*Prop1*$^{df/df}$) (SORNSON et al., 1996). The mutation of the *Prop1* gene in mice triggers dwarfism through lack of GH production due to a compromised development of anterior pituitary cells. Additionally, as a result of this mutation, df/df mice are also characterized by a deficiency of both prolactin (PRL) and thyroid stimulating hormone (TSH); yet as mentioned earlier, the mice exhibit an extended lifespan and healthspan (BARTKE, 2000; SORNSON et al., 1996). Ames dwarf mice appear normal at birth however they develop slower in contrast to their wild type littermates and also gain only one-third of the regular adult body weight (Fig. 1). Due to PRL deficiency, their sex-related maturation is delayed; with females being unable to maintain healthy pregnancy unless a PRL replacement therapy is provided throughout the pregnancy (BARTKE, 1964; BARTKE, BROWN-BORG, 2004). Ames dwarf mice have reduced levels of IGF-1 and thyroid hormones, and lower plasma glucose and decreased levels of insulin. Moreover, they exhibit enhanced insulin sensitivity as well as increased hepatic sensitivity to insulin as well as enhanced antioxidant defenses, that goes along with an improved resistance to oxidative stress (BARTKE, BROWN-

BORG, 2004). They present with a decreased body temperature (HUNTER et al., 1999), however their food intake as well as their oxygen intake per gram of body weight are elevated (MATTISON et al., 2000; WESTBROOK et al., 2009). Ames dwarf mice live considerably longer and much healthier lives than their N siblings. These GH-deficient mice are very insulin sensitive, glucose tolerant, and do not show a diabetic phenotype. Furthermore, df/df mice are less susceptible to cancer (MASTERNAK, BARTKE, 2012). Ames dwarf mutants display a number of qualities of CR mice such as decreased body size, suppressed IGF-1, insulin, glucose and lower body temperature, however they are not CR mimetics. When subjected to CR, they exhibit a further increase in both longevity and insulin sensitivity (MATTISON et al., 2000).

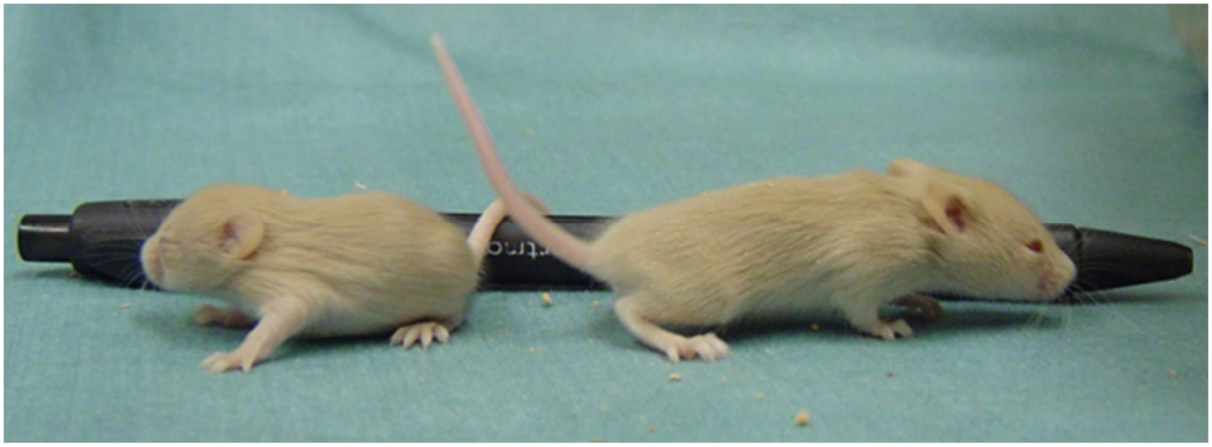


Figure 1 - Ames dwarf and Normal littermate control mouse at an age of two weeks.
(Ames dwarf on the left, Normal littermate control on the right).

1.4 Calorie Restriction

Calorie Restriction (CR) is a well-established dietary intervention to extend the lifespan by a reduction in calorie intake (WEINDRUCH et al., 1986). Early rodent studies have shown that a 30-60% decrease in calorie consumption significantly enhances life expectancy and improves healthspan by delaying the onset of and also protecting animals from variety of age related conditions (ANDERSON et al., 2009) (Fig. 2).

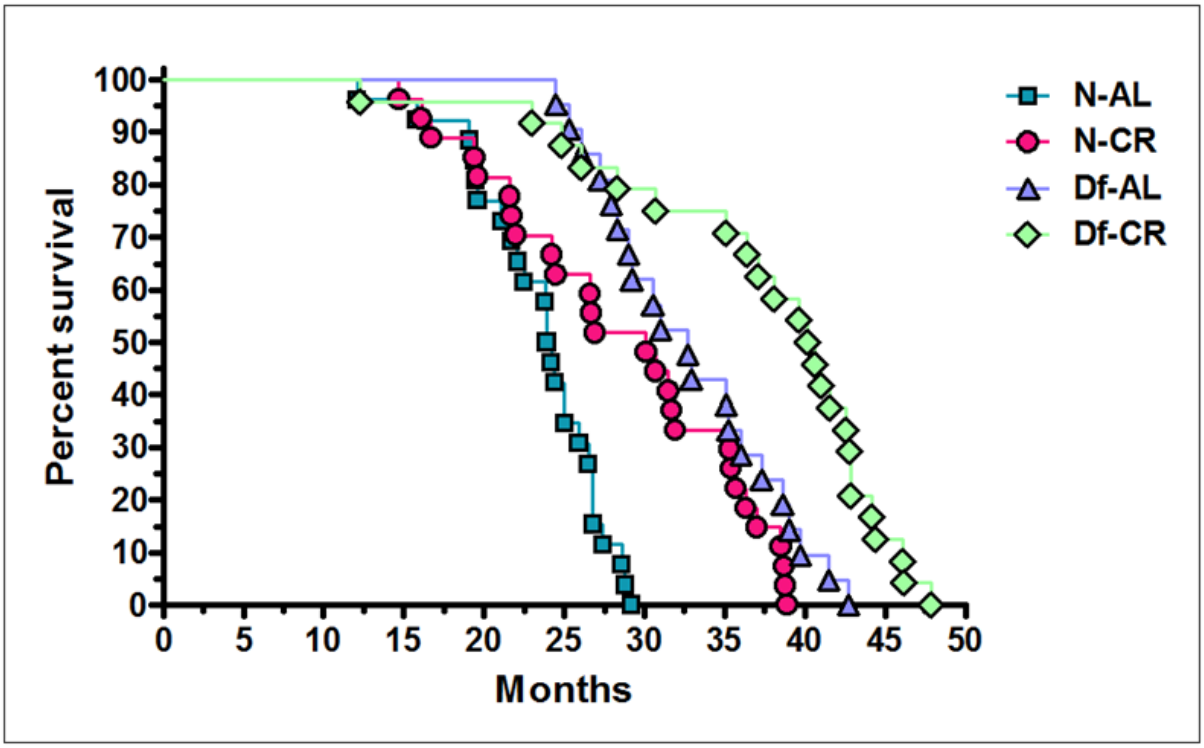


Figure 2 – Kaplan-Meier survival plot presenting long-living Ames dwarf (df/df) and normal littermate control (N) mice subjected life-long calorie restriction (30% CR) or fed ad libitum (AL). Figure taken from Bartke et al. (BARTKE et al., 2001b).

The survival graph in Fig. 2 presents the maximum life expectancy expansion of df/df-AL and N-AL mice and also in df/df-CR and N-CR mice. These data indicate that CR increases the lifespan to the longevity maintained by long-living df/df animals, yet the dwarf mice showed additional lifespan extension in response to CR indicating that the mechanism of CR and Ames dwarfism is not identical (Fig. 2). This implies that CR influences different pathways than that seen in df/df mice which leads to an increase in longevity (BARTKE et al., 2001b). The reduction of body fat as well as the enhancement in insulin sensitivity are some positive effects of CR. CR also reduces the levels of plasma glucose and insulin, correlating to improved insulin sensitivity (MASTERNAK et al., 2009). Various animal studies have shown that CR slows aging and also reduces levels of cholesterol and blood pressure (OMODEI, FONTANA, 2011). In particular, studies with different rodents including mice and rats support the concept of CR causing delayed aging, and the onset of various age-related diseases such as type 2 diabetes or cancer. It has been shown that longevity can be greater than 40% when mice are subjected to CR; Moreover, it could be demonstrated that CR increases the lifespan not only in rodents but also in other animals such as rhesus monkeys, *Drosophila melanogaster*, *Caenorhabditis elegans* or yeast (MASORO, 2006). Additionally, it was also be shown that in non-mammalian models i.e., yeast, worms and flies, the lifespan extension is even more pronounced (FONTANA et al., 2010; WEINDRUCH et al., 1986). Several studies have shown that animals

subjected to CR, with the same chronological age, are physiologically younger than their N controls (MASORO, 2006). Studies with rats indicated that CR reduces the rate of the age-related mortality (FINCH, HAYFLICK, 1977; MASORO, 2006). In addition, patient-based studies revealed multiple positive effects including bodyweight and adiposity reduction as well as significant improvement in blood metabolites (RACETTE et al., 2006), protection against atherosclerosis (FONTANA et al., 2004), improvement of memory in older individuals (WITTE et al., 2009) and also enhancement of heart functionality (MEYER et al., 2006). Existing data suggests that the impact of CR on the aging process may be regulated through a decrease in glucose, by means of the glycation reactions resulting in advanced glycosylation end-products, and thus altering protein and DNA stability (MASORO et al., 1989). Furthermore, the influence of CR on the aging process may also be regulated through oxidative damage, or even adjustments in certain gene expression (MORLEY et al., 1988).

1.5 Insulin sensitivity in long-living Ames dwarf mice

Previous studies revealed that improved insulin activity as well as glucose homeostasis appears to be a crucial aspect for preserving healthy aging (BARBIERI et al., 2003; MASTERNAK et al., 2009; WIJSMAN et al., 2012). A disrupted glucose and insulin homeostasis are often associated with increased fat content in the body. Aging is known to increase the risk for metabolic disorders including resistance to insulin, which is one of well recognized causes of type 2 diabetes mellitus, cardiovascular diseases and chronic inflammations. Moreover, those diseases have been recently connected to the development of cancer (KINTSCHER et al., 2008; LAGO et al., 2007; NELSON et al., 2004; PAN et al., 2006; PRADHAN et al., 2001; WEISBERG et al., 2003; WILLERSON, RIDKER, 2004). Earlier research study has shown that alterations in insulin as well as insulin-like growth factor-1 (IGF-1) signaling expands the life expectancy and also reduces aging in rats, nematodes, as well as in flies (TATAR et al., 2003) mentioning that this signaling pathway is therefore a primary factor in the regulation of healthy aging. Notably, the impact of this signaling pathway on aging seems also to be applicable in humans (FONTANA et al., 2010). It is well known that glucose tolerance decreases with advanced age. In humans, there is a decrease in glucose resistance which starts in the third or fourth decade and also proceeds throughout the whole adult lifespan (DEFRONZO, 1981). The main problem of this age-related damage in glucose metabolism is that the cells become resistant to the insulin effect. Increasing insulin resistance caused rising glucose levels known as glucose intolerance (CHANG, HALTER, 2003). Many factors possibly contribute to insulin resistance, including decreased physical activity as well as reduced muscle mass. However, the aging process seems to have its own deleterious effect on the

tissue sensitivity to Insulin (DEFRONZO, 1981). Since ages, scientists correlate human long life with a reduced level of insulin resistance (BARBIERI et al., 2003). That supports the idea that remarkably long-living individuals are insulin delicate during their whole life expectancy, and maybe even genetically shielded from an age-related decrease of insulin activity. Ames dwarf mice live much longer than their N siblings due to lack of development of the anterior pituitary cells, and reveal many symptoms of delayed aging (BARTKE, BROWN-BORG, 2004). One of the most essential factors is, that these GH-deficient mice display an enhanced response to administered insulin, they exhibit a reduced fasting insulin as well as glucose concentrations. They have also a high concentrations of serum adiponectin (WIESENBORN et al., 2014b). Additionally, they are healthier along with getting older and also show equivalent functions to those discovered in centenarians (BARBIERI et al., 2003; MASTERNAK et al., 2009; MASTERNAK et al., 2010). Analysis of genes and proteins regulating insulin action in various organs and also basic glucose and insulin tolerance examinations sustains the hypothesis that enriched insulin signaling in long-living df/df mice is actually strongly associated along with lifespan expansion (MASTERNAK et al., 2004; MASTERNAK et al., 2005; MASTERNAK, BARTKE, 2007, 2012; MASTERNAK et al., 2009; MASTERNAK et al., 2010; WANG et al., 2006). Along with a variety of other mutations, there are well-known interventions that expand the life expectancy in lab animals (BARTKE, BROWN-BORG, 2004). One of the most highly effective intervention in research laboratory animals is CR which enhances the insulin sensitivity and also prolongs the lifespan (BARTKE, BROWN-BORG, 2004; BARTKE et al., 2001b; MASTERNAK et al., 2009). There is not sufficient evidence whereby CR can increase the lifespan in humans. Nonetheless, it is well examined that a decrease of calorie consumption improves the level of insulin sensitivity, glucose homeostasis and also sustains a healthy metabolic process in human beings. In addition, a pharmacological study with rapamycin indicated significant suppression of mammalian target of rapamycin gene (mTOR) and displayed successful lifespan extension in mice (HARRISON et al., 2009; MILLER et al., 2011). Noticeably, long life therapy with rapamycin does not enhance the insulin level of sensitivity (MILLER et al., 2008) however it might instead foster a glucose intolerance as well as insulin resistance (BLAGOSKLONNY, 2011). Extended treatment with rapamycin advertises a metabolic altering and also adjustments by enhancing level of insulin sensitivity and additionally expanding long life (FANG, BARTKE, 2013). These studies imply that healthy insulin and glucose metabolism is crucial for long life. However, it is not yet established whether systemic or tissue-specific insulin sensitivity is crucial for healthy aging. In this study, it was investigated if enhanced insulin signaling in df/df mice correlates with improved tissue-specific insulin activity *in vivo* (WIESENBORN et al., 2014a). Hyperinsulinemic-euglycemic clamps along with tracer techniques were carried out to analyze whole-body and also tissue-specific

insulin activity *in vivo* for its very first time in long-living df/df mice (WIESENBORN et al., 2014a). The hyperinsulinemic-euglycemic clamp is well-established as a "gold standard" procedure to assess insulin responsiveness *in vivo* (AYALA et al., 2011; BERGLUND et al., 2008; MCGUINNESS et al., 2009; TAM et al., 2012). Throughout the clamp study, insulin is infused at a steady rate to accomplish physiological or pharmacological hyperinsulinemia. Through injecting glucose at a variable rate and also a continual measuring of blood glucose level alongside following adjustment of the glucose infusion rate, euglycemia is achieved. Thus, this technique enables the measurement of insulin activity independent of modifications in circulating glucose. To analyze insulin action in different insulin target organs, including subdue of hepatic glucose manufacturing and also stimulation of organ specific glucose uptake, radioactively-labeled glucose analogs have been applied.

1.6 The Gastrointestinal tract

The gastrointestinal (GI) tract is an organ system in animals and humans, which is responsible for the uptake, digestions and resorption of food, to absorb nutrients as well as energy and to dispense the resulting waste as feces. The GI tract is composed of mouth, esophagus, stomach, small intestine (duodenum, jejunum, ileum) and large intestine (cecum, colon, rectum) (Fig. 3) (HOWELL, WELLS, 2011).

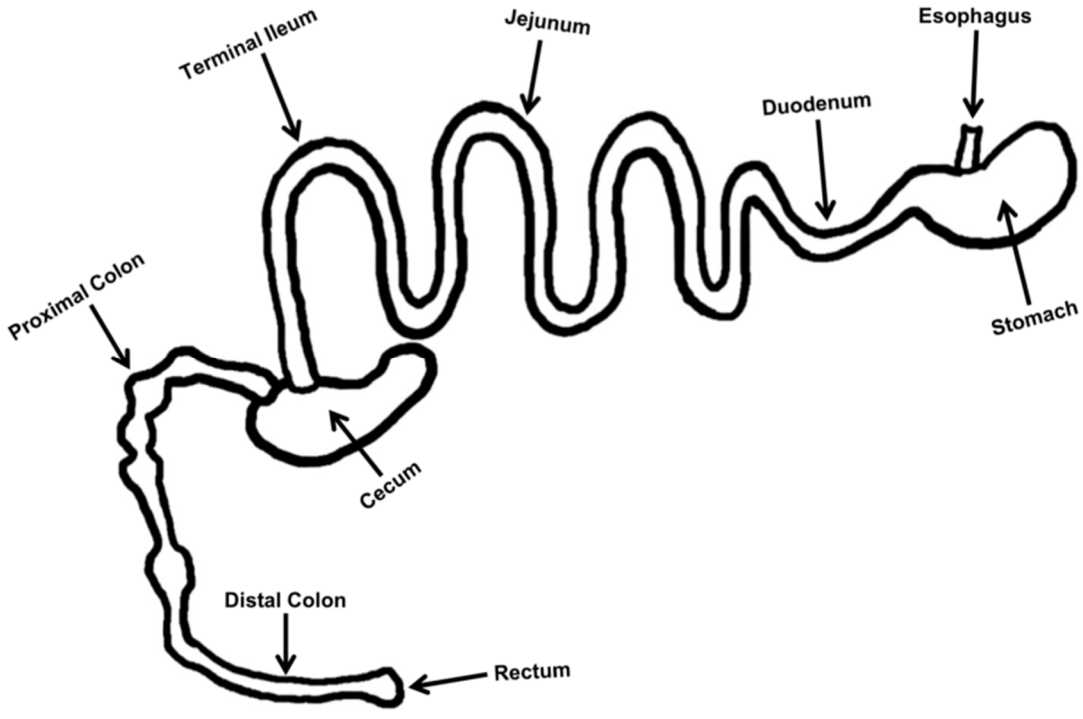


Figure 3 - The Gastrointestinal tract

In addition, there are different glandular systems associated with the GI tract that support digestion, such as the tongue, salivary glands, pancreas, liver and gallbladder. The GI tract contains all the organs between the mouth and the anus, creating a constant passageway that encompasses the main digestive organs, such as the stomach, small and large intestine. Furthermore, besides being the largest digestive and endocrine organ the GI tract is also the largest immune organ in the human body (PIERRE et al., 2016). The lymphatic system and its lymphocytes are very important for the body's defense. There is a large amount of lymphatic tissue in the gut. This is clearly visible in the small intestine i.e., as Peyer-Plaques, papilliform elevations on the antimesenterial side of the intestine. Additional lymphatic tissue is found in the mesentery in form of lymph nodes closely associated with the GI tract, whose function is primarily the supply of blood as well as lymphatic liquid. The tubular hollow organs, small intestine and large intestine as well as the esophagus, stomach and cecum are made up of different tissue layers, which directly connect the inner layer of tissue to the food. the external muscle layers consist of muscle, neural, connective and the lymphatic tissue. The various muscle layers in the intestine are responsible for different types of contraction. The entire human GI tract is about 9 meters long and harbors trillions of microbes, with about 4,000 specific bacterial strains having various functions in immune health and metabolism maintenance. There is a large amount of lymphatic tissue in the intestine. The average ratio of the intestinal surface to body surface area is comparable in between mice and also humans (CASTELEYN et al., 2010), however this ratio varies considerably among both species over various sections of the intestine. The human cecum is comparatively small, when compared to rodents, and has an attached appendix (NGUYEN et al., 2015). Interestingly, latest work has indicated that the appendix plays a function in intestinal recolonization after natural flora (e.g., through antibiotics) becomes degraded (NGUYEN et al., 2015; SMITH, 2013). The small and large intestine wall is made up of functionally distinct layers. The outer layer is composed of the serosa and the smooth muscle, which participate in peristalsis. The middle layers consist of the submucosa and muscularis mucosae, the innermost layers of the lamina propria, and the plain columnar epithelium that lines the luminal surface. (HOWELL, WELLS, 2011). This basic intestinal epithelium borders the luminal surface area. Within the small intestine, the surface area is composed of villi and crypts. The villi harbor mainly absorptive cells, while crypt cells are usually considered as secretory. Almost all of the transfer of nutrients takes place in the small intestine, while the colon is mainly responsible for the resorption of liquids and electrolytes (KIELA, GHISHAN, 2016). By the mechanism of cell division, maturation and migration the epithelial cells can constantly be renewed every 4 to 5 days. This renewal is based on proliferative cells (stem cells) which are located around the bottom of the epithelial crypts and that have the potential to develop to new epithelial (progenitor) cells (CLEVERS,

BATLLE, 2013). After the new cells have been created at the base, they move upwards and also out of the crypt and start maturing along the way. At some point, they go through apoptosis as well as being shed into the lumen of the intestine (VAN DER FLIER, CLEVERS, 2009). The cellular lining of the gut is continuously renewed while the number of cells composing the epithelial layer remains constant. In this area, the epithelium is accountable for supplying an enormous cell turnover as well as the epithelial stem cell protective niche (UMAR, 2010). The dominant intestinal epithelial cell types in the small and as well in the large intestine are the enterocytes. Their primary function is the intake of nutrients and liquids. Paneth cells are specialized secretory epithelial cells which are physiologically located at the bottom positions of small intestinal crypts (GASSLER, 2017). Those cells secrete abundant antimicrobial proteins, like lysozyme, and through disrupting of the Paneth cell secretion may lead to inflammatory diseases (BEL et al., 2017). Antimicrobial peptides, especially α-defensins which are also expressed by Paneth cells, control the composition of microbiota and play a major role in intestinal barrier function and homeostasis (EHMANN et al., 2019). These have been shown to be relevant to the intestinal stem cells, since they are implicated in the provision of the microenvironmental niche in the base of the crypts (HOWELL, WELLS, 2011). Goblet cells which are accountable for mucus secretion occur more frequently in the large intestine than in the small intestine. Enteroendocrine cells (e.g., enterochromaffin cells) comprise lower than 1% of all intestinal epithelial cells, but due to their secretion of GI hormones, they are an important endocrine organ (HOWELL, WELLS, 2011; MAYER, 2011). With respect to immunity, it is important to consider the Microfold cells or M cells which are located in the gut-associated lymphoid tissue (GALT) of the Peyer's patches in the small intestine and as well in other areas of the intestine in the mucosa-associated lymphoid tissue (MALT). These kinds of cells are recognized to cause mucosal immunity reactions on the apical membrane of the M cells and also enable microbes and particles to be transported throughout the epithelial cell layer from the intestinal lumen to the lamina propria where the interactions along with immune cells may occur (MABBOTT et al., 2013). It is necessary to have a coordinated interaction between the intestinal layers to perform physiological tasks of the gut (HOWELL, WELLS, 2011). The GI tract is a very complex organ system which performs a variety of vital functions including digestion, absorption, secretion, mixing, segmentation, excretion, propulsive movements (peristalsis and migrating motor complexes), as well the immune defense (UESAKA et al., 2016). Several of these intestinal functions are mediated by intrinsic neurons of the enteric nervous system (ENS) and/or by extrinsic sympathetic, parasympathetic, and sensory neurons (UESAKA et al., 2016).

1.7 The gut microbiota

The first essential step of human microbiota is beginning already in the mother's womb. The GI tract is basically clean and sterile at birth, however its own colonization through various bacterial strains starts during the course of birth. Then they will develop into the largest bacterium population in the human body which reaches up to 10^{11} to 10^{12} cells/g of luminal contents in the colon (DAVE et al., 2012). The human microbiome establishes itself after birth within the first few years until a certain degree of stability and high diversity is achieved. However, especially in the first three years of life, numerous environmental influences, such as nutrition, diseases (especially infections), antibiotics and other medical therapies, can lead to pronounced, sometimes persistent microbiome shifts, some of which can have considerable health consequences. The intestine of an adult individual host hundreds to thousands of bacterial species (DONALDSON et al., 2016). The amount of bacteria rises from the small to the large intestine (GERRITSEN et al., 2011). Within the small intestine, the composition of the bacteria also varies from that of the large intestine. It is reasonable to assume that the physiology of the intestine plays an important role in this process, since there are pH and oxygen gradients from the small to the large intestine and also a various availability of nutrients (DONALDSON et al., 2016). Also, the much shorter transit period in the small intestine was suggested to have an influence on adherence and therefore the colonization of the small intestine. Since the levels of oxygen are higher in the small intestine, the bacteria which are colonizing this habitat are facultative anaerobes that can tolerate a lower pH (e.g., Lactobacillaceae) (DONALDSON et al., 2016; GU et al., 2013). In comparison, in the colon the availability of straightforward carbon sources is extremely small meaning that the bacteria have to have the ability to assimilate "resisting" polysaccharides. This encourages the development of fermentative anaerobes (e.g., Bacteroidaceae) (DONALDSON et al., 2016). In the GI tract all components are covered by the mucous layer. Basically, mucus consists of water and highly glycosylated gel-forming mucins, that are made and also constantly renewed by goblet cells (JOHANSSON et al., 2011; KELLY et al., 2015; PELASEYED et al., 2014). In fact, in the colon, the mucus layer is a lot thicker as in the small intestine and it consists of basically two layers (JOHANSSON et al., 2011) which are described as loosely outer as well as firmly inner layer (ATUMA et al., 2001; JOHANSSON et al., 2011). Both the thickness and the microbiota composition in between of these two layers are distinct (DONALDSON et al., 2016; JOHANSSON et al., 2011). Additionally, the thickness of these layers is really variable and is also suggested to be affected due to the existing bacteria. As it is not very easy for bacteria to enter the mucus, the microbial population inhabiting the mucus has to have unique colonization capabilities (DONALDSON et al., 2016). The usage of mucin glycans is one of the products which is frequently expressed in the mucosa-associated microbiota (PNG et al., 2010). In

addition to the distinct bacterial composition of the inner and outer mucus layer, the colon has strong spatial heterogeneity (ZHANG et al., 2014). Furthermore, the digesta-associated colonic community is distinguished from the mucosal microbial community (ECKBURG et al., 2005). Therefore, the microbial characterization of fecal samples is thus now regarded more critical (DONALDSON et al., 2016). In terms of this aspect, it is particularly important to consider the impact of diet on the microbiota. Although microbial community profiling in fecal samples provide acceptable data with respect to the intestinal lumen communities, it should be noted that the mucosal-associated bacteria may be slightly less influenced by, or require longer periods of, intervention for modifications to come to be apparent (DONALDSON et al., 2016).

1.8 Factors that affect the intestinal microbiota

Several factors that have an effect on the intestinal microbiota have been identified. In reality, the first crucial step of human microbiota is beginning already in the mother's womb. In spite of previous claims that the mother's placenta is free of germs, new research studies has found that the maternal microbiota also impacts the child's microbiota (AAGAARD et al., 2014; COLLADO et al., 2015; RODRIGUEZ et al., 2015). For this reason, deliberate modifications of diet as well as overall life style of the mother have currently been advised as a therapeutic approach for the development of healthy fetal microbiota and also as an assistance to lessen the risks of specific diseases in the unborn child (COLLADO et al., 2015; RODRIGUEZ et al., 2015). The delivery process is actually the next important step for the microbiota of the child. In these findings, it is very clear that the Cesarean section affects the microbiota in a detrimental manner, rendering the infant more vulnerable to diseases, like but not restricted to asthma, celiac disease and obesity (BARROS et al., 2012; COLLADO et al., 2015; DECKER et al., 2011; THAVAGNANAM et al., 2008). Breast milk as a first food is strongly involved in the development of the intestinal microbiota of the infant (DONALDSON et al., 2016). This result is attributable to the fact that there are bacteria in the milk, as properly as milk oligosaccharides, which work as carbon sources for some microbes, and also maternal antibodies, that act as defense and thereby encourage homeostasis (DONALDSON et al., 2016; FERNANDEZ et al., 2013; ROGIER et al., 2014; YU et al., 2013). While the microbiota is also an adaptable mechanism in later stages of life, it is especially important to develop a healthy microbiota during the childhood (BAUER et al., 2016; BORRE et al., 2014; RODRIGUEZ et al., 2015). The lifestyle, medications, environment, diet and as well supplements (e.g., pre-and probiotics) have an effect on the microbiota during the entire life (GERRITSEN et al., 2011; RODRIGUEZ et al., 2015). Whereas the abovementioned factors

can often be affected by the host, various other factors do not lead to alterations, such as sex and age which often shape the intestinal microbiota (GERRITSEN et al., 2011; MARKLE et al., 2013). It is currently believed that the diet has the greatest effect on the microbiota (DAVID et al., 2014; DONALDSON et al., 2016). However, the immune system also affects and imposes selective pressure on the intestinal microbiota (DONALDSON et al., 2016).

1.9 Gut microbiota in health and aging

The gut microbiome describes a population of microorganisms that inhabits the GI tract, including bacteria, viruses, archaea and eukaryotic microbes. The GI system can host approximately 1000 microbial varieties, much of which encode protein features that are missing from the human genome (LI et al., 2014). Consequently, the gut microbiome is capable of impacting metabolic functions, modulating the host immune system and protecting against pathogens (SHREINER et al., 2015). In addition to that, it is known that the microbiome has an influence on aging processes and a "well-balanced and healthy" microbiota may possess a defensive effect that protect organism from the onset of different diseases during aging. Numerous research studies have actually revealed that the aging process reduced microbiome selection and also boosted frailty, which has actually been related to age-related bowel problems as well as low-level chronic inflammation (CANDELA et al., 2014). Diet regimen along with environmental factors are mostly identified as the major components fit microbiome composition, recommending that regulating as well as preserving a healthy diet plan might assist to induce a microbiome that enhances health and wellness as well as expands life-span (CLAESSON et al., 2012). Notably, modifications in the microbiome as well as microbial metabolites throughout aging have actually been related with a greater risk of Alzheimer's (SHOEMARK, ALLEN, 2015) and Parkinson’s condition (SCHEPERJANS et al., 2015; UNGER et al., 2016), which could be associated to modifications in mucosal barrier function (SCHWIERTZ et al., 2018). Several studies reported that a dietary treatment, such as Calorie Restriction (CR), extends the lifespan in a number of different animal models. Experimental studies revealed that CR regimen in mice may expand life-span through approximately 40%, while in non-mammalian models, the impacts of CR was actually more pronounced/clear (FONTANA et al., 2010; WEINDRUCH et al., 1986). This dietary treatment decreases the levels of body weight and plasma glucose and insulin, while enhancing the tolerance of insulin in laboratory animals and overall health (ANDERSON et al., 2009). Long living df/df mice exhibit many of the attributes of CR mice, but the mechanism of action is not the same, as previous studies have provided evidence that CR additionally expands not only life expectancy but also improves insulin sensitivity in these long-living animals (MATTISON et al., 2000).

Additionally, it is also well known that blood pressure and cholesterol rates are likewise lowered by CR (FONTANA et al., 2004). Dietary CR studies in human beings have actually shown numerous health advantages consisting of decreased body weight and obesity (RACETTE et al., 2006), minimized atherosclerosis (FONTANA et al., 2004), and enhanced heart functionality (MEYER et al., 2006). In fact, the incidence for age-related diseases like type 2 diabetes and cancer is reduced by CR (EVERITT et al., 2006). Considering that the df/df mutation as well as CR treatment both prolong the lifespan and also healthspan in mice, the effect of this particular mutation and as well nutritional routine on the arrangement of intestine microbiome has been analyzed. The objective was to figure out if the hereditary mutation that prolongs life expectancy in mice will certainly switch microbiome composition in contrast to genetically N littermates stemming from the same moms and dads which were kept according to the exact same environmental conditions. Therefore, when comparing df/df and N mice, it has been examined whether nutritional treatments with CR could cause different changes in the gut microbiota.

2. Specific Aims

Growth hormone and insulin signaling pathways are important regulators of healthy metabolism and aging. Importantly, GH-deficient df/df mice are characterized by increased longevity, which is strongly associated with improved insulin sensitivity due to suppression of GH action. Additionally, external factors such as food and associated microbiota plays an important role in regulating insulin and glucose metabolism. Based on this the general hypothesis that Ames dwarfism mutation has significant impact on improvement of insulin sensitivity and maintaining health gut microbiota was proposed.

Specific Aim 1: Determine the tissue-specific insulin action in long-living Ames dwarf mice using the hyperinsulinemic-euglycemic clamp technique.

Previously published data indicate a strong association between insulin sensitivity and expected lifespan (BARTKE et al., 2002). Several Studies with different organisms suggested that maintaining low insulin levels and moderate hypoglycemia predicts longer lifespan. Additionally experiments with basic insulin tolerance tests (ITT) in long-living df/df mice showed a significant association between ITT and longevity (MASTERNAK et al., 2009). However, beside basic ITT and glucose tolerance test (GTT) analysis and extensive studies of insulin signaling pathway, physiological studies have been performed that indicate *in vivo* responses. Based on the actual literature and previous studies of insulin signaling pathways in different insulin responsive organs I hypothesize that enhanced insulin sensitivity in df/df mice is mainly regulated through enhanced hepatic responses to insulin and increased glucose clearance by skeletal muscle and adipose tissue. To test this hypothesis, a hyperinsulinemic-euglycemic clamp was performed for the first time in these mice. Until now this approach was not realized due to difficulties related to the extremely small size of these animals.

Specific Aim 2: Determine the impact of Prop1df lifespan extending mutation and the effect of CR on development of gut microbiota.

Studies showing that the gut microbiome has an impact on aging processes and metabolic functions, and it can also protect against several diseases. The gut microbiome of an organism is relatively stable in healthy person over time, yet there is increased variability during aging. The composition of the microbiome can be also influenced by diet and other environmental factors (SHREINER et al., 2015). In fact, many studies have shown that the process of aging

or specific diets (i.e., calorie restriction, high fat diet etc.) can have a strong impact on health and variety of the gut microbiome (NAGPAL et al., 2018).

Based on latest studies of the gut microbiome related to aging I hypothesize that life-extending df/df mutation and CR causes improvement in gut microbiota and that the bacterial composition in the gut will be affected differently, that presumably contributes to the phenotypic differences and may be beneficial to improved health- and lifespan. To test this hypothesis, the microbiome analysis in these long-living genetic and dietary animal models was performed for the very first time.

3. Material and Methods

3.1 Approach

Specific Aim 1: Determine the tissue-specific insulin action in long-living Ames dwarf mice using the hyperinsulinemic-euglycemic clamp technique.

Long-living df/df mice have a GH, PRL and TSH deficiency and they are characterized by an extended life- and healthspan. Furthermore, they are protected from variety of age-related diseases, including metabolic complications such as glucose intolerance or insulin resistance. Multiple studies show that df/df mice display a better insulin signaling in various insulin-target organs, thus suggesting that this is a potential lifespan mechanism. Though, it is not known if enhanced insulin signaling in df/df mice leads to a better insulin action on the production of hepatic glucose and tissue glucose uptake. These mice have reduced circulating levels of fasting glucose and insulin. They also show increased glucose tolerance and they are hypersensitive to injected insulin. For the first time, a hyperinsulinemic-euglycemic clamp study was conducted to investigate tissue-specific insulin activity *in vivo* in these long-living df/df mice. These findings showed that the rate of glucose infusion needed to sustain euglycemia was 2-fold higher in df/df mice than in N control mice. In df/df mice this improved role of insulin action and glucose homeostasis can play a major role in promoting healthy aging and lifespan extension.

3.1.1 Research Design and Animals

All experiments were approved by the Animal Care and Use Committee at the Sanford-Burnham Medical Research Institute at Lake Nona. Ames dwarf and also N mice were provided by NIA Aged Rodent Colonies (http://www.nia.nih.gov/research/dab/aged-rodent-colonies-handbook) at the age of 12 months. Clamp study (n=4/sex/genotype) was conducted on N and df/df males and females mice. The mice were obtained and placed for 1 week prior to surgery on a sterilized rodent diet (Harlan Teklad LM-485, #7912; Harlan Teklad, Madison, WI). Ames dwarf mice and their normal siblings were housed with a 12-hour light and dark cycle under controlled temperature at 23°C with free access to food and water. The mice were catheterized after anesthesia with sodium pentobarbital (70 mg/kg body wt) at least 5 days prior to the experiments (NISWENDER et al., 1997). A two-part catheter consisting of PE-10 (inserted into the artery) and silastic (0.025 outer diameter [OD]) was used to catheterize the left common carotid artery for sampling. For infusions with a silastic catheter (0,025 OD), the right jugular

vein was catheterized. Afterwards, the free catheter ends were tunneled to the back of the neck under the skin and attached to tubing made of Micro-Renathane (0.033 OD) through stainless steel connectors. The tubing was externalized and closed with plugs made of stainless steel. In N and df/df mice was only a catheter in the right jugular vein implanted due to their tiny body size. Every day the lines were flushed with ~50 µl of saline consisting of 200 units/ml heparin and 5 mg/ml ampicillin. The animals were housed separately after surgery, and their body weight was recorded on a daily basis. The animals were excluded from the study if the bodyweight were not within the 10% of their presurgery weight until day 5 after postsurgery (AYALA et al., 2006).

3.1.2 Hyperinsulinemic-Euglycemic Clamp

The hyperinsulinemic-euglycemic clamp, also called insulin clamp, was used to measure the insulin sensitivity *in vivo*. N and GH deficient df/df mice had a catheter implanted in the right jugular vein for infusions (AYALA et al., 2006) (Fig. 4). However, due to their tiny body size as well as the challenge of the surgical procedure, blood sampling was not performed by accessing the carotid vein. Instead, blood was collected from unrestrained mice through the tail tip during the clamp (Fig. 4). The following experimental setup and timeline for the hyperinsulinemic-euglycemic clamp are shown in Figure 5. Complying with a 5-day recuperation from the surgery, mice were fasted at t=0 min. for 5 hours before the beginning of a hyperinsulinemic-euglycemic clamp. At t=-90 min, mice got a prepared, constant infusion of HPLC-purified [3-^{3}H]glucose (1µCi prime, 0.05 µCi/min continuous) during the course of a 90 min. equilibration time period. For measurements of fasting glucose, insulin and plasma [3-^{3}H]glucose, blood samples were taken at -15 min (25 µl) and -5 min (50 µl). A continuous infusion of insulin (16 or 300 mU/kg bolus followed by 2.5 mU·kg^{-1}·min^{-1}; Humulin R; Eli Lilly, Indianapolis, IN) and a variable infusion of glucose were started at t=0 min. Blood glucose was determined every 10 minutes (Accu-Chek Aviva, 1 µl samples) and also the glucose infusion rate was readjusted as necessary to keep euglycemia (~160 mg·dL^{-1}). For measurements of steady-state clamp glucose and plasma [3-^{3}H]glucose, blood samples of 50 µl were collected at t=80, 90, 100, 110 and 120 min and processed to evaluate glucose specific activity. Blood samples were used to measure clamp insulin levels at t=120 min (additional 25 µl of blood was taken). Then, a 12µCi bolus of 2[^{14}C]deoxyglucose (2[^{14}C]DG) was given to measure the tissue-specific glucose uptake. Blood samples (25 µl) were collected at t=2, 15, 25, and 35 min. after administration of the bolus for measurements of plasma 2[^{14}C]DG. After these interventions (Fig. 5) the mice were anesthetized with sodium pentobarbital (70 mg/kg body wt). Anesthetized animals were euthanized and various tissue samples such as soleus,

gastrocnemius, superficial vastus lateralis, gonadal adipose tissue, brain and heart were collected, immediately flash frozen in liquid nitrogen and preserved at -80ºC until analyzed.

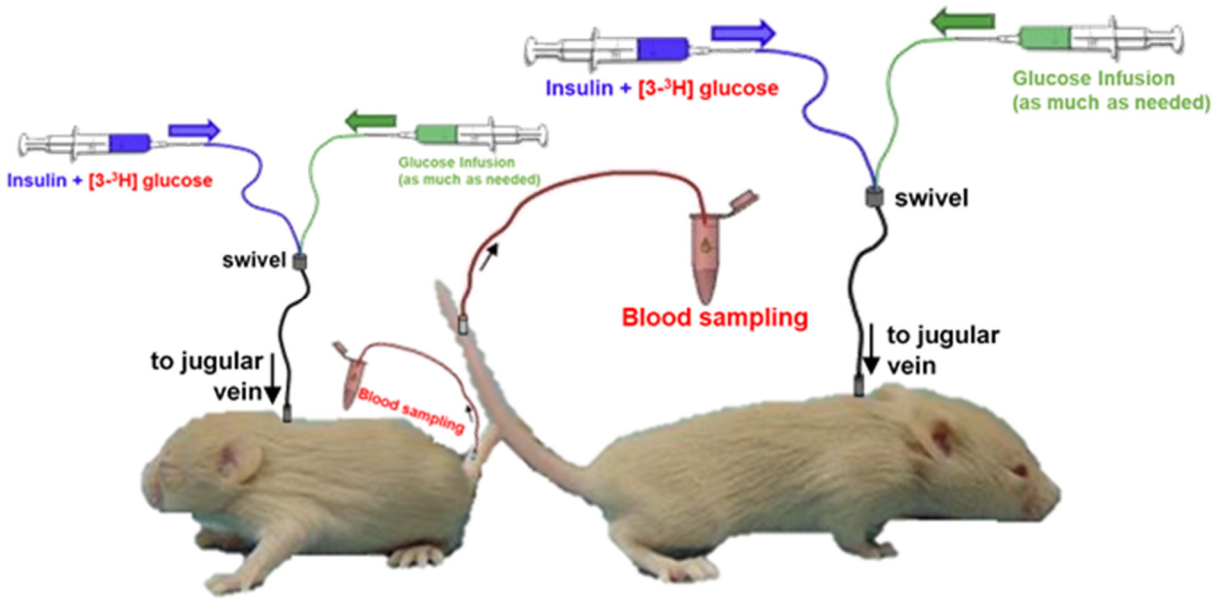


Figure 4 - Depiction of catheterized Ames dwarf (df/df) and normal (N) mice for the insulin clamp experiment.

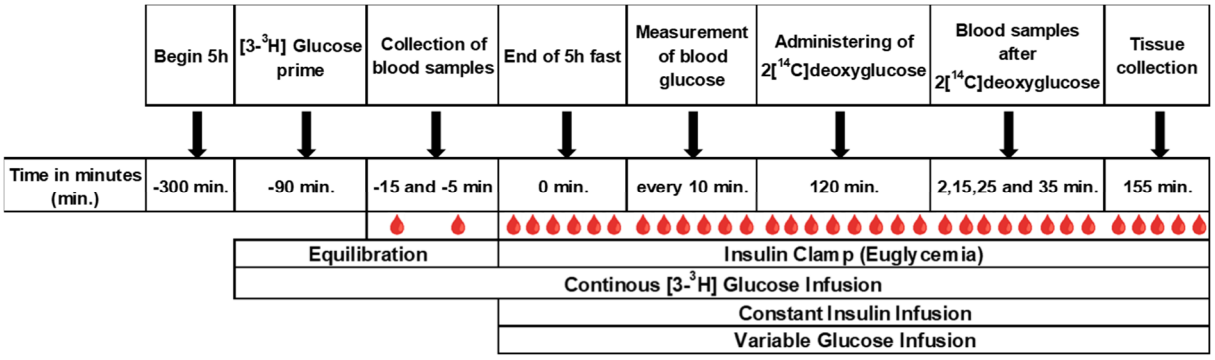


Figure 5 - A timeline of the procedures for setting up and performing the hyperinsulinemic-euglycemic clamp.

3.1.3 Processing of plasma and tissues

The radioactivity of plasma [3-^{3}H]glucose and 2[^{14}C]DG was determined through liquid scintillation counting (Packard TRI-CARB 2900TR) with Ultima Gold (Packard) as scintillant after the previous deproteinization with barium hydroxide [$Ba(OH)_2$, 0.3N] and zinc sulfate [$ZnSO_4$, 0.3 N] by the method of Somogyi (AYALA et al., 2006). To determine radioactivity, one aliquot of 2[^{14}C]DG and 2[^{14}C]DG-6-phosphate (2[^{14}C]DGP) was immediately counted. To remove 2[^{14}C]DGP and any tracer inserted into glycogen, a second aliquot was treated with $Ba(OH)_2$ and $ZnSO_4$ and then counted for the determination of 2[^{14}C]DG radioactivity. The accumulation from 2[^{14}C]DGP has been normalized to the tissue weight in all studies (AYALA et al., 2008).

3.1.4 Calculations

Steele's non-steady state equations were used to calculate the rate of glucose appearance (R_a) and disappearance (R_d) (DEBODO et al., 1963; STEELE et al., 1956). The endogenous glucose rate (endogenous R_a; mg · kg^{-1} · min^{-1}) was calculated through subtracting the glucose infusion rate (GIR) from total R_a. The calculation of the glucose metabolic index (R_g), which is an index of glucose uptake, was performed by using the equation (AYALA et al., 2007; KRAEGEN et al., 1985):

$$R_g = \frac{2[^{14}C]DGP_{tissue}}{AUC\ 2[^{14}C]DG_{plasma}} \text{ X [arterial glucose]}$$

R_g has been normalized to the tissue weight. Where 2[^{14}C]DGP_{tissue} is the 2[^{14}C]DGP radioactivity in the muscle (in dpm/g), the AUC 2[^{14}C]DG_{plasma} is the region under the plasma 2[^{14}C]DG disappearance curve (in dpm · min · ml^{-1}) and the [arterial glucose] is the average blood glucose (in mmol/l) from t = 80–120 min during the experimental clamp period.

3.1.5 Statistical analysis

A one-tailed student's t-test was performed for in-between group analyses followed by F-statistics. For the significance level, alpha was set to $P < 0.05$. All statistical data have been carried out using Prism 5.04 (GraphPad Software, San Diego CA.). All data throughout the text and figures are reported as mean ±Standard Error of the Mean (SEM).

3.2 Approach

Specific Aim 2: Determine the impact of Prop1df lifespan extending mutation and the effect of CR on development of gut microbiota.

The gut microbiome (GM) is a large and very complex ecosystem consisting of many strains of microorganisms. The impact of GM health in diverse diseases, including cancer, diabetes, cardiovascular diseases, and aging, is of considerable interest. During the lifetime the GM changes and is strongly linked with different diseases associated with aging. Ames dwarf (df/df) mice are distinguished by an extended life- and healthspan, but their microbiome has not been studied yet. The focus here was on studying the changes in the GM in df/df and N littermate mice in order to assess the role of microbiota in longevity animal models, while comparing parents prior mating and littermate mice at three different time points through early life stages (WIESENBORN et al., 2019). In addition, it was analyzed the effects of 6-month CR, which is the most powerful intervention for extending the lifespan (WIESENBORN et al., 2019). Throughout early life development, the data indicate significant changes in GM composition, and also differences in the abundance of some bacteria between df/df and N mice in early life were observed. All in all, there were significant differences in the variability of the microbiota through genotype, time-point, and breeding pair. In addition, CR led to significant modifications in the microbiota by GI-location (distal colon, ileum and cecum), genotype as well as diet (WIESENBORN et al., 2019). Nevertheless, the total effect of the genotype was more pronounced than that of the CR. In summary, these results suggest that the intestinal microbiota plays a key role in postnatal development of long-living df/df mice, and that CR dietary treatment can significantly alter the GM.

3.2.1 Experimental Design of Ames dwarf mice

Ames dwarf (df/df) mice were bred and kept, under light- as well as temperature-controlled conditions, with a 12-hour light and 12-hour dark cycle and also a continuous temperature of 20-23°C at the Burnett School of Biomedical Sciences, University of Central Florida. UCF's Vivarium is a special pathogen-free barrier facility which is approved by AAALAC. PPE should always be worn by every staff worker entering the animal facility including shoe coverings, one-time lab coat, face and hair cover as well as gloves. Every person needs to go through the air-shower barrier to access the facility. The shoe cover and gloves were treated with chlorine dioxide for disinfection before and after using the air shower as well as before entering the room with the animals. Mice used in the research were maintained with automatic watering in laminar flow racks in Tecniplast filtered cages. Prior to use, all caging systems were

autoclaved. In order to remove any possible contamination, food and water were irradiated. In addition to the high standard of treating animals with comprehensive use of PPE, cages were put in a laminar flow workstation for any animal handling and the entire cage was sprayed with chlorine dioxide before opening. Moreover, every six months the PCR analysis showed that for: Aspiculuris 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, mice were negative. Homozygous Ames dwarf (df/df) male and phenotypic normal (N) heterozygous female mice were mated to breed df/df and N littermates. In the study, male as well as female offspring were used and all planned experiments were approved by the Animal Care Committee of the UCF Laboratory.

At 4 specific time points, fecal pellets were gathered: Fecal pellets of non-pregnant female (N) and male mice (df/df) were collected prior to mating at Collection 1 (C1). (C1; males n=4 and females n=4). Fecal pellets of individual N and df/df pups were taken on day 22 at Collection 2 (C2). (C2; one day after weaning of N mice; N males n=7 and N females n=6). At day 21, only N pups were weaned at C2, while df/df pups had to stay with their mothers until day 41, following breeding protocol, as a result of their slower growth and development, at the time when collection was performed at C3. On day 70, fecal pellets from individual N and df/df pups were gathered at Collection 4 (C4). (C4; N males n=7, N females n=6, df/df males n=4 and df/df females n=6). At the final collection on day 70, mice were anesthetized with isoflurane, bled by cardiac puncture and sacrificed by cervical dislocation to collect the last fecal samples from distal colon, terminal ileum and cecum. Moreover, for additional studies, all samples were snap frozen in liquid nitrogen and stored at −80°C.

3.2.2 Experimental Design of Calorie Restriction

Long-living df/df and N littermate control mice were kept in single cages at a controlled temperature between 20-24°C with an uninterrupted 12-hour light and dark cycle. An ad libitum (AL) diet was fed to the mice (Rodent Laboratory Chow 5001; not autoclaved; 23.4% protein, 4.5% fat, 5.8% crude fiber; LabDiet PMI Feeds, Inc., St. Louis, MO). Ames dwarf and also N male mice were split right into 4 various groups (n= 10 mice/group) at the age of 3 months:

1. N mice fed ad libitum (N-AL), 2. N mice subjected to 30% CR (N-CR), 3. df/df mice fed ad libitum (df/df-AL) and 4. df/df mice subjected to 30% CR (df/df-CR). Mice chosen for dietary treatment underwent steady CR by minimizing food consumption to 90% throughout initial week, 80% throughout the second week and also 70% from the third week to completion of

the research study. The weekly food intake of AL mice has been calculated to change the food consumption dose in CR groups. Ames dwarf as well as N mice on CR were fed daily. Mice were anesthetized with isoflurane, following about 6 months of CR (mice were 9 months of age) and afterwards bled by cardiac puncture and sacrificed by cervical dislocation. For additional studies, fecal pellets from distal colon, terminal ileum and also cecum were gathered and snap frozen in liquid nitrogen as well as stored at −80°C.

3.2.3 16S rDNA Amplification, Sequencing and Microbiome Analysis

For the identification of microbiome, the amplification of the 16S rRNA gene's V4 region (F515/R806) was carried out as described in earlier protocols (CAPORASO et al., 2011). For every 30µL PCR reaction, 25ng DNA was utilized for DNA-based amplicon sequencing. The PCR was composed of first denaturation for 30 s at 98°C, adhered to by 25 cycles of 10 s at 98°C, 20 s at 55°C and also 20 s at 72°C. Each sample was amplified in triplicate as well as merged together. Afterwards, the PCR amplicons were then sequenced on the Illumina MiSeq platform (PE250). By using Usearch 8.1 software package (http://www.drive5.com/usearch/), the reads were put together, quality checked as well as grouped. In short, the reads were merged utilizing -fastq_mergepairs with -fastq_maxdiffs 30 and the filtering of quality with fastq_filter (-fastq_maxee 1) and a minimal read length of 200 bp was performed. The UPARSE algorithm (EDGAR, 2013) was used to identify OTU (Operational taxonomic unit) clusters and representative sequences followed by taxonomy allocation using the Silva database v128 (QUAST et al., 2013) and the RDP Classifier (WANG et al., 2007) with bootstrap confidence cut off of 80%. An Operational Taxonomic Unit (OTU) is used to classify the groups of organisms which is currently be studied (JONES et al., 1973). Utilizing the phyloseq package (MCMURDIE, HOLMES, 2013), the OTU absolute abundance table and mapping file were made use of for statistical analyses as well as data visualization in the R statistical programming environment (TEAM, 2008).

4. Results

4.1 Effects of whole-body insulin sensitivity in long-living Ames dwarf mice

There were no variations in insulin sensitivity and as well glucose regulation between males and females observed, corresponding to the lack of sex differences in the lifespan in these animals (BARTKE, BROWN-BORG, 2004; BROWN-BORG et al., 1996). However, this has made it possible to merge and compare male and female results. The initial tests showed significantly lower levels of fasting insulin in df/df mice relative to N littermates (P<0.0005; F=5.329; df=14), whereas fasting glucose levels between genotypes revealed only a trend. (P<0.08; F=2.826; df=14) (Fig. 6).

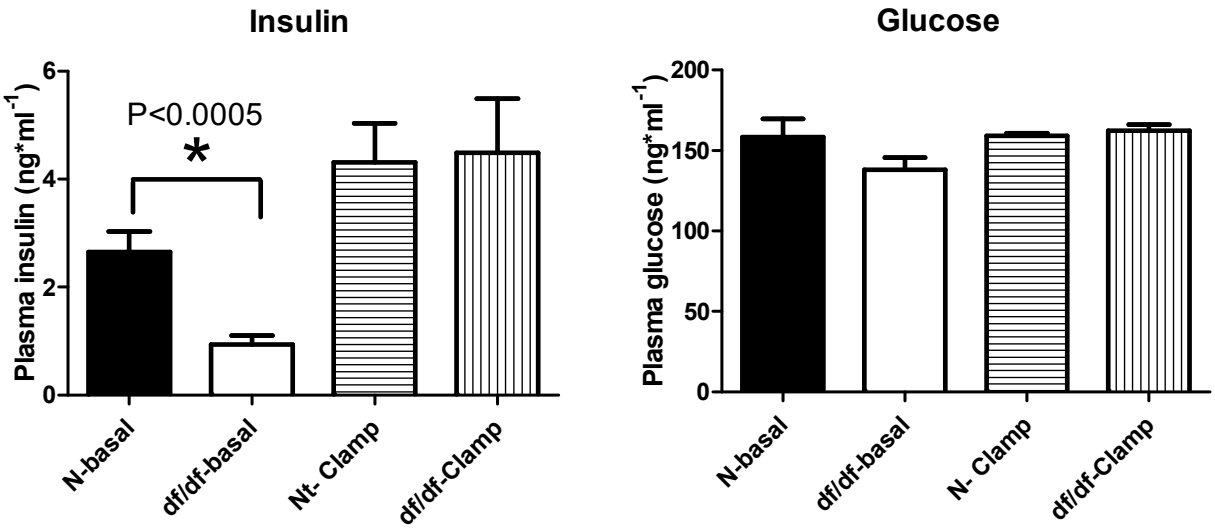


Figure 6 - Insulin and glucose levels before and during clamp of N and df/df mice. N= 8 animals for each genotype. *P<0.05

The glucose infusion rate needed to sustain euglycemia (~160mg•dL-1) (Fig. 7A) was about 2-fold greater in GH-deficient df/df mice compared to N littermate controls (Fig. 7B) throughout a hyperinsulinemic-euglycemic clamp, indicating that glucose clearance is much greater in these long-living Ames dwarf mice when compared with N animals.

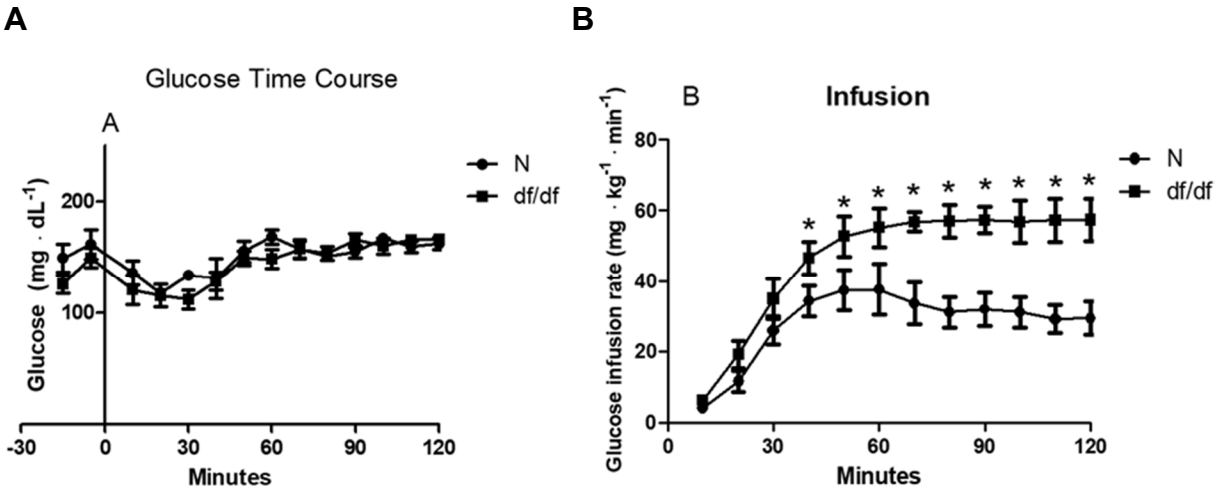


Figure 7 - **(A)** Arterial glucose in long-living Ames dwarf (df/df) and normal (N) mice during insulin clamping. **(B)** Glucose Infusion Rate in N and df/df mice during insulin clamping. Each genotype contained n= 8 mice. *P<0.05

During fasted, baseline conditions, the rate of endogenous glucose appearance (endoRa), which indicates the production of hepatic glucose, did not differ between df/df and N mice. Indeed, endoRa was entirely suppressed throughout the clamp in df/df mice while in N littermate controls it was just reduced by ~ 60% (Fig. 8).

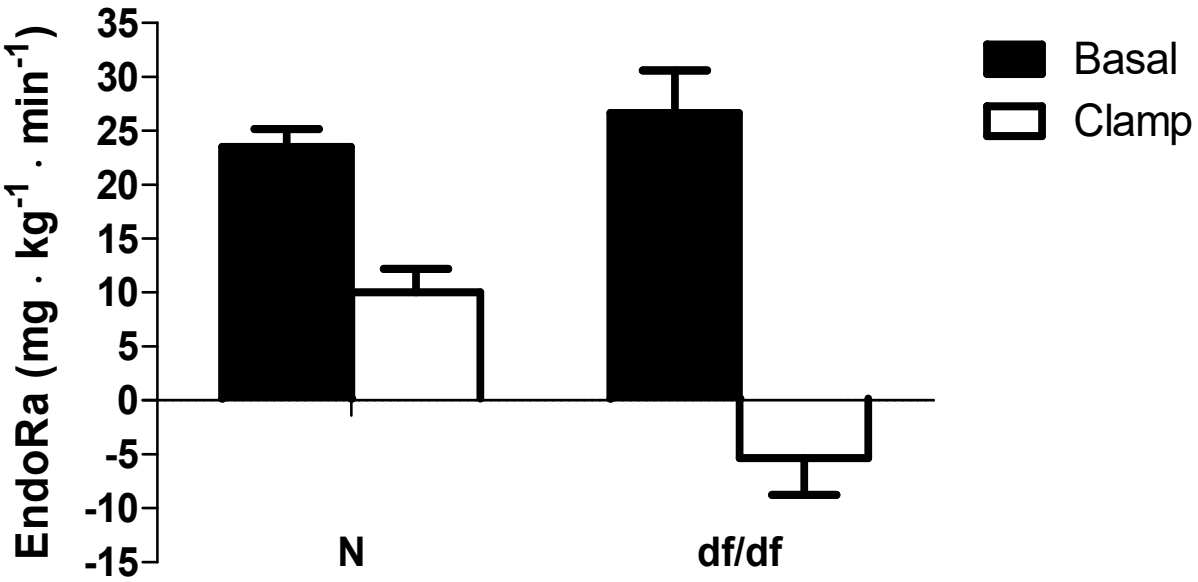
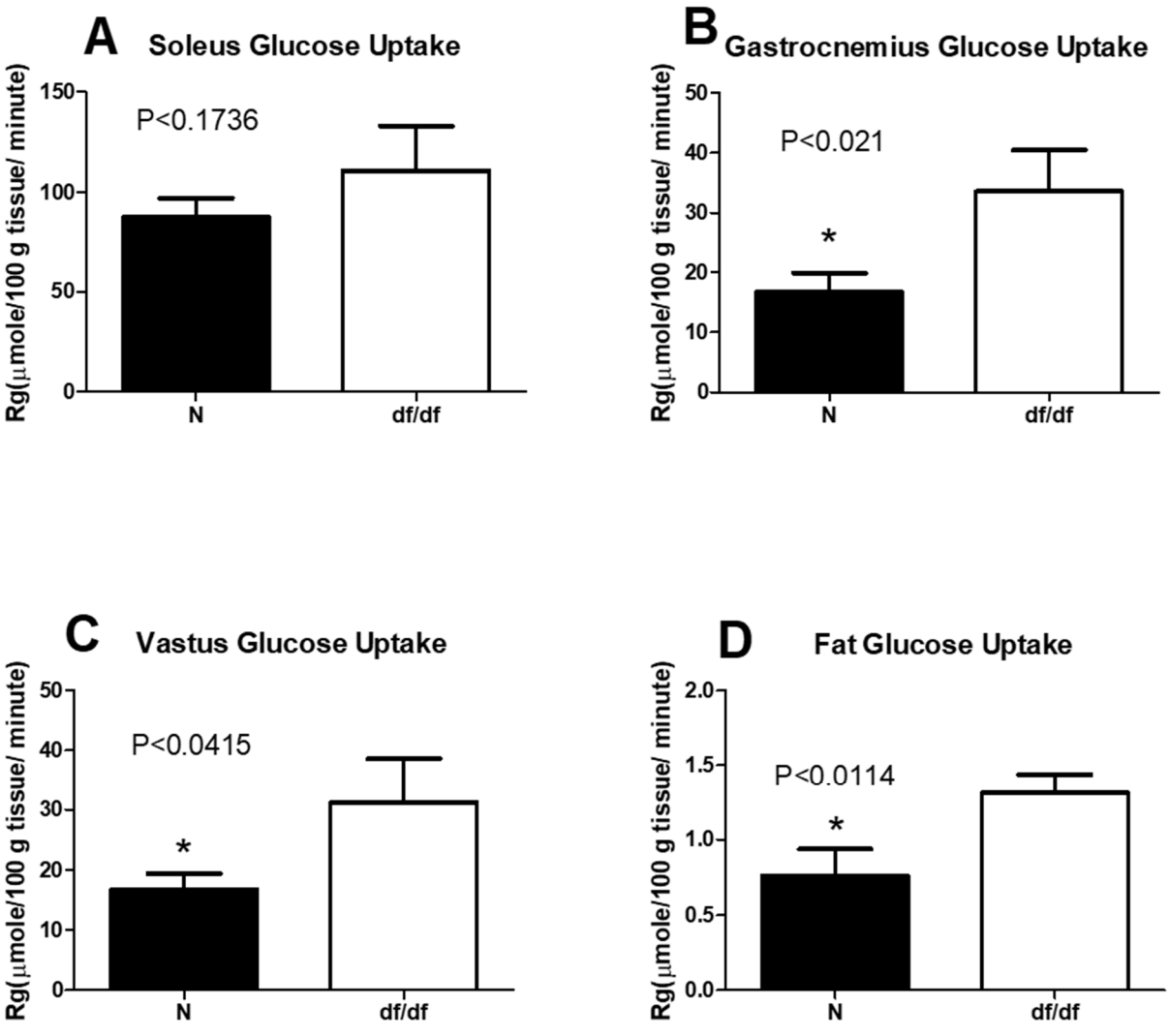


Figure 8 - EndoRa for normal (N) and Ames dwarf (df/df) mice. Black bars present EndoRa under basal condition, while white bars represent insulin clamp.

These greater hepatic response during clamp condition revealed that long-living df/df mice are characterized by increased hepatic insulin sensitivity by much more rapid responses to changing insulin and glucose levels in the body. Furthermore, *in vivo* stimulation of skeletal muscle in tested mice indicated that there is increase glucose uptake in gastrocnemius and superficial vastus lateralis in long-living df/df mice relative to N littermate controls (P<0.021; F=4.588; df=14 and P<0.0415; F=7.850; df=14, respectively) (Fig. 9). Moreover, when studying df/df and N control mice, there was no distinction in soleus muscle and also in heart glucose uptake (P<0.1736; F=5.23; df=14 and P<0.2065; F1.435; df=14, respectively). Interestingly, review of visceral adipose tissue analysis suggested a higher glucose uptake in df/df mice relative to N mice (P<0.0114; F=1.911; df=14) (Fig. 9). Remarkably, in df/df mice the uptake of glucose in the brain was decreased in comparison to N littermate control mice (P<0.0001; F=1.403; df=14) (Fig. 9).



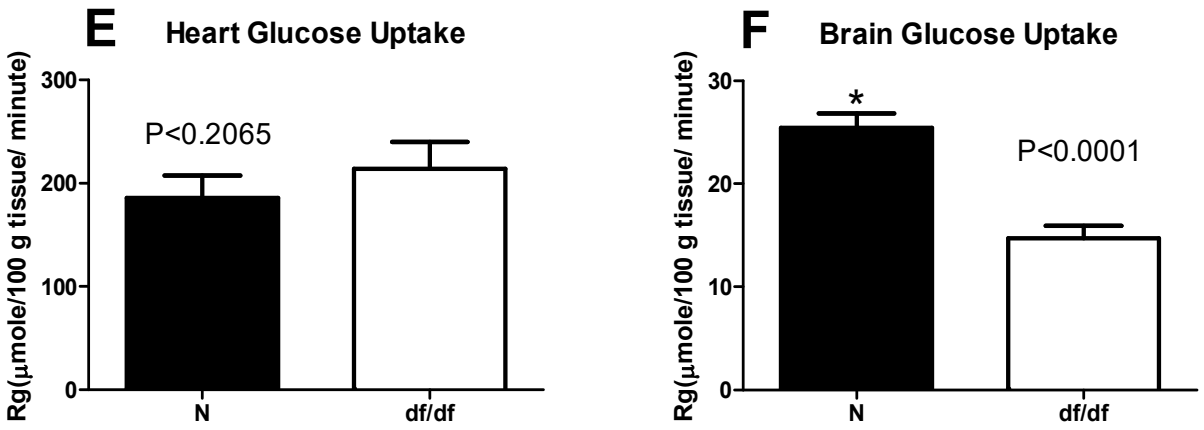


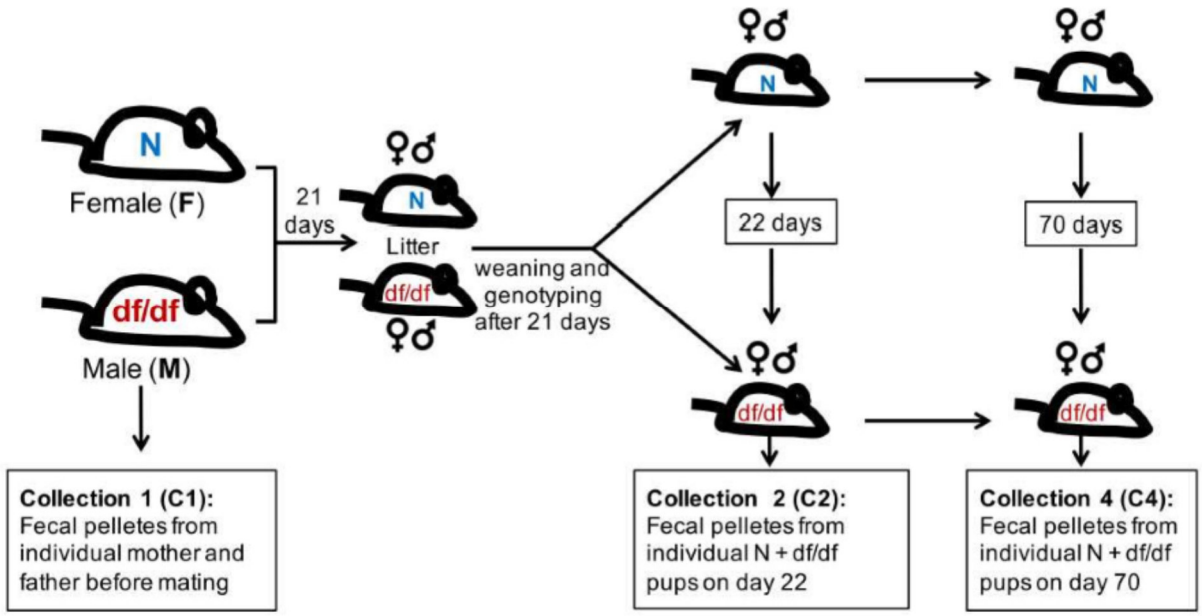
Figure 9 - Glucose metabolic index (Rg) during insulin clamps in different organs from normal (N) and Ames dwarf (df/df) mice: (A) soleus, (B) gastrocnemius, (C) superficial vastus lateralis, (D) fat, (E) heart and (E) brain. n= 8 mice for each genotype. *P<0.05

4.2 Long living GH-deficient df/df mice have a different microbiota composition

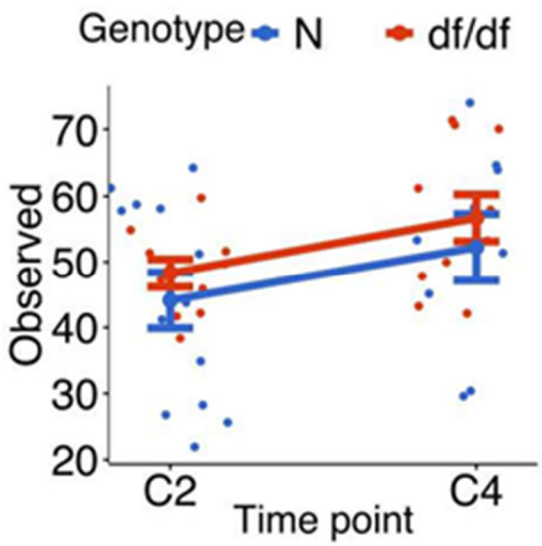
A longitudinal littermate control analysis according to Stappenbeck & Virgin 2016 (STAPPENBECK, VIRGIN, 2016) (Fig. 10A) was conducted to determine whether GH deficiency affects the intestinal microbiota of df/df mice. This kind of study was required as it was demonstrated that family transmission and environmental factors had a strong influence on the composition of microbiota in laboratory mice (MOELLER et al., 2018). Subsequently, Ames dwarf (df/df) male mice were crossed to female heterozygous (N) mice. After the offspring were born, the microbiota composition was examined through 16S rRNA gene sequencing after 22 days (feces), 42 days (feces) and 70 days (content of distal colon) in offspring (df/df and N) mice. Examination of alpha diversity showed a comparable variety of identified species (Observed species) (Fig. 10B) and a similar evenness (Shannon index) (Fig. 10C) at both times between df/df and N littermate control mice. Noticeably, examination of beta diversity by using Bray–Curtis dissimilarity and non-metric multidimensional scaling (NMDS) readily showed distinctions in between df/df as well as N mice at both time points (Fig. 10D). Statistical analysis of permutational multivariate ANOVA (Adonis) was conducted to determine the contribution of genotype, breeding pair as well as gender to variability within the microbiota (Fig. 10D). This study showed that even after weaning both breeding pair (R2= 0.195, p= 0.06) and genotype (R2= 0.130, p= 0.013 *) added to the observed variability. The results showed that after 70 days (C4) there was an even greater genotype effect (R2= 0.314, p= 0.001 ***) (Fig. 10D). In order to examine distinctions among the microbiota composition between mice, weighted Unifrac distances were utilized in addition to Bray-Curtis dissimilarity. The weighted

Unifrac distances among df/df mice were minor in relation to the distances in between df/df as well as N mice after 70 days. This result proves the advancement of genotype-dependent modifications in the composition of the microbiota (Fig. 10E). However, the ratio between Bacteroidetes and Firmicutes (B/F ratio) to further characterize these disparities was quantified. Remarkably, the B/F ratio during weaning did not differ, but the B/F ratio at 10 weeks showed considerably differences (0.25 vs 0.5, $p< 0.001$) in between df/df and also N mice (Fig. 10F). Studies of microbiota composition on family level using beta-diversity analysis revealed in both groups the prevalent existence of commensal bacteria such as Muribaculaceae, Lachnospiraceae as well as Lactobacillaceae (Fig. 10G). LEfSe (Linear discriminant analysis Effect Size) analysis was conducted using standard parameters (Linear Discriminant Analysis [LDA] score > 3.0) (SEGATA et al., 2011) to determine which bacterial taxa showed significantly changed relative abundances between both genotypes at the age of 10 weeks. In general, LEfSe identifies the characteristics most likely to clarify the distinctions between the groups (organisms, clades, operational taxonomic units, genes, or functions) by combining standard statistical significance tests with additional biological consistency and effect relevance encoding tests (SEGATA et al., 2011). LEfSe analysis showed that families of Muribaculaceae (BacteroidalesS24_7 group), Enterococcaeceae, Enterobacteriaceae, Clostridiales vadinBB60group which belongs to the Phylum Firmicutes, and Porphyromonadaceae were found enriched in df/df mice, and in comparison, to littermate control mice a decrease of Lactobacillaceae, Rikenellaceae, and Planococcaceae (Fig. 10H) could be seen. In order to identify significant differences on the levels of individual OTUs, DESeq2 (LOVE et al., 2014) which uses various statistical approaches and models, was applied in addition to LEfSe. These results display in df/df mice an enrichment of OTUs belonging to the Muribaculaceae (=Muribaculum S24-7 group), Enterococcus spp., and Klebsiella spp., as well as a decrease of an OTU belonging to Lactobacillus spp., in comparison to N mice (Fig. 10H). In summary, all this research data supports the theory that throughout post-natal development, GH deficiency in df/df mice influences the microbiota composition.

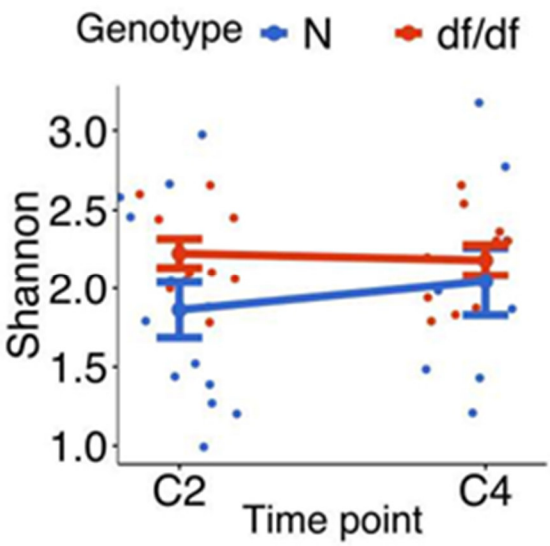
A


N
Female (F)
df/df
Male (M)
21 days
N
Litter
df/df
weaning and genotyping after 21 days
22 days
70 days
Collection 1 (C1): Fecal pelletes from individual mother and father before mating
Collection 2 (C2): Fecal pelletes from individual N + df/df pups on day 22
Collection 4 (C4): Fecal pelletes from individual N + df/df pups on day 70

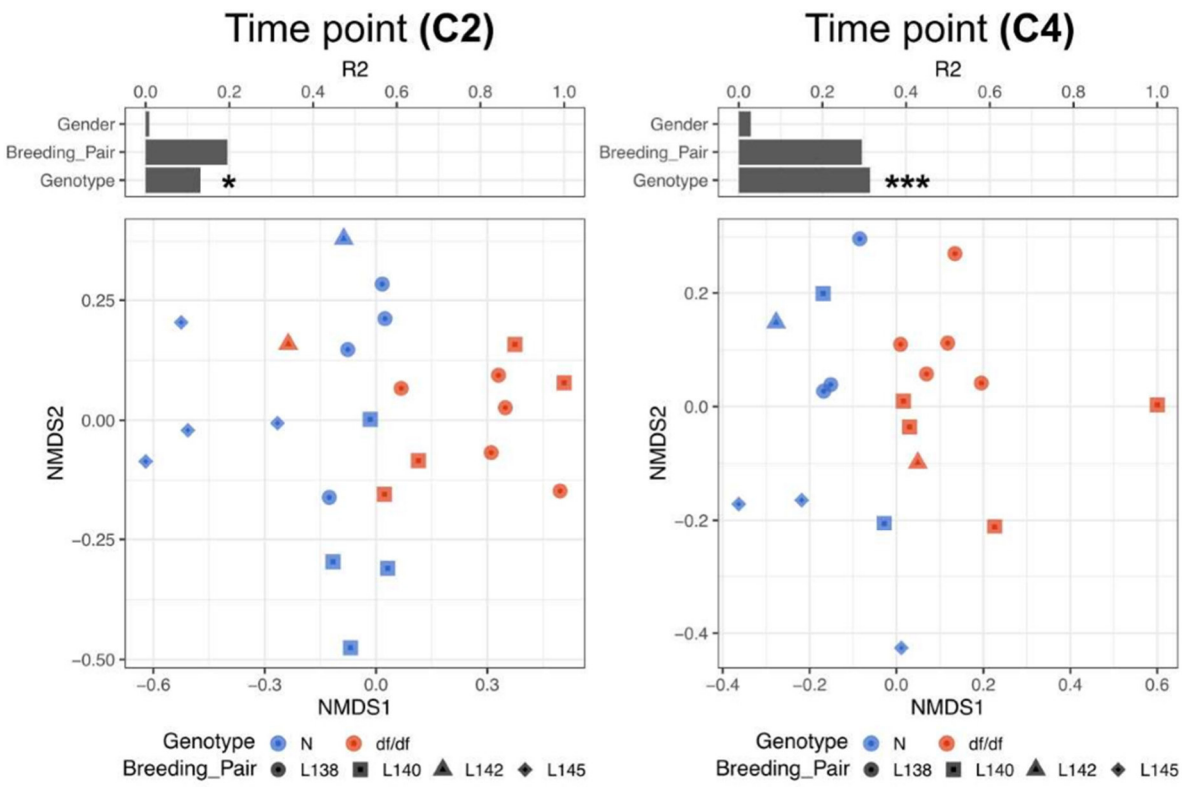
B


Genotype N df/df
Observed
70
60
50
40
30
20
C2
C4
Time point

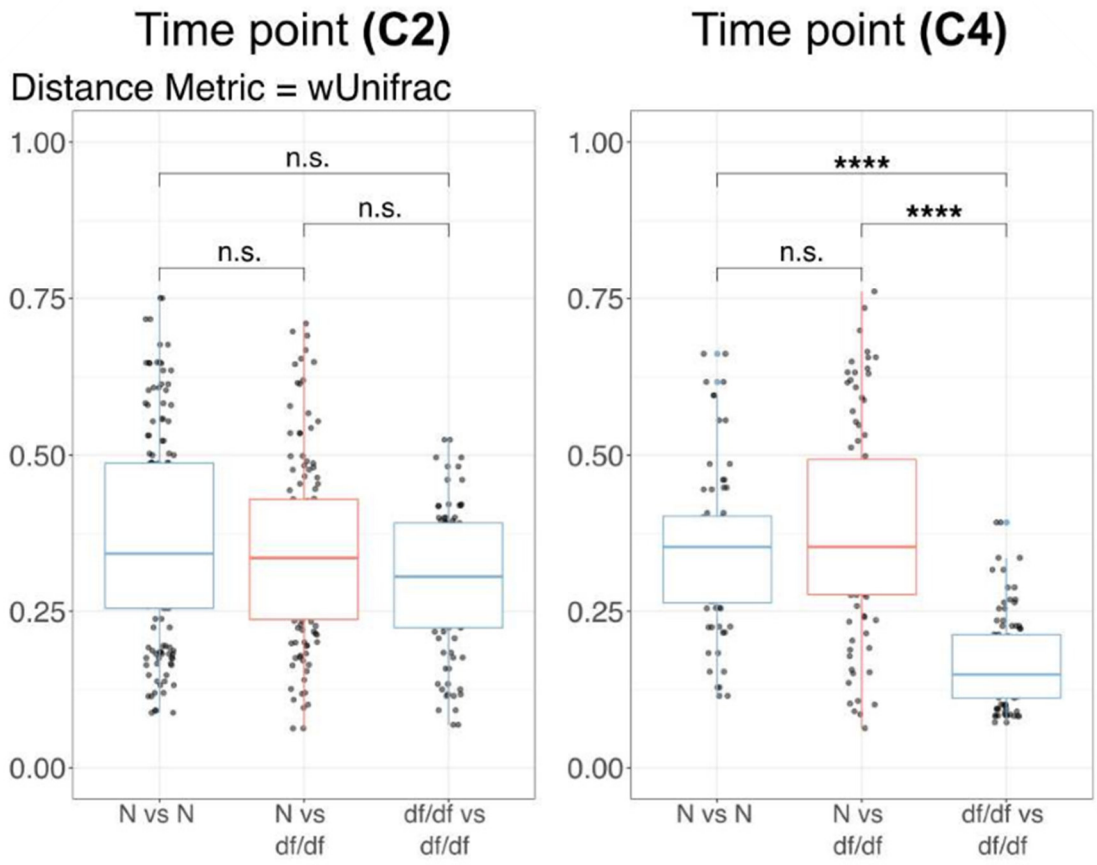
C


Genotype N df/df
Shannon
3.0
2.5
2.0
1.5
1.0
C2
C4
Time point

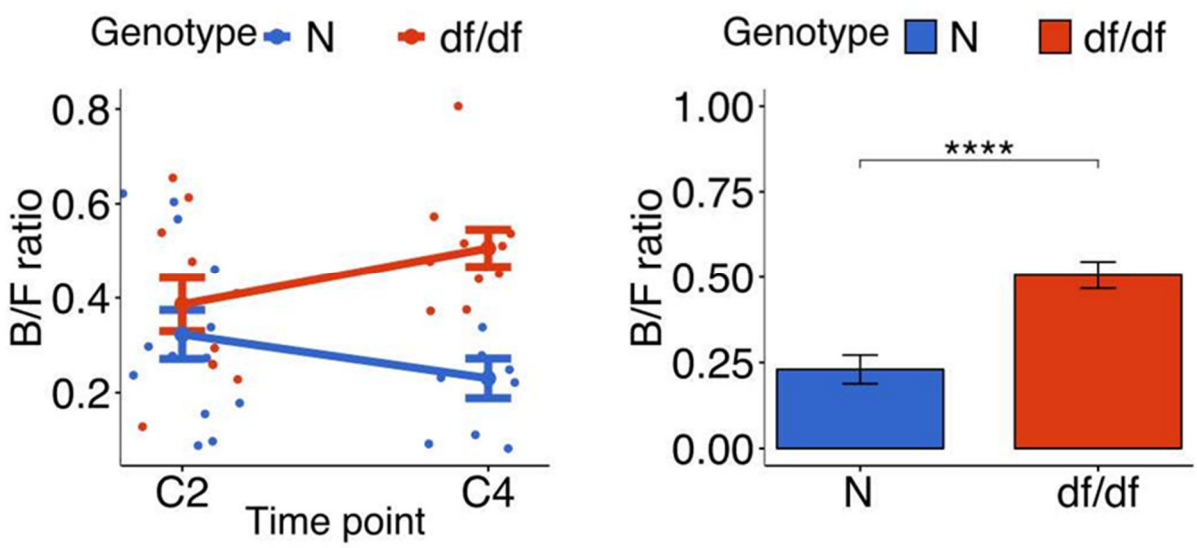
D


Time point (C2)
Time point (C4)
R2
Gender
Breeding_Pair
Genotype
*
***
NMDS1
NMDS2
Genotype N df/df
Breeding_Pair L138 L140 L142 L145

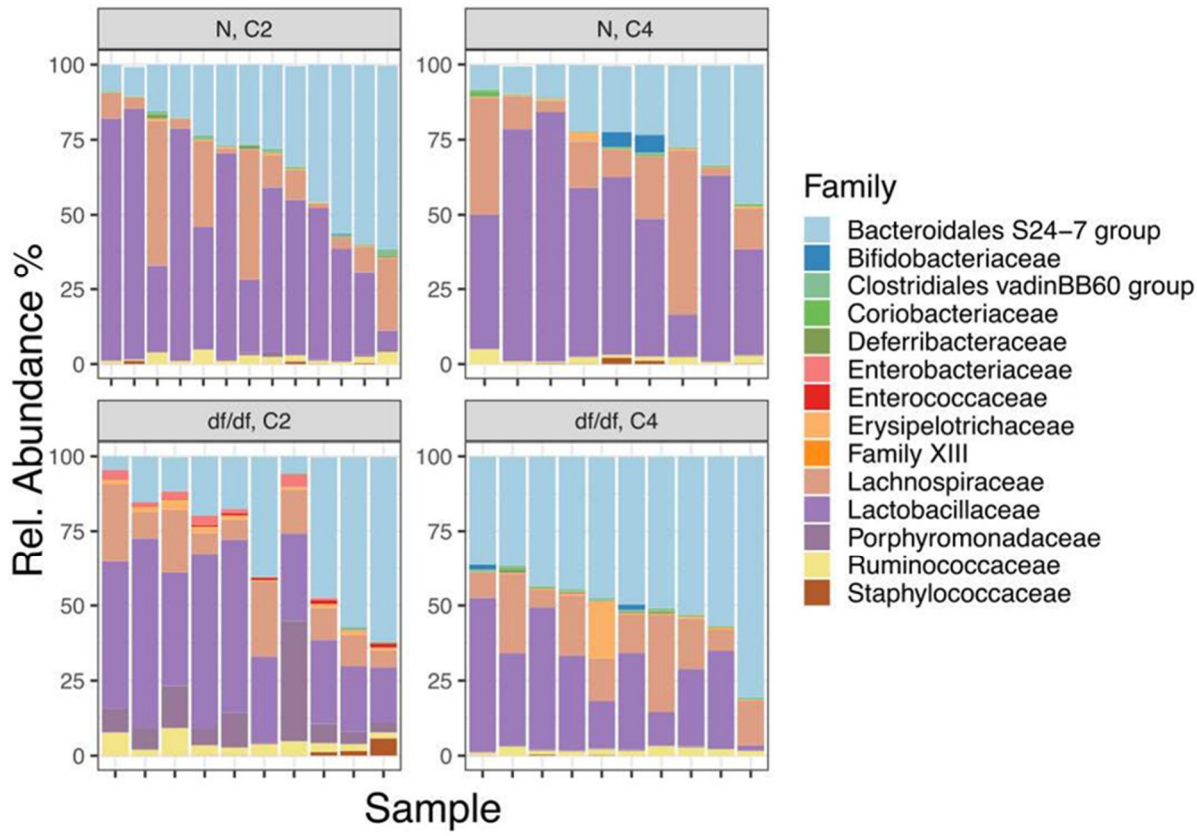
E


Time point (C2)
Time point (C4)
Distance Metric = wUnifrac
n.s.
****
N vs N
N vs df/df
df/df vs df/df

F


Genotype N df/df
B/F ratio
0.8
0.6
0.4
0.2
C2
C4
Time point
Genotype N df/df
1.00
0.75
0.50
0.25
0.00
****
N
df/df

G


N, C2
N, C4
df/df, C2
df/df, C4
Rel. Abundance %
100
75
50
25
0
Sample
Family
Bacteroidales S24–7 group
Bifidobacteriaceae
Clostridiales vadinBB60 group
Coriobacteriaceae
Deferribacteraceae
Enterobacteriaceae
Enterococcaceae
Erysipelotrichaceae
Family XIII
Lachnospiraceae
Lactobacillaceae
Porphyromonadaceae
Ruminococcaceae
Staphylococcaceae

H

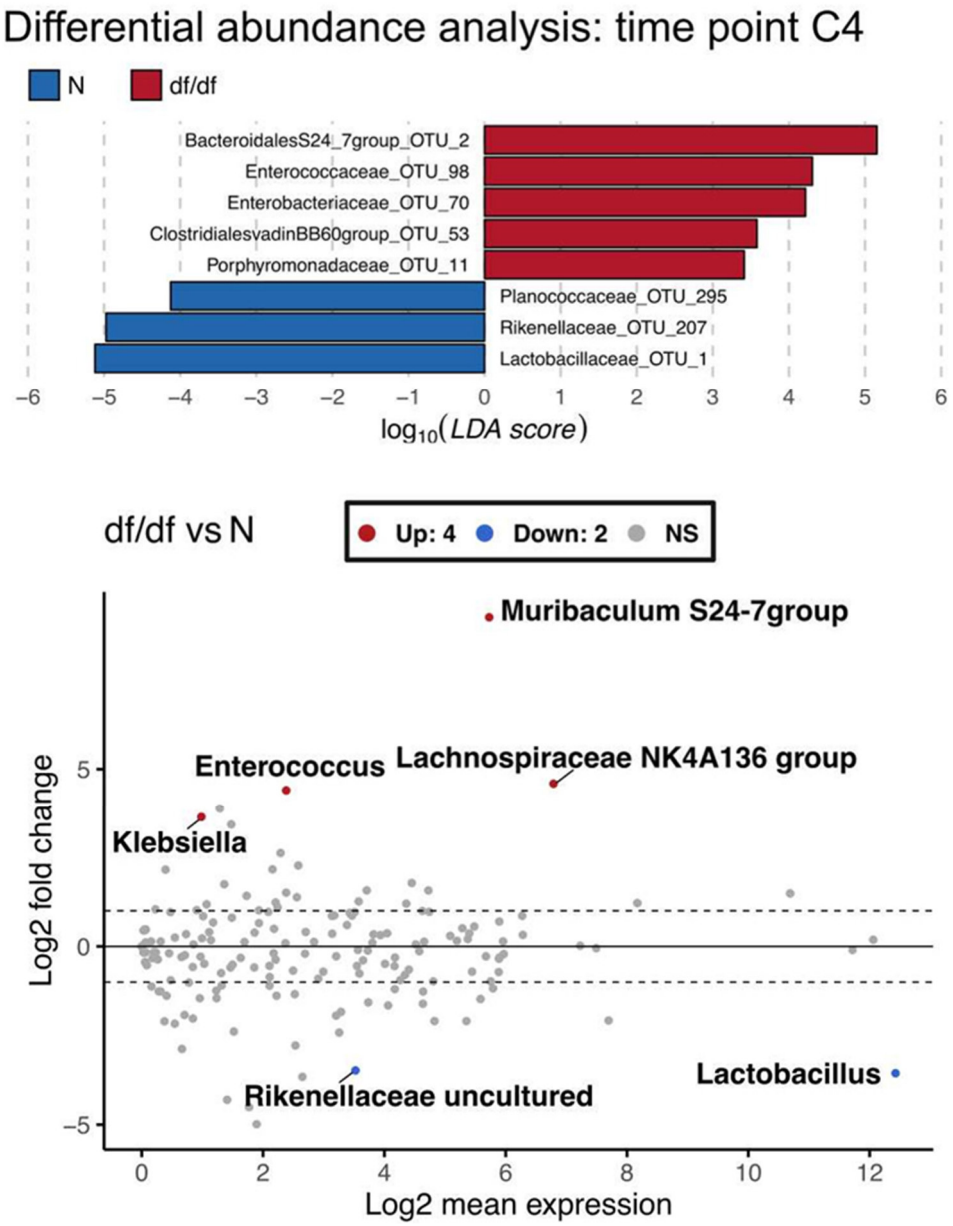


Figure 10 - The variability of the gut microbiota in N and df/df littermates is described through genetics and breeding pair

(A) Experimental design by using Normal (N) and Ames dwarf mice (df/df) littermates for microbiome normalization. For DNA isolation the fecal samples were collected from parents **(P-N, P-df/df)** and offspring C2 (**2nd week**) and C4 (10th -week) after weaning. At C4, luminal samples were taken of indicated locations (GI: Ileum, Cecum and Distal Colon).

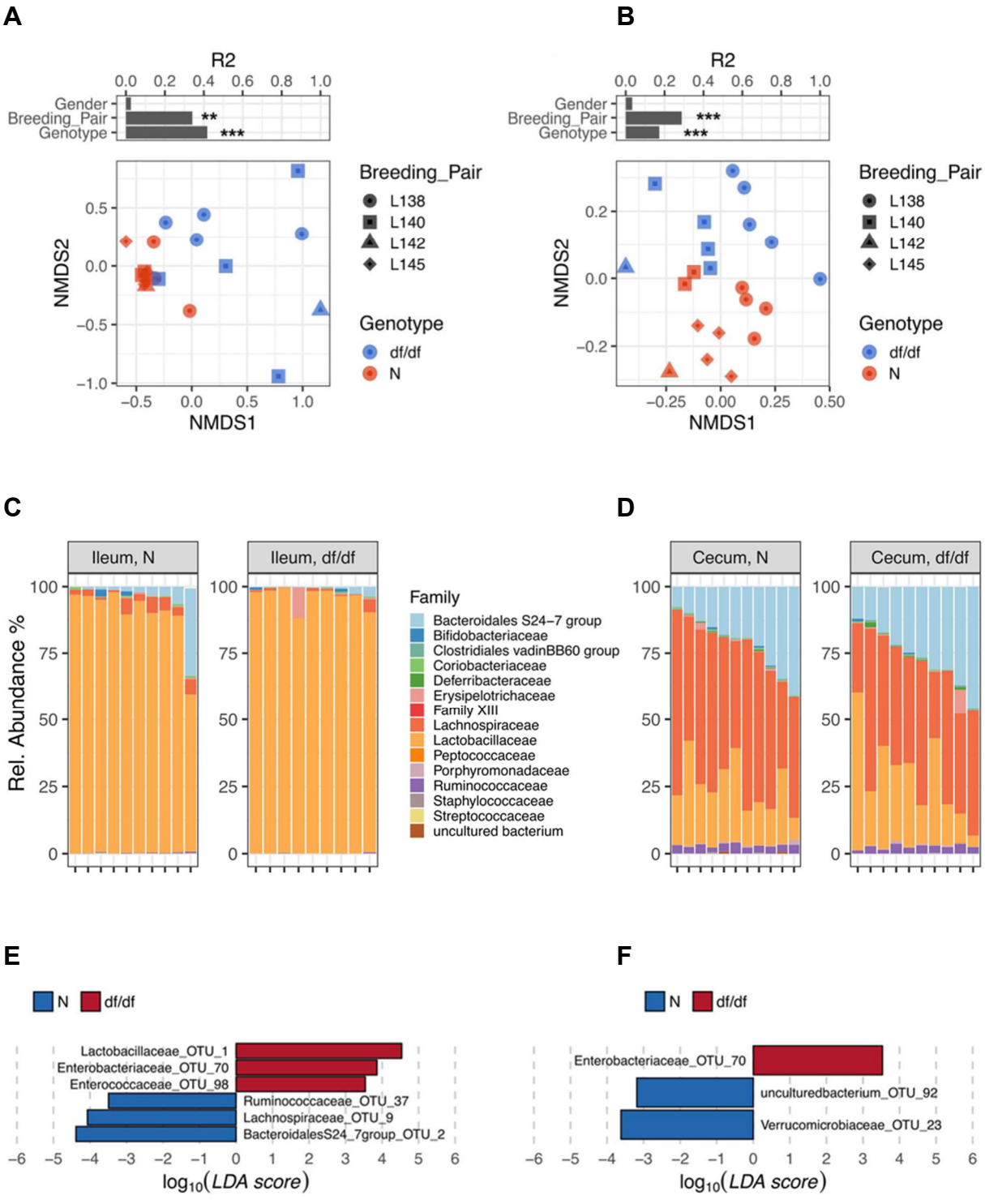
(B) Alpha-diversity in regard to offspring fecal microbiome at C2 as well as C4 by using observed-richness and **(C)** Shannon index. The Bacteroidetes/Firmicutes ratio (calculated to be the relative abundance of Bacteroidetes / total (Firmicutes + Bacteroidetes)) at timepoint C2 and C4.

(D) Analysis of Beta-diversity of the gut microbiota composition at different timepoints C2 and C4. Variance effect size of "genotype", "breeding pair" and “gender” by using NMDS ordination analysis and Bray-Curtis distances of colonic samples at timepoint C2 and C4.
(**E**) Weighted UniFrac distances of samples according to genotype at timepoint C4. In blue, are shown the comparisons among the same category and in red, the inter-group comparison.
(F-G) Taxonomic bar plot of dominant bacterial families for each sample (top 14 Families) sorted through Bacteroidetes/Firmicutes ratio.
(**H)** Differential abundance analysis (DA) in between df/df and also N mice utilizing LEfSe as well as DESeq2. The upper chart represents the comparison of LEfSe at Family level (log10(LDA score) > 3.5). Below the upper chart, MA plots displays DA OTUs (Up: red; down: blue; P values < 0.05 after correction for several tests). A significant effect was due to the ADONIS test when the 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.

4.3 Alterations in gut microbiota are not restricted to the colonic microbiome

In order to determine if df/df mice have also changed the composition of the microbiota in the individual segments like small intestine and cecum, a comprehensive analysis of microbial communities in these organs was carried out. An analysis of beta diversity in the ileum and cecum, being consistent with data from the distal colon, showed distinctions among mice from the two genotypes for both organs (Fig. 11A and B). A permutational multivariate ANOVA analysis provided evidence that the observed variability was driven by both the breeding pair (R2= 0.341, p= 0.004 **, Ileum) (R2= 0.288, p= 0.001 ***, Cecum) and the genotype (R2= 0.417, p= 0.001 ***, Ileum) (R2= 0.172, p= 0.001 ***, Cecum). According to earlier studies in further mouse lines (GALVEZ et al., 2017), the microbiome in the small intestine of df/df and also N mice is mainly dominated through Lactobacillaceae (Fig. 11C). The Lactobacillaceae, Enterobacteriaceae and Enterococcaceae families were identified as being overrepresented in the ileum of df/df mice through LEfSe (LDA score > 3.0), whereas the relative abundance of Muribaculaceae, Lachnospiraceae and Ruminococcaceae families were decreased (Fig. 11E). Remarkably, just one OTU, Lactobacillus spp., was determined, with enhanced abundance, while a number of various other OTUs with low abundance were reduced (Fig. 11G). The cecal microbiota of df/df and also N mice is mostly controlled by Muribaculaceae, Lachnospiraceae and Lactobacillaceae in both groups which is comparable to the microbiota in the distal colon (Fig. 11D). LEfSe determined the Enterobacteriaceae family to be elevated in df/df mice in cecum, that correlates with the enhanced relative abundance of this family in the distal colon (Fig. 11F). Furthermore, several bacterial families that were increased in the distal colon, like Muribaculaceae, were not changed in the cecum. Remarkably, in both the cecum as well as distal colon of df / df mice, it was found the same OTU (OTU 2) from the Muribaculaceae family

indicating the existence of common changes in the cecum and distal colon (Fig. 11H). The relative abundance of Lactobacillaceae was not as prominently modified in the cecum as it was in the distal colon, implying the existence of site-specific genotype-dependent alterations in the microbiome. Based on these evidences GH deficiency in df/df mice impacts the intestinal populations in colon and cecum distinctively.



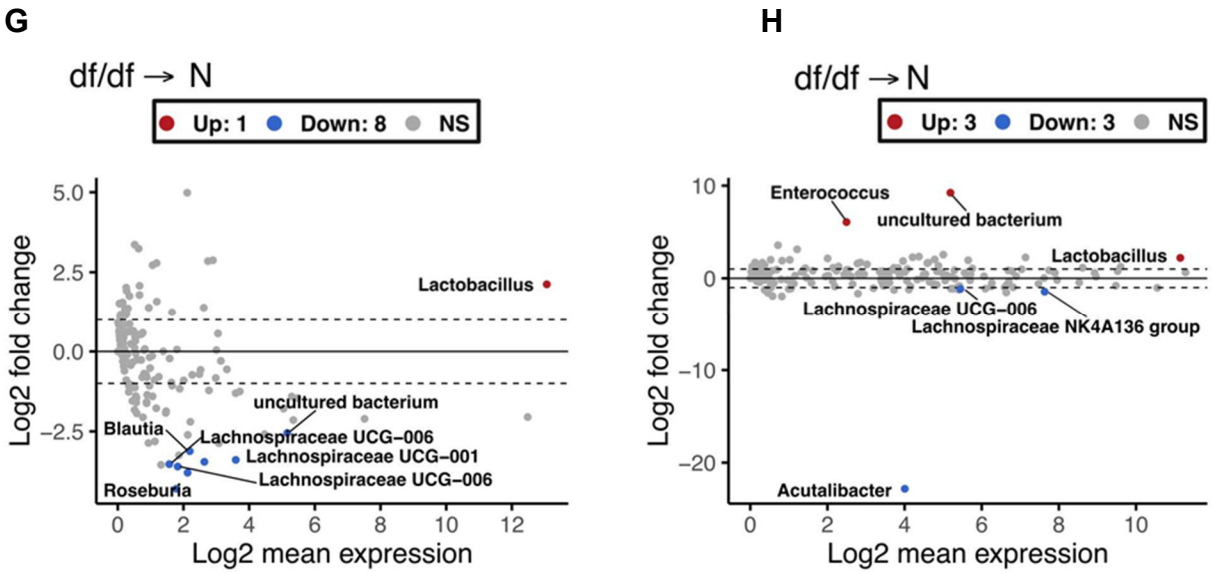


Figure 11 – Different microbiota composition in Ileum and Cecum in df/df mice.

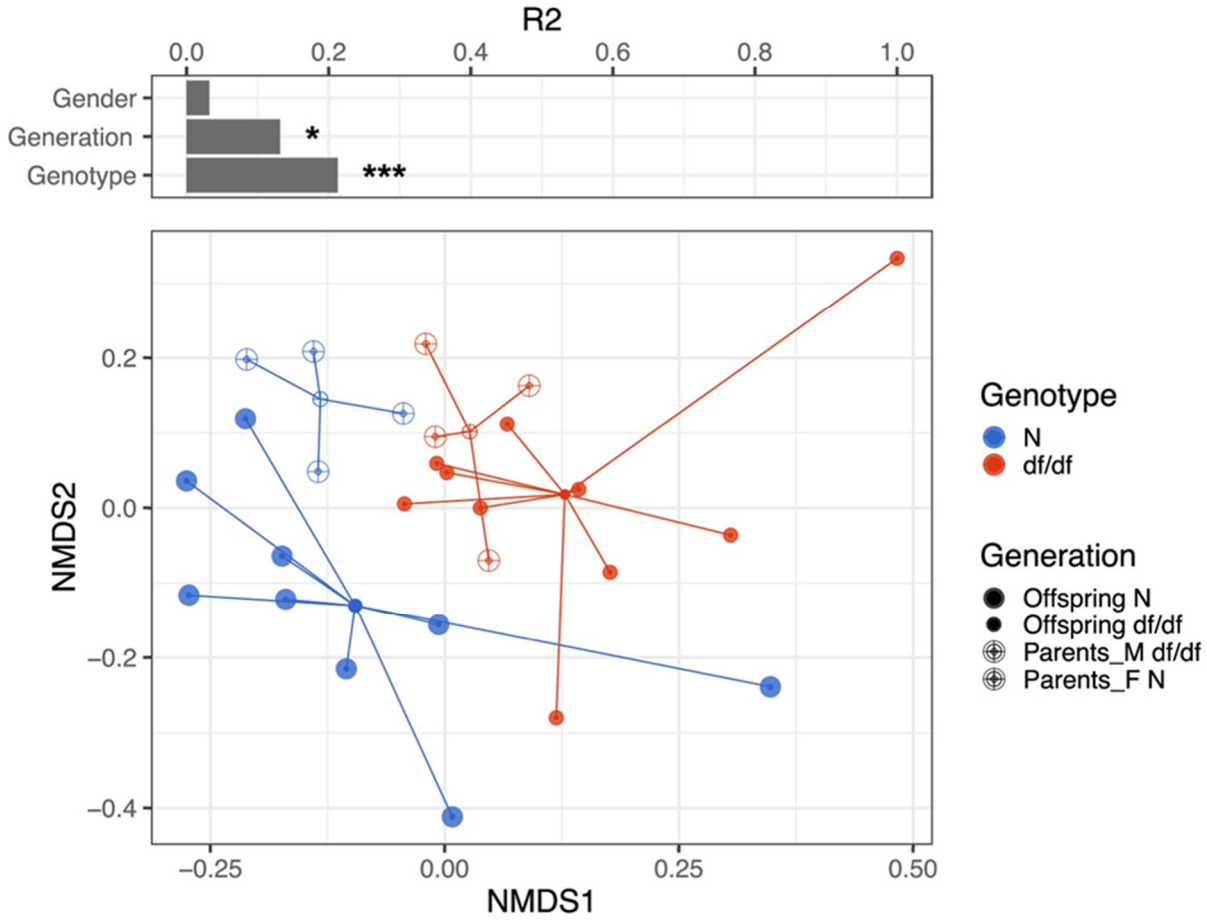
Analysis of Beta-diversity of intestinal microbiota from different intestinal segments at timepoint C4. Variance effect size using NMDS ordination analysis in **(A)** Ileum, and **(B)** Cecum.
Taxonomic bar plot of the dominant bacterial families for each sample (top 14 Families) sorted through Bacteroidetes/Firmicutes ratio **(C-D)**.
(E-H) Differential abundance analysis (DA) in between df/df and also N mice by using LEfSe as well as DESeq2. The upper chart represents the comparison of LEfSe at Family level (log10(LDA score) > 3.5). Below the upper chart, MA plots displays DA OTUs (Up: red; down: blue; P values < 0.05 after correction for several tests). 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.

4.4 The vertical inheritance of microbiota in df/df mice

After having an effect of GH deficiency on microbiota in the GI tract, it was next examined if changes in df/df offspring mice are characteristic over generations when comparing the microbiota from parents (F0, genotype: mother: N/df; father: df/df) and offspring (F1, genotypes: N and df/df). Beta diversity analysis showed distinct pattern that were affected by both genotype (Adonis, R2= 0.212, p= 0.001 ***) and generation (R2= 0.131, p= 0.022 *) (Fig. 12A). In order to further assess the influence of the genotype, it was conducted a comparison of the microbial communities through measuring weighted Unifrac distances between individual mice of the various groups. When genotype-dependent effects are maintained across generations, it would predict smaller Unifrac weighted differences between the mice of the same genotype relative to mice of the same generation but just another genotype. In line with this hypothesis, comparing the distances between the F1 mice showed significant

differences depending on the genotype (Fig. 12B and C). In particular, the F0 males, sharing the very same genotype, were the closest related group, meaning the next shortest distance, for the F1 df/df mice (Fig. 12B). The nearest related group for the F1 N/df mice were the F0 mothers, who shared the genotype similarly (Fig. 12C). This all together gives additional proof of distinctive genotype-dependent changes in GH-deficient df/df mice relative to N mice.

A



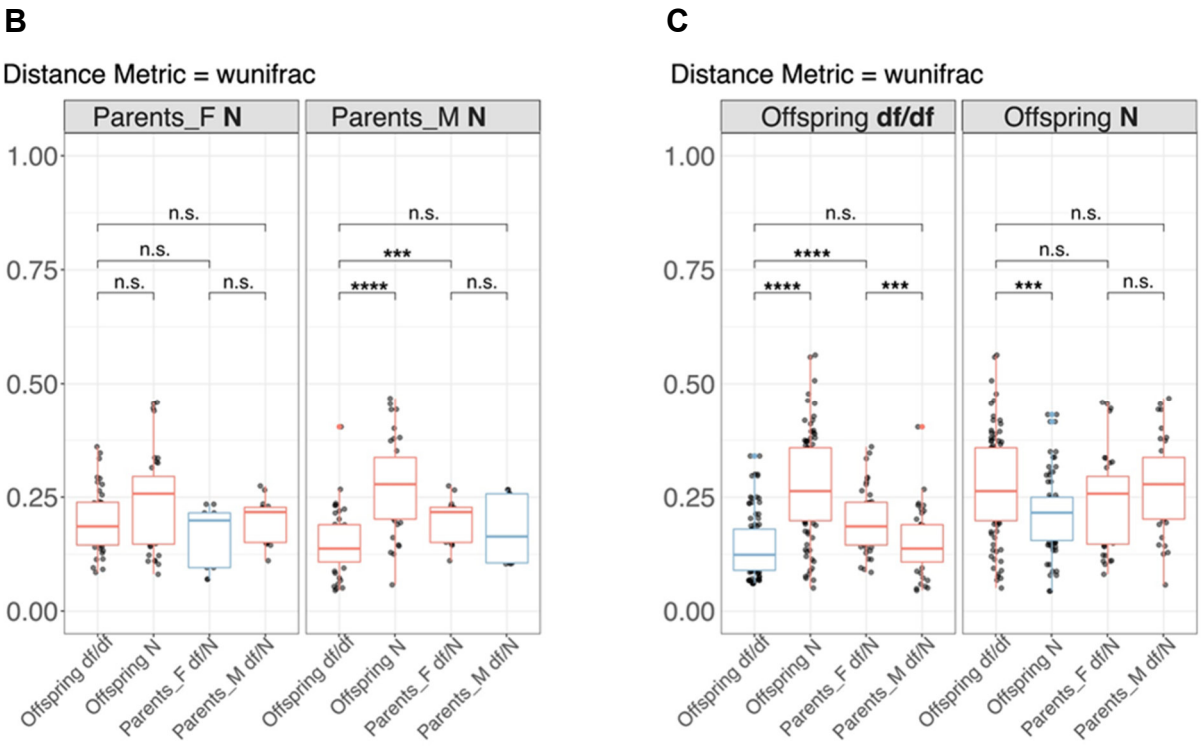


Figure 12 - The offspring of df/df mice demonstrates a genotype-associated composition of the microbiota

(A) The analysis of Beta-diversity of offspring vs. parents (homozygotes df/df and heterozygotes N). The variance effect size of "Genotype", "Gender" as well as the interaction among “Generation and genetics” of colonic samples.
(B-C) The comparison of weighted UniFrac distances from homozygotes df/df as well as heterozygotes N offspring mice against parents. In blue, are shown the comparisons within the same category and in red, the inter-group comparison.

4.5 The effects of Calorie restriction on bodyweight and on fasting blood glucose in N and df/df mice

As expected, subjecting animals to 30% CR showed that the body weight of N-CR and df/df-CR mice were significantly decreased in comparison to their ad libitum (AL) fed littermate controls ($p < 0.0001$*** and $p = 0.0016$**) (Fig. 13). Additionally, the fasting blood glucose levels were also measured and as anticipated from previous observations df/df-AL mice were characterized through lower levels of fasting blood glucose in contrast to N-AL mice.

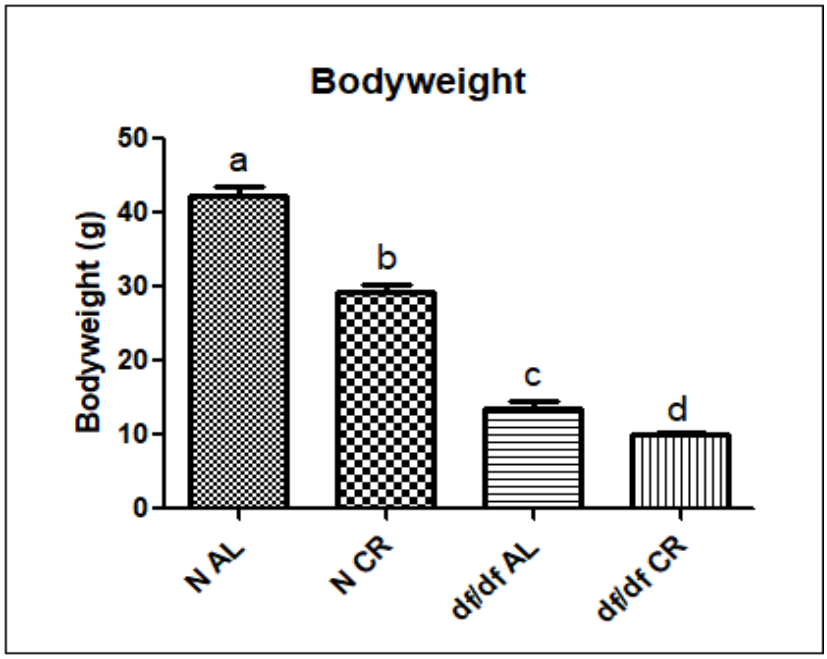


Figure 13 - Bodyweight comparison of Normal (N) and Ames dwarf (df/df) mice fed ad libitum (AL) or subjected to 30% calorie restriction (CR). Different letters indicate statistical significance.

As anticipated from previous observations df/df-AL mice were characterized through lower levels of fasting blood glucose in contrast to N-AL mice (p= 0.0343*). The CR treatment caused a significant decrease in blood glucose levels in N-CR mice in comparison to N-AL littermate control mice (p< 0.0001***) and also led to an additional decrease in blood glucose levels in df/df-CR mice relative to df/df-AL mice (p= 0,0042**), (Fig.14).

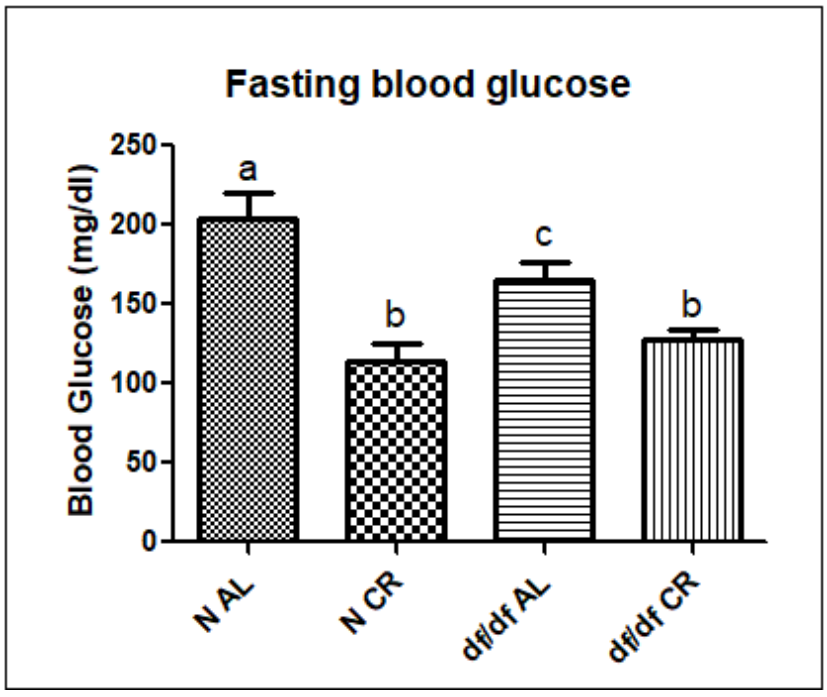
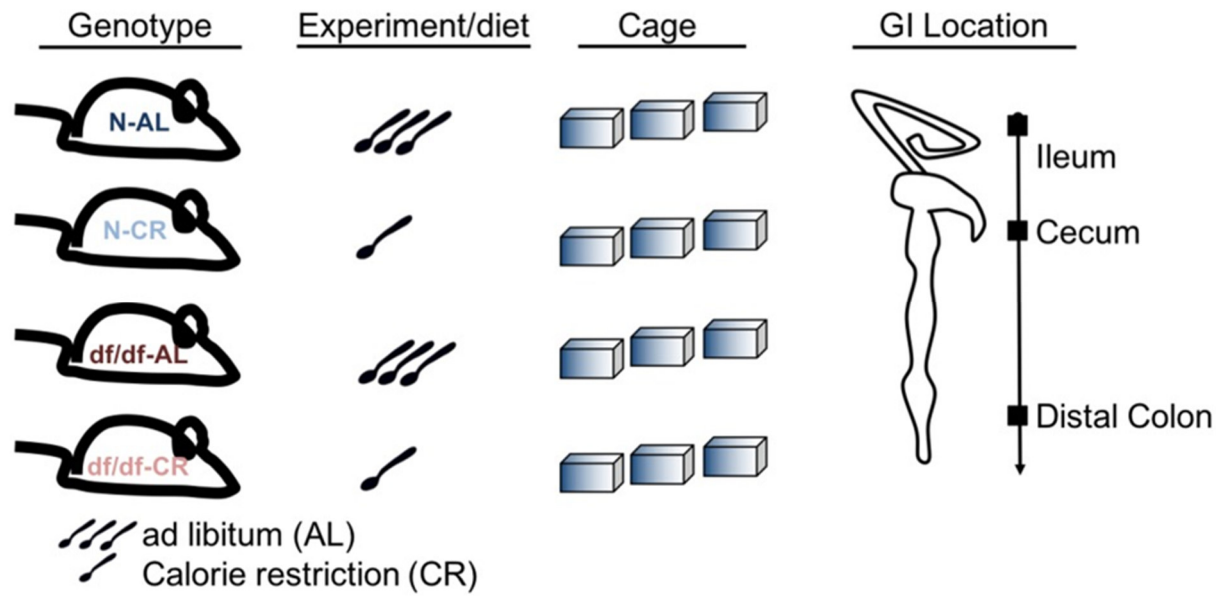


Figure 14 - Fasting blood glucose levels of Normal (N) and Ames dwarf (df/df) mice fed ad libitum (AL) or subjected to 30% calorie restriction (CR). Different letters indicate statistical significance.

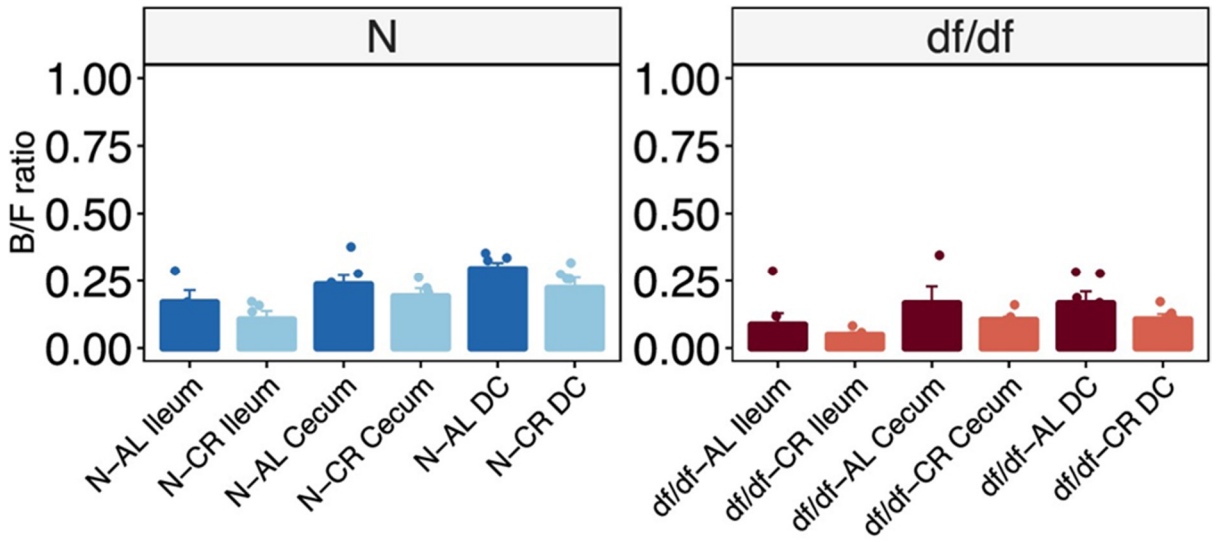
4.6 Calorie restriction intervention changes the microbiota in df/df and N mice

In the next step, it was examined if diet causes similar or divergent changes in the microbiota of df/df and N mice. Thus, df/df and N mice underwent a 30% CR beginning at 3 months of age and after 6 months of dietary treatment, the microbiome analysis was carried out (Fig. 15A). First, alpha diversity in ileum, cecum and distal colon of df/df-AL, df/df-CR, N-AL as well as N-CR mice was investigated. However, despite a greater number of species observed in the cecum of N-AL in comparison to N-CR mice, there were no significant distinctions noted among mice of different genotype or diet (Fig. 15B-D). By comparison, analysis of beta diversity using Bray-Curtis dissimilarity as well as non-metric multidimensional scaling (NMDS) showed distinctions amongst groups for the individual sample sites (Fig. 15E-G). Alterations between df/df-AL and N-AL mice (data not shown) were observed as with sampling times in previous experiments, demonstrating that there are genotype-dependent modifications in the microbiota at various time stages. Additionally, to the influence of genotype, the total evaluation of variability identified a large cage-driven effect as well as small impacts of the variable diet (Fig. 15E-G). The analysis of the microbiota composition among N-AL and N-CR mice utilizing LEfSe at genus level showed that CR caused significant alterations in the intestinal microbiota at all examined sites (Fig. 16A). In the ileum was an overabundance of various members of Lachnospiraceae (OTU_184 and OTU_21) and Ruminococcaceae (Acutalibacter OTU_71) (Fig. 16B). Remarkably, the Erysipelotrichaceae (Turicibacter OTU_6) family in cecum and colons showed significant enrichment in N-AL mice, which indicates that this class of bacteria is CR-responding (Fig. 16C-D). By comparison, LEfSe only detected minor CR induced changes in the microbiota of df/df mice (Fig. 16E-G). This result implies that CR-induced changes differ among the two genotypes, because in N mice was seen this strong CR-induced decrease in the family Erysipelotrichaceaen but not in df/df mice. In summary, this data develops that CR affects the composition of microbiota in N mice in a different way than in df/df mice.

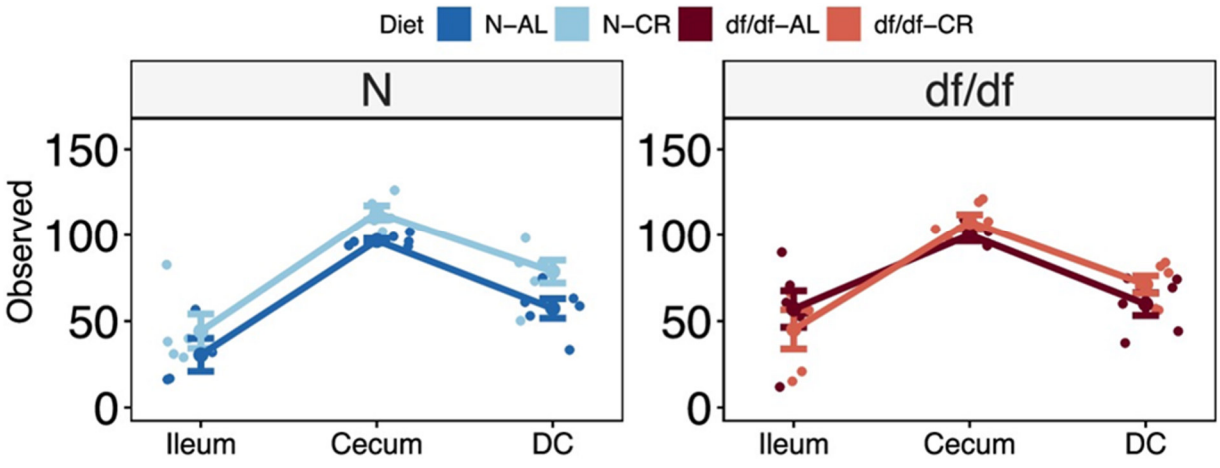
A


Genotype
Experiment/diet
Cage
GI Location
N-AL
N-CR
df/df-AL
df/df-CR
Ileum
Cecum
Distal Colon
ad libitum (AL)
Calorie restriction (CR)

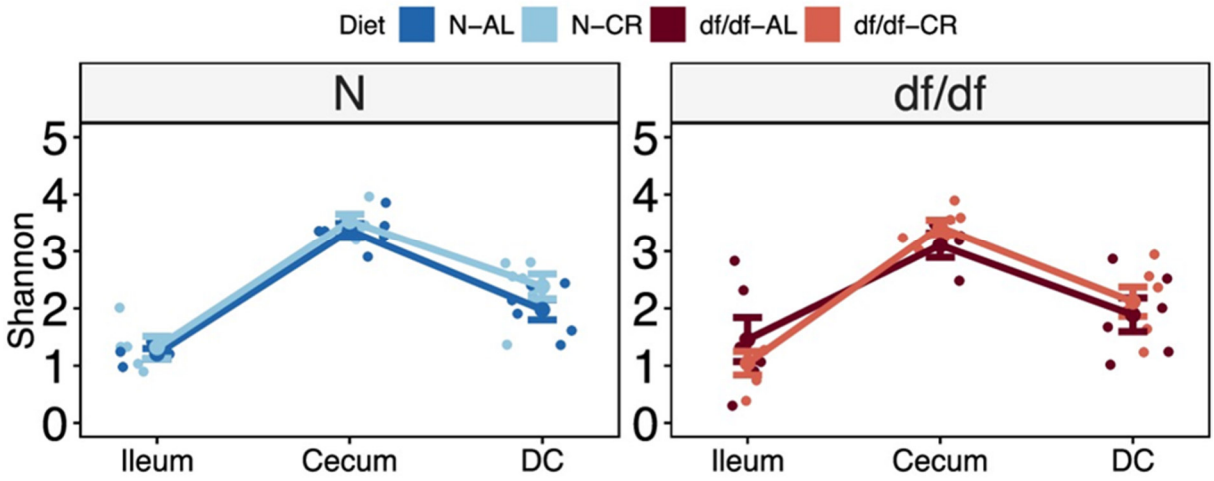
B


N
df/df
B/F ratio
1.00
0.75
0.50
0.25
0.00
N–AL Ileum
N–CR Ileum
N–AL Cecum
N–CR Cecum
N–AL DC
N–CR DC
df/df–AL Ileum
df/df–CR Ileum
df/df–AL Cecum
df/df–CR Cecum
df/df–AL DC
df/df–CR DC

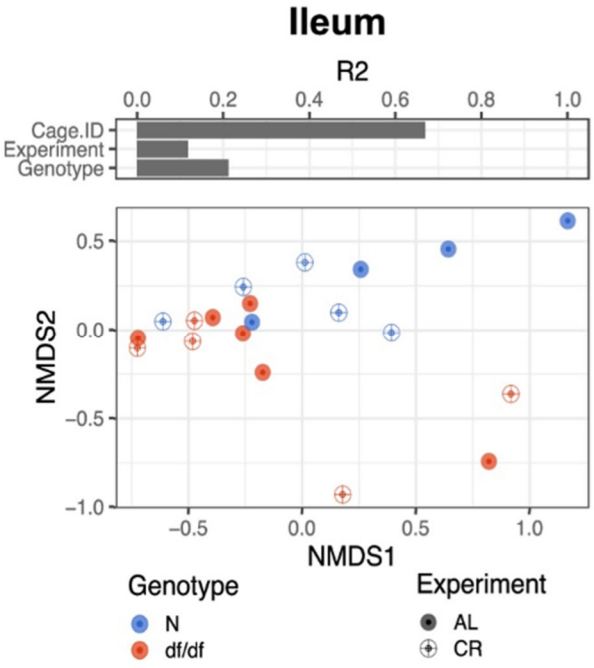
C


Diet
N–AL
N–CR
df/df–AL
df/df–CR
N
df/df
Observed
150
100
50
0
Ileum
Cecum
DC

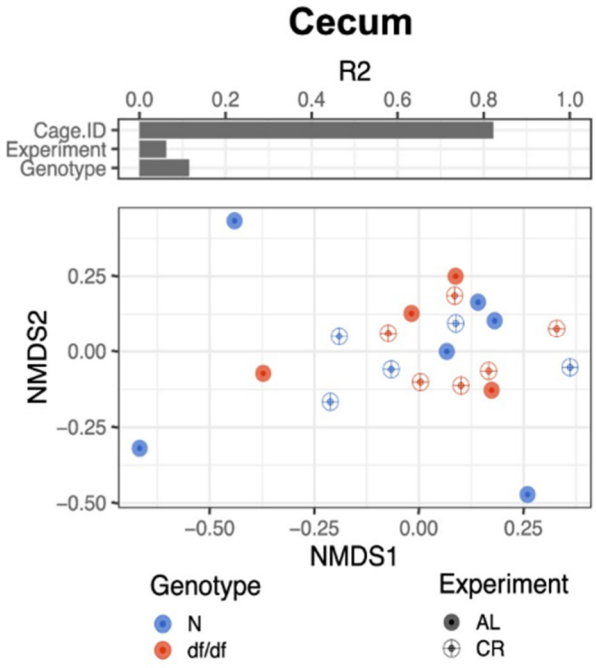
D


Diet
N–AL
N–CR
df/df–AL
df/df–CR
N
df/df
Shannon
0
1
2
3
4
5
Ileum
Cecum
DC

E


Ileum
R2
0.0
0.2
0.4
0.6
0.8
1.0
Cage.ID
Experiment
Genotype
NMDS2
NMDS1
0.5
0.0
−0.5
−1.0
Genotype
N
df/df
Experiment
AL
CR

F


Cecum
R2
0.0
0.2
0.4
0.6
0.8
1.0
Cage.ID
Experiment
Genotype
NMDS2
NMDS1
0.25
0.00
−0.25
−0.50
Genotype
N
df/df
Experiment
AL
CR

G

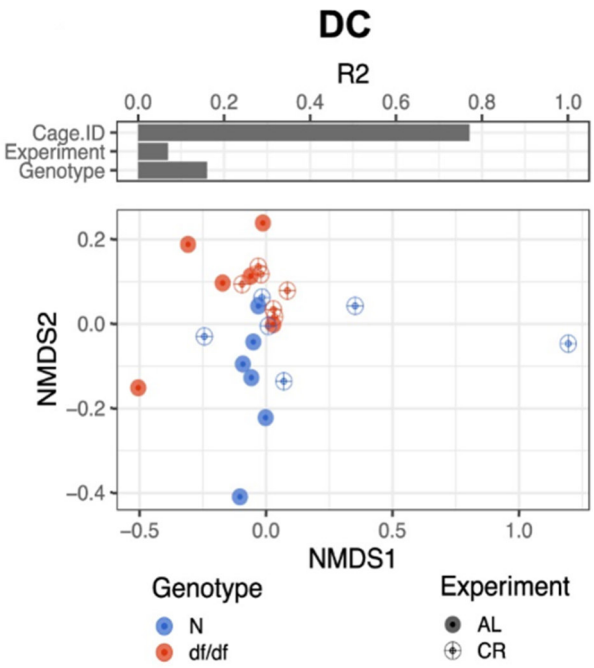
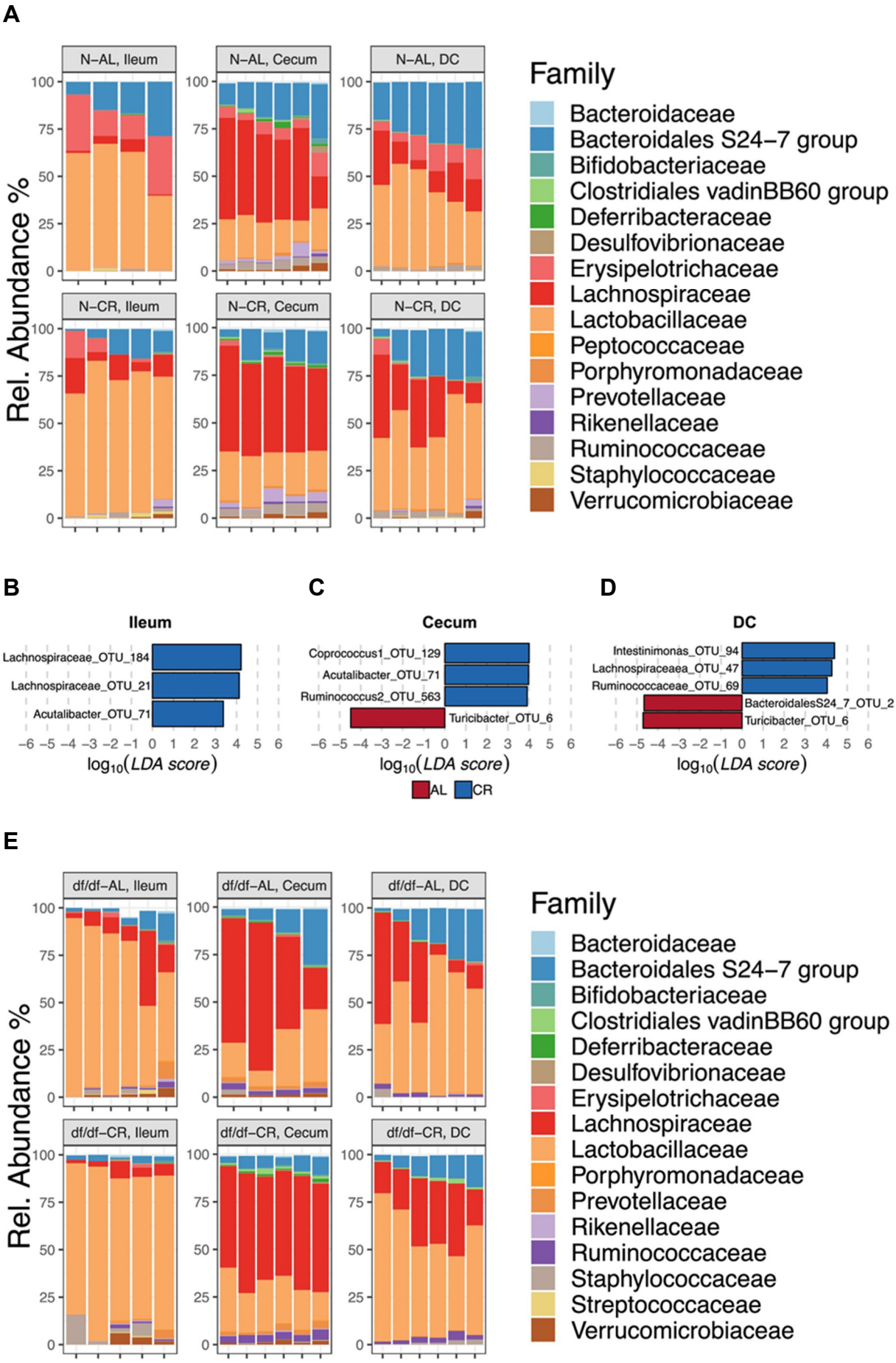


Figure 15 - The gut microbiota composition in relation to calorie restriction in long-living df/df mutants and N littermate controls mice.

(A) Experimental design to normalize the microbiome by using littermates Normal (N) as well as Ames dwarf mice (df/df). From parents **(N-AL, N-CR, df/df-AL, df/df-CR)** were drawn fecal samples for DNA isolation. After mice were euthanized, the luminal samples were taken from the described locations (GI locations: Ileum, Cecum and Distal Colon).

(B-D) Estimation of alpha-diversity by using the observed-richness, Shannon index and as well Firmicutes/Bacteroidetes ratio.

(E-G) Analysis of Beta-diversity of intestinal microbiota on different anatomical sites. Variance effect size by using ADONIS test as well as NMDS ordination analysis using Bray-Curtis distances coming from luminal samples.


A
N–AL, Ileum
N–AL, Cecum
N–AL, DC
N–CR, Ileum
N–CR, Cecum
N–CR, DC
Rel. Abundance %
Family
Bacteroidaceae
Bacteroidales S24–7 group
Bifidobacteriaceae
Clostridiales vadinBB60 group
Deferribacteraceae
Desulfovibrionaceae
Erysipelotrichaceae
Lachnospiraceae
Lactobacillaceae
Peptococcaceae
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Ruminococcaceae
Staphylococcaceae
Verrucomicrobiaceae
B
Ileum
Lachnospiraceae_OTU_184
Lachnospiraceae_OTU_21
Acutalibacter_OTU_71
log10(LDA score)
C
Cecum
Coprococcus1_OTU_129
Acutalibacter_OTU_71
Ruminococcus2_OTU_563
Turicibacter_OTU_6
log10(LDA score)
AL
CR
D
DC
Intestinimonas_OTU_94
Lachnospiraceaea_OTU_47
Ruminococcaceae_OTU_69
BacteroidalesS24_7_OTU_2
Turicibacter_OTU_6
log10(LDA score)
E
df/df–AL, Ileum
df/df–AL, Cecum
df/df–AL, DC
df/df–CR, Ileum
df/df–CR, Cecum
df/df–CR, DC
Rel. Abundance %
Family
Bacteroidaceae
Bacteroidales S24–7 group
Bifidobacteriaceae
Clostridiales vadinBB60 group
Deferribacteraceae
Desulfovibrionaceae
Erysipelotrichaceae
Lachnospiraceae
Lactobacillaceae
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Ruminococcaceae
Staphylococcaceae
Streptococcaceae
Verrucomicrobiaceae

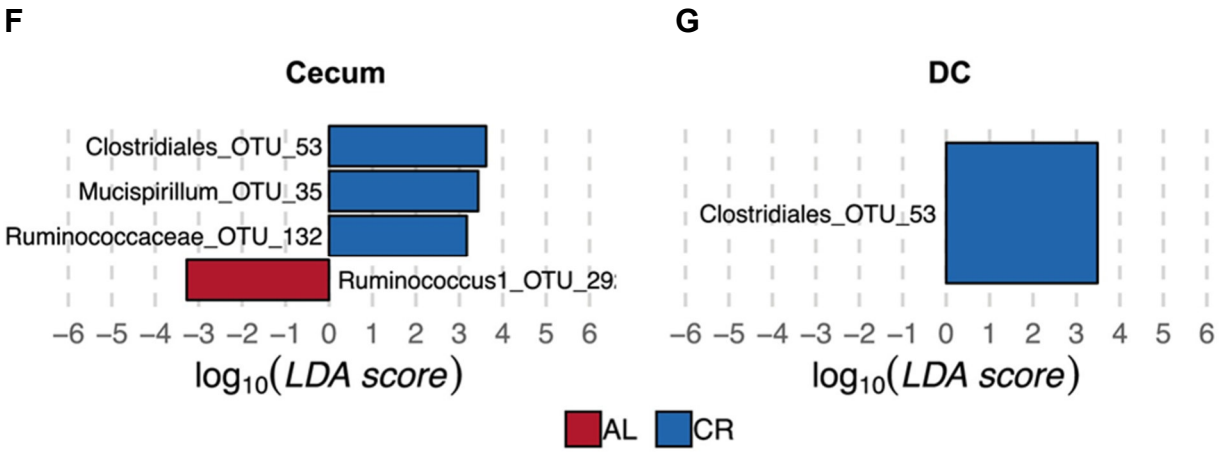


Figure 16 - The impact of Calorie Restriction on the gut microbiota in N and df/df mice

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

(B-D) Differential abundance analysis (DA) in between df/df and also N mice by using LEfSe comparison at Genus level (log10(LDA score) > 3.5).

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

(F-G) Differential abundance analysis (DA) in between df/df and N mice by using LEfSe comparison at Genus level (log10(LDA score) > 3.5).

5. Discussion

Insulin sensitivity in Ames dwarf mice

Enhanced longevity of GH-deficient df/df mice makes them a suitable model for aging research (BARTKE, BROWN-BORG, 2004; BROWN-BORG et al., 1996; MASTERNAK et al., 2004; MASTERNAK et al., 2005; MASTERNAK, BARTKE, 2007, 2012; MASTERNAK et al., 2009; MASTERNAK et al., 2010; TATAR et al., 2003; WANG et al., 2006). Moreover, there is strong evidence that these mice have enhanced insulin signaling, making them a valuable model for researching insulin as well as glucose metabolism (MASTERNAK et al., 2009; WANG et al., 2006). Previously published data reveal that the replacement of GH in these animals reverses their enhancement in insulin signaling and also reduces their life expectancy (MASTERNAK et al., 2010; PANICI et al., 2010). However, this strengthens the hypothesis that the main factor controlling lifespan and the metabolic environment of df/df mice is GH deficiency rather than PRL or TSH deficiency. As displayed in Fig. 5, df/df mice have significantly reduced fasting insulin levels compared to N mice, however they are still capable to sustain normal levels of fasting glucose. Earlier findings suggest that there is no distinction among glucose levels in df/df and N control mice throughout the young life as well as adult years. With aging of these animals, the GH-deficient df/df mice sustain lower glucose levels compared to their N control mice (LOUIS et al., 2010; MASTERNAK et al., 2004; MASTERNAK et al., 2005; MASTERNAK et al., 2009; MASTERNAK et al., 2010; MENON et al., 2014; PANICI et al., 2010). Healthy people also have no issues with increasing levels of fasting glucose, glucose intolerance or resistance to insulin. Nevertheless, there is a slight decrease in glucose tolerance in normal aging populations from the third decade of life, except of centenarians, which maintain a high insulin sensitivity throughout their whole lifetime (BARBIERI et al., 2003; DEFRONZO, 1981). Results of the clamp study revealed that df/df mice needed a ~2-fold higher glucose infusion rate to maintain glycemic levels similar to that of N control mice, suggesting improved insulin sensitivity and a greater suppression of glucose appearance in these long-living animals. In long-living df/df mice are the most remarkable metabolic phenotypes their enhanced insulin regulation and glucose homeostasis (BARTKE et al., 2013). The simultaneous decrease in both insulin and glucose levels demonstrates an improvement in the ability of insulin to clear glucose and shows a great protection from development of metabolic syndrome and diabetes. The explanations for their enhanced insulin sensitivity may be due in part attributable to improvements in the expression levels of hepatic genes which are associated with glucose metabolism (AL-REGAIEY et al., 2005) as well as improved insulin signaling in skeletal muscle. Importantly, it was shown that Ames dwarf mice have tendency to get obese, yet fat also help with greater clearance of glucose in df/df mice when compared with N control animals.

Furthermore, the study by Menon et al. showed that while removal of visceral fat in N mice improved insulin sensitivity and glucose tolerance the same procedure had rather negative effects on glucose metabolism in healthy obese df/df animals indicating important role of all, liver, skeletal muscle and fat in maintaining high insulin sensitivity and improved glucose metabolism in df/df mice (MENON et al., 2014). The use of tracer techniques revealed that long-living df/df mice have a better hepatic insulin action than their N controls, which was shown through a complete suppression from endogenous rates by glucose appearance. However, these results sustain previous findings that show enhanced insulin sensitivity at the levels of insulin receptor (IR) and IR substrate-1 (IRS1) in the liver of long-living df/df mice (LOUIS et al., 2010; MASTERNAK et al., 2004; MASTERNAK et al., 2009; MASTERNAK et al., 2010; PANICI et al., 2010). After acute insulin stimulation, the phosphorylation of the IR was significantly greater in df/df mice, however this effect was attenuated by the replacement of GH (MASTERNAK et al., 2010; PANICI et al., 2010). These results demonstrate that the liver plays a major role in controlling insulin sensitivity in df/df mice, which can add to a prolonged longevity. In the systemic regulation of glucose and lipid metabolism, the liver plays a major role and an aberrant hepatic insulin action is known to be a main driver of the insulin resistance, in which higher circulating insulin levels are required to regulate blood glucose levels adequately (SANTOLERI, TITCHENELL, 2019). Nonetheless, as already demonstrated before, the reactions to injected insulin (MASTERNAK et al., 2009) as well as the efficiency of glucose clearance (MASTERNAK et al., 2010; MENON et al., 2014) in these mice most likely require multiorgan action. The current research studies likewise show improved insulin-stimulated gastrocnemius and also vastus muscle glucose uptake in df/df mice. This can additionally play an essential key role in preserving normal glucose levels in df/df animals identified by extremely reduced circulation insulin levels as the skeletal muscle stands for the main organs accountable for glucose clearance. It is also important in glucose metabolism regulation as it is well known that metabolic complications and insulin resistance often starts with resistance of insulin sensitivity in skeletal muscle and observed high glucose uptake in df/df skeletal muscle indicate greater protection form metabolic complications. Notably, adipose tissue of df/df mice also displayed an enhanced uptake of glucose relative to adipose tissue from N animals in response to insulin stimulation. This supports the notion that adipose tissue plays an important role in regulating whole body levels of insulin sensitivity through GH's response. As previously revealed that surgical elimination of visceral fat enhances insulin sensitivity in N mice, whereas the exact same treatment either impaired insulin sensitivity or had no impact in neither GH-deficient df/df nor GH-resistant Laron dwarf mice (MASTERNAK et al., 2012; MENON et al., 2014). This presents an essential function of visceral fat, which is referred to as "bad" metabolic body fat, in keeping a well-balanced healthy glucose level as

well as high insulin sensitivity in these long-living mutants. The study further indicates that the repression of GH signaling substantially changes adipose tissue function on increased insulin sensitivity, which is possibly be mediated through impacts on adipose tissue glucose uptake. The results of previous research showed improved IR and IRS1 insulin-stimulated phosphorylation of df/df mouse in adipose tissue (MASTERNAK et al., 2010), that indicates more effective activation of insulin signal pathway in adipose tissue at cellular levels. This improved insulin signaling pathway is actually required to sustain a healthy glucose level along with a chronically reduced insulin level in long-living df/df mice. Surprisingly, the rates of brain glucose uptake in df/df mice were decreased. Usually, decreased brain glucose levels are linked to enhanced hepatic glucose production and as well reduced muscle and adipose glucose uptake, which was not seen in df/df mice. In addition, a reduced brain glucose supply is also linked to cognitive deficits, while df/df mice actually retain their cognitive function longer in comparison to their control mice (KINNEY et al., 2001). Therefore, the mechanism as well as the impacts of lowered brain glucose uptake in df/df mice needs still to be studied. However, it can be assumed that it is an adjustment to low glucose levels in df/df species. Notably, it is not required to support whole-body insulin sensitivity through increased insulin signaling action in all target insulin organs. It might offer the proof that some blockade or suppression of the cellular signal can be advantageous for health and aging. The over-expression of the Klotho gene has been mentioned to prolong the lifespan of mice however at the same time it also generates insulin resistance (KURO-O et al., 1997; KUROSU et al., 2005). In a similar way, rapamycin therapy prolongs lifespan and inhibits insulin sensitivity and glucose tolerance instead of increasing it, which means that suppression of insulin signaling or parts of the signal may also be advantageous for healthy aging at certain stages of life (BARTKE, 2006; FANG, BARTKE, 2013; KURO-O et al., 1997; KUROSU et al., 2005). It was hypothesized that the adjustment to low glucose uptake in df/df brain could be similar to the condition existing throughout CR or starvation which might be important to maintain the healthy brain function throughout the lifespan as well as aging. As this interesting phenomenon should be further investigated in the future, one might speculate that it could be related to preservation rather than over activation of PI3K/AKT and mTOR signaling pathway in the brain.

To sum up, hyperinsulinemic-euglycemic clamp studies show enhanced tissue-specific insulin sensitivity in long-living df/df mice. This effect is mediated through changes on insulin action in hepatic, muscle and as well fat tissue. These practical data are related to the past studies, presenting increased insulin signaling in these tissues responsive to insulin (LOUIS et al., 2010; MASTERNAK et al., 2004; MASTERNAK et al., 2005; MASTERNAK et al., 2009; MASTERNAK et al., 2010; MENON et al., 2014; PANICI et al., 2010; WANG et al., 2006). Taken all together, these results point to the fact that enriched insulin sensitivity in various

organ systems and the ensuing enhancement in total metabolic status can lead to prolonged life span of df/df mice (BARTKE, WESTBROOK, 2012; MENON et al., 2014; WESTBROOK et al., 2014; WESTBROOK et al., 2009).

Impact of Ames dwarfism and CR on gut microbiota

Considering that the gut is one of the main entrances to the body, it is well equipped with a complex intrinsic immune system. Actually, the gut is also one of the most important organs in regulating food processing, nutrient intake and immune system function. One of the most essential requirements for healthy aging as well as extended lifespan is preserving a healthy gut microbiome. Ames dwarf mice live longer and healthier lives than their N-littermate controls, however, the gut microbiota has never been studied before in these long-living df/df mice. In this study the changes in the gut microbiome of GH-deficient df/df mice relative to their N littermate control mice were assessed. This study was carried out in both parents and offspring, considering gender and also genotype. In addition, three different GI locations (Ileum, Cecum, and Distal Colon) changes in the intestinal microbiota in CR-treated in comparison with the AL-fed N and df/df mice were assessed. Throughout the study, significant changes could be demonstrated in the composition of gut microbiota in long-living df/df mice relative to N mice. Taking into account the same parents, the same diet, and an environment which is free of pathogens as formerly defined, indicates that the bacterial composition in the gut of those mice is regulated by GH deficiency and that cecum and colon intestinal communities are variably affected by genotype, which indicate that genetic alterations might be more impactful on the microbiome than just environment and diets. Furthermore, these findings indicated that the composition of microbiota between df/df mice and their N controls was strongly modified at 70 days of age, and there were minor variations in the composition of microbiota among df/df mice relative to the overall difference between df/df and N mice. These outcomes support the importance of genotype-dependent alterations in the microbiota composition. In this current research analysis, the ratio among Bacteroidetes and Firmicutes (B/F ratio), also well-known as indicator for a healthy microbiota, was quantified (BRUSSOW, 2013; LEY et al., 2006). These two major bacterial phyla, Bacteroidetes and Firmicutes which were described above, are the most dominant bacteria in the intestine with an abundance of more than 99%. (DIBAISE et al., 2008). Human studies have demonstrated that the ratio of Bacteroidetes and Firmicutes is associated with body weight. In overweight subjects, it was discovered that the intestinal microbiota used to have a higher proportion of Firmicutes and lower population of Bacteroidetes when contrasted to lean subjects, also the proportion of Bacteroidetes was higher on a lower calorie diet with weight loss (LEY et al., 2006).

Furthermore, the B/F ratio in ob/ob mice has also changed (LEY et al., 2005). Most notably, these results indicate that the B/F ratio of df/df mice compared with the N littermate controls is significantly higher. Research studies were also associated with changes in the B/F ratio with aging in humans (MARIAT et al., 2009) and also in younger mice the B/F ratio is higher compared to older mice (SPYCHALA et al., 2018). Hence, for these long-living df/df mice, this special composition of the intestinal microbiota in df/df mice can therefore be considered as advantageous. Muribaculaceae, which was formerly referred to the Bacteroidales S24-7, is a member of the phylum Bacteroidetes and the Lachnospiraceae, Ruminococcaceae, Lactobacillaceae as well as Erysipelotrichaceae belongs to the phylum Firmicutes (SMITH et al., 2019). In both genotypes, the bacteria of Muribaculaceae, Lachnospiraceae and Lactobacillaceae were recognized. In particular, df/df mice showed a higher number of Muribaculaceae, Enterococcaeceae, Enterobacteriaceae, Clostridialesvadin BB60 group, and Porphyromonadaceae, while the number of Lactobacillaceae, Rikenellaceae, and Planococcaceae were lower relative to their N littermate controls. The Bacteroidales diversity is known to increase in humans through childhood (ENCK et al., 2009; HOPKINS, MACFARLANE, 2002) and reaches adult levels at approximately 17 years of age (BALAMURUGAN et al., 2008). Through the entire life, the amount of Bacteroides remains nearly stable, but decreases throughout aging (MARIAT et al., 2009). In this research study, it was shown an accumulation of the Muribaculaceae bacterial family, which belongs to the order Bacteroidales, in df/df mice. In previous studies, it has been revealed that the Rikenellaceae family was detected in the microbiome of older people (CLAESSON et al., 2012). Remarkably, the quantity of this bacteria family is reduced in long-lived df/df mice whereas it is enhanced in N mice. It might indicate that the Rikenellaceae family is playing a crucial role in the regulation of lifespan. This research study showed that GH deficiency impacts the intestine microbiota early throughout postnatal development. The composition of microbiota can be influenced by many factors such as diet or environmental conditions. Remarkably, in df/df mice one OTU Lactobacillus with an enhanced abundance in this research study. In previous studies, it has been formerly reported that raised levels of Lactobacilli as well as Enterococci were found in older people relative to young adults (SALAZAR et al., 2017). Nonetheless, others showed that Lactobacilli were expressed in lower quantities in the elderly compared to young adults. Moreover no variations were identified in the levels of Bacteroides and Eubacterium (HOPKINS et al., 2001). In addition, it has been stated that the composition of the bacteria in the gut is really variable in elderly people (CLAESSON et al., 2011; CLAESSON et al., 2012). Current studies have consistently demonstrated lower Bacteroides and Eubacterium levels in elderly people contrasted to younger individuals (HARMSEN et al., 2000; HILL et al., 2016). In general, throughout early development, the data showed significant modifications in the

microbiota. In the literature, there is strong evidence that shifts in microbiota are even greater in humans as well as animals during advanced aging (MAFFEI et al., 2017). Animals were not tracked throughout the entire aging process, but it was plainly shown how a distinct microbiome evolves in extended longevity models during early life. It is believed that it might be also relevant and insightful to investigate the microbiota throughout early postnatal development regarding the impact on aging and in particular in its intersection with GH signaling. In recent studies it was shown that a short treatment with GH for 6 weeks using GH-deficient df/df mice throughout early development pre-programmed the lifespan of these mice by shortening their longevity (PANICI et al., 2010). This result suggests that physiological changes based on GH during early development could stand for an important pre-programming factor in the aging process. In general, this may indicate that throughout early development the distinctions in gut microbiota in GH-deficient mice may represent important changes in bacteria populations, which promote healthy lifespan during aging. Nonetheless, further follow-up studies are needed in these long-lived dwarf mice to investigate longitudinal changes in gut microbiome. It is also required to follow-up with translational research studies in order to offer a better relevance of these results to human aging. As mentioned earlier, several important similarities exist, i.e., low abundance of Rikenellaceae in long-living df/df mice already at a young age as compared to N mice, whereas in humans these bacteria are known to be increased during aging. This could imply that from early life on, df/df mice are shielded from overpopulations of these bacteria. Nonetheless, until further longitudinal studies are available, this can only be hypothesized. Most importantly, in order to achieve a better significance for these results and their translational significance, it would be of great benefit to follow up on microbiota studies in humans with IGHD type 1B, caused by a mutation of the GHRH receptor gene or as well individuals which are affected by Laron dwarfism (AGUIAR-OLIVEIRA, BARTKE, 2019). As reported in previous studies, the genus Lactobacillus was found to be significantly enhanced in CR (FRAUMENE et al., 2018). The increase in relative abundance of Lactobacillus, sets well to lower levels of cholesterol and triglycerides as a result of CR (FRAUMENE et al., 2018), while it is not clear which modifications occurs first. Calorie restriction is a widely known experimental approach which can result in lowered bodyweight, lowered plasma insulin and also glucose levels, as well as improved insulin level of sensitivity, health span and also life expectancy (ANDERSON et al., 2009). There is limited knowledge about the impact of CR on gut microbiota; nonetheless, previous studies revealed that diet regimen is an important aspect influencing the intestinal microbiota throughout aging (YATSUNENKO et al., 2012). The gut microbiota plays an essential function in regulating the impacts of a diet regimen treatment consisting of weight-loss, maintaining a high basal metabolism rate and reducing blood glucose and serum cholesterol levels (WANG et al., 2018). In fact, antibiotic treatment has

been shown to remove the metabolic-regulatory roles of the bacteria in the intestine as well as leads to loss of the health benefit of the intestinal microbiota (WANG et al., 2018). In this study, it was intended to explore how the CR treatment in df/df as well as N control mice would influence the composition of the bacteria in three various GI locations. The impact of CR was strongly marked on the microbiome in N in contrast to df/df mice. Furthermore, an overabundance of various members of the order Bacteroidales in ileum in N-AL mice and a significant improvement of the Erysipelotrichaceae family in cecum as well as colon was seen. Nevertheless, the bacteria family Erysipelotrichaceae did not show any alterations in the CR experiments. However, Erysipelotrichaceae plays an important role in the development of metabolic disorders (KAAKOUSH, 2015). In addition, in obese individuals there were greater amounts of this bacteria family found (ZHANG et al., 2009). A current research revealed that a depletion of gut microbiota made mice resistant to CR-induced loss of body weight, leading to enhanced fat mass (WANG et al., 2018), which indicates that the microbiota could be the crucial mediator of CR effects. Actually, the results revealed that df/df mice take profit from the impacts of CR (BARTKE et al., 2001a). Nevertheless, changes in body weight are not as marked as in N mice (MASTERNAK et al., 2004), as well as some impacts on insulin signaling are actually eliminated through GH substitute (GESING et al., 2014). This data is consistent with the current observations that the microbiota diversity in df/df mice is actually much less influenced through CR as in N mice. Due to these and earlier findings, not every mouse model has a benefit of CR effects on the lifespan. As a result, a detailed investigation of the microbiota in humans subjected to long-term CR would be beneficial for future functional research studies, which may be followed up with ongoing human CR studies or with the support of volunteers participating in CRON-diet investigations (Calorie Restriction with Optimal Nutrition).

Summarizing, this research study highlights that GH-deficient df/df mice have a distinct gut microbiota in comparison to N animals. During the postnatal development, there were significant differences in the B/F ratio as well as genotype-dependent changes in the microbiota composition. Additionally, results revealed that modifications of the microbiota from df/df mice were related with parental vertical transmission. Moreover, it was found that alterations in intestinal microbiota were more apparent in N than df/df mice after a dietary treatment, indicating the different impacts of CR on the various starting microbial communities. In order to better understand the function of GH deficiency and CR in the regulation of the gut microbiome, more studies are needed. However, one could hypothesize that the composition of the gut microbiome probably conduces to the phenotypic differences and health benefits observed in long-lived df/df mice and it could be considered as new potential hallmark of age-related and metabolic modifications.

6. Conclusions

These results of the Hyperinsulinemic-euglycemic clamp study showed improvements in whole-body insulin sensitivity in long-living df/df mice, which is primarily driven by a hepatic effect with a smaller contribution from muscle and fat glucose uptake, which was published by our team (WIESENBORN et al., 2014a). Based on these findings it may be inferred that the increased insulin sensitivity in the different insulin-responsive tissues leads to a significant improvement in metabolism, which in turn could lead to a longer lifespan of df/df mice.

These findings regarding to the development of the gut microbiota composition during early life displayed significant differences in df/df and N mice. Already during this early aging process, distinctions in the abundance of various bacteria between df/df and N mice were found. Another interesting finding in the present study with additionally CR treatment concerned the significant differences of the gut microbiome in the GI location, genotype and as well the diet experiment, both findings were published by our team (WIESENBORN et al., 2019). Based on these results it can be concluded that the gut microbiota plays a key role already during early life regarding to longevity in df/df mice. In addition to that CR could even regulate the lifespan by affecting the gut microbiota. Since the gut microbiota in long-living df/df mice was studied for the first time, more studies would be needed to better understand the function of GH deficiency and CR in the regulation of the intestinal microbiota.

7. References

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8. Authors List of Publications

2019 The Role of Ames Dwarfism and Calorie Restriction on Gut Microbiota.

***Wiesenborn DS**, Gálvez EJC, Spinel L, Victoria B, Allen B, Schneider A, Gesing A, Al-Regaiey KA, Strowig T, Schäfer KH, Masternak MM. J Gerontol A Biol Sci Med Sci. 2020 Jun 18;75(7):e1-e8. doi: 10.1093/gerona/glz236.*

2017 The role of transplanted visceral fat from the long-lived growth hormone receptor knockout mice on insulin signaling.

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2016 A Long-lived Mouse Lacking Both Growth Hormone and Growth Hormone Receptor: A New Animal Model for Aging Studies.

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2015 Thyroxine modifies the effects of growth hormone in Ames dwarf mice.

*Do A, Menon V, Zhi X, Gesing A, **Wiesenborn DS**, Spong A, Sun L, Bartke A, Masternak MM. Aging (Albany NY). 2015 Apr;7(4):241-55.*

2014 The effect of calorie restriction on insulin signaling in skeletal muscle and adipose tissue of Ames dwarf mice.

***Wiesenborn DS,** Menon V, Zhi X, Do A, Gesing A, Wang Z, Bartke A, Altomare DA, Masternak MM. Aging (Albany NY). 2014 Oct;6(10):900-12.*

2014 Insulin sensitivity in long-living Ames dwarf mice.

***Wiesenborn DS**, Ayala JE, King E, Masternak MM. Age (Dordr). 2014;36(5):9709. doi: 10.1007/s11357-014-9709-1. Epub 2014 Aug 29.*

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