Insight into the Role of Gut Microbiota in Duchenne Muscular Dystrophy

An Age-Related Study in Mdx Mice

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Dystrophin deficiency alters the sarcolemma structure, leading to muscle dystrophy, muscle disuse, and ultimately death. Beyond limb muscle deficits, patients with Duchenne muscular dystrophy have numerous transit disorders. Many studies have highlighted the strong relationship between gut microbiota and skeletal muscle. The aims of this study were: i) to characterize the gut microbiota composition over time up to 1 year in dystrophin-deficient mdx mice, and ii) to analyze the intestine structure and function and expression of genes linked to bacterial-derived metabolites in ileum, blood, and skeletal muscles to study interorgan interactions. Mdx mice displayed a significant reduction in the overall number of different operational taxonomic units and their abundance (α-diversity). Mdx genotype predicted 20% of β-diversity divergence, with a large taxonomic modification of Actinobacteria, Proteobacteria, Tenericutes, and Deferribacteres phyla and the included genera. Interestingly, mdx intestinal motility and gene expressions of tight junction and Ffar2 receptor were down-regulated in the ileum. Concomitantly, circulating inflammatory markers related to gut microbiota (tumor necrosis factor, IL-6, monocyte chemoattractant protein-1) and muscle inflammation Tlr4/Myd88 pathway (Toll-like receptor 4, which recognizes pathogen-associated molecular patterns) were up-regulated. Finally, in mdx mice, adiponectin was reduced in blood and its receptor modulated in muscles. This study highlights a specific gut microbiota composition and highlights interorgan interactions in mdx physiopathology with gut microbiota as the potential central metabolic organ.

DMD development and the links between the affected or-
tients DMD pathophysiology and offer improvement in the pa-
ment, prevention of secondary processes that exacerbate
decade of life. However, in the absence of a curative treat-
patients with DMD can now expect to live into their fourth
processes, altered calcium homeostasis, oxidative
antibiotics leads to a decrease in skeletal muscle endur-
depletion of gut bacteria by treatment with broad-spectrum

dysfunction and quality in patients with DMD (a
mary therapies (to restore or partially restore functional
disease, and myoblast transplantation. Many other therapies are being evaluated
inflammation markers [IL-6, IL-4, monocyte
skeletal muscle in
variants (eg, obesity, type 2 diabetes, insulin resistance).

cachexia (ie, the cancer-associated loss of skeletal muscle

defects, and impaired autophagy, as well as declines in mitochondrial number and function.

Actual therapies can be divided in two categories: pri-
primary therapies (to restore or partially restore functional
dystrophin protein) and other therapies (to improve muscle
ceptors FFAR3 and FFAR2 promote insulin sensitivity and
modulate glucose uptake. These findings suggest that gut
microbiota composition/function in DMD might be altered, with possible effects on the host’s health and myopathy. However, very few exploratory data are available on intesti-
nal smooth muscle in DMD, although dystrophin is expressed in smooth muscle, and intestinal function is altered in patients with DMD. Moreover, gastrointestinal allo-
ations, including aerophagia, gastrolesophageal reflux, and constipation (affecting nearly 70% of patients for this last item), have been documented in these patients.

The term gut microbiota describes the 10^{14} bacteria housed in the digestive tract and classified into different
species, families, and phyla. This bacterial community plays an important role in the host metabolism and health. Due to the functional crosstalk with other organs (eg, brain, heart, liver, adipose tissue), perturbations of the gut microbiota composition and function (ie, dysbiosis) have been associated with many diseases, such as brain disorders (eg, depression, autism, Alzheimer disease) and metabolic dis-
orders (eg, obesity, type 2 diabetes, insulin resistance). Moreover, several studies have shown the implication of the intestinal microbiota in antitumor treatment and in cachexia (ie, the cancer-associated loss of skeletal muscle and adipose tissue). Indeed, Bindels et al found that the gut microbiota was depleted in a leukemic and cachexic mouse model. Interestingly, when the gut microbiota was normalized by oral supplementation of probiotics, the concentration of inflammation markers [IL-6, IL-4, monocyte chemoattractant protein-1 (MCP-1), and granulocyte colony-stimulating factor], autophagy markers (LC3 and cathepsin L), and proteolysis markers (atrogin1 and MAFb) in the muscles decreased. Probiotics prevented skeletal muscle inflammation and atrophy. Since this first study suggesting a gut microbiota—skeletal muscle axis, accumulating evidence has highlighted the potential influ-
ce of gut microbiota on the skeletal muscle phenotype. Muscle mass is reduced in germ-free mice that lack microbiota compared with control mice. Gut bacteria are necessary to optimize skeletal muscle function. Indeed, depletion of gut bacteria by treatment with broad-spectrum antibiotics leads to a decrease in skeletal muscle endur-
and to an alteration in glucose homeostasis, as

indicated by the decreased expression of short-chain fatty acid chain (SCFA) and glucose transporters in the ileum, and reduced glycogen content in muscle. This phenotype is normalized after natural reseeding. Similarly, Yan et al observed that after transfer of gut microbiota from obese or lean pigs to germ-free mice, mice replicated the donor’s skeletal muscle fiber profile.

Different pathways might be involved in the gut microbiota—skeletal muscle axis. Several circulating medi-
tors, such as pro-inflammatory cytokines, SCFA, and branched-chain amino acids, in relation with the gut micro-
biota composition, have recognized effects on skeletal mus-
cle. For example, microbiota-derived SCFAs (eg, acetate, butyrate, propionate) produced during a gut microbial fermentation process could drive skeletal muscle toward an oxidative metabolism. Similarly, in mice fed a high-fat diet, butyrate supplementation is associated with improved insulin sensitivity, increased peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and AMP-activated protein kinase (AMPK) activity (regulation of energy metabolism), and a higher proportion of type 1 fibers in skeletal muscle. Moreover, the muscle-specific SCFA recep-
tors FFAR3 and FFAR2 promote insulin sensitivity and
modulate glucose uptake. These findings suggest that gut microbiota composition/function in DMD might be altered, with possible effects on the host’s health and myopathy. However, very few exploratory data are available on intesti-
snal smooth muscle in DMD, although dystrophin is expressed in smooth muscle, and intestinal function is altered in patients with DMD. Moreover, gastrointestinal allo-
ations, including aerophagia, gastrolesophageal reflux, and constipation (affecting nearly 70% of patients for this last item), have been documented in these patients.

Gut bacterial community alteration in DMD could be potentiated by the fact that this chronic disease is associated with conditions that promote intestinal dysbiosis in other pathologic contexts. For instance, a sedentary lifestyle has deleterious effects on microbial composition, and treatments prescribed to patients with DMD, particularly antibi-
totics to treat respiratory infections, deplete the intestinal microbiota. Therefore, to exclude these extrinsic factors, gut microbiota composition can be studied in mdx mice, an animal model of DMD. Some studies report that, similar to patients with DMD, mdx mice exhibit increased intestinal peristalsis and reduced fecal excretion, particularly due to an alteration of the migrant motor complex responsible for mobility in the interdigestive period.

The aims of the current study were: i) to characterize the gut microbiota composition in mdx mice and its changes during disease development by comparing the α- and β-
diversity of taxonomic profiles and gut microbiota abundance (16S RNA gene metagenomic analysis) in mdx mice and control littermates at 8 weeks, 12 weeks, 6 months, and 1 year of age, ii) to measure plasma biomarkers that could mediate the skeletal muscle—gut microbiota crosstalk, and iii) to investigate the intestine structure and contractile

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properties, as well as the expression in the ileum and muscle, of genes linked to bacterial-derived metabolites to identify microbiota–muscle interactions in the mdx phenotype.

Materials and Methods

Animal Care

Male mdx mice (C57BL10SnSc-DMD\textsuperscript{mdx}/J) and wild-type mice [B10: wild-type C57BL10SnSc raised in the laboratory’s animal facility (F2 generation)] were used at different ages (8 weeks, 12 weeks, 6 months, and 1 year) \((n = 10\) per group per age). Mice were housed in ventilated cages (20°C to 22°C, 12:12 hour light–dark cycle) with food and water \textit{ad libitum}. The diet (3395 kcal/kg) was standardized and identical for all groups (SAFE A03; SAFE, Augy, France). It included 69.2% cereals, 20.2% vegetal protein, 6.0% animal protein, and 4.6% of a mineral and vitamin cocktail. The following is a general description of the macronutrient composition: 61.3% protein carbohydrates, 25.2% protein, and 13.5% lipids. The study experimental protocols complied with the European directives on animal experimentation (86/609/EEC) and were approved by the French National Ethics Committee (APAFIS#19430-2019022513523628v2).

Stool Collection

Feces were collected directly from the anus of the mice at 8 weeks, 12 weeks, 6 months, and 1 year of age. Samples were then immediately frozen in liquid nitrogen before storage at −80°C.

Euthanasia and Sample Collection

Mice were fasted for 12 hours and then euthanized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). Blood was collected via inferior vena cava sampling and centrifuged at 2500 × g for 15 minutes; the plasma supernatant was snap-frozen and stored at −80°C. Intestinal tissue samples (duodenum, jejunum, ileum, and colon), cecum content, and soleus and tibialis anterior (TA) samples were collected, weighed, and immediately frozen in liquid nitrogen and stored at −80°C. Second TA samples were collected and placed directly in isopentane cooled in liquid nitrogen and stored at −80°C. Both extensor digitorum longus (EDL) muscles were carefully removed and tendons tied with braided surgical silk for future \textit{ex vivo} contractility.

TA Staining by Immunohistochemistry

Serial transverse sections (10 μm thick) from liquid nitrogen–cooled isopentane TA muscle samples embedded in optimal cutting temperature medium were obtained using a cryostat at −25°C and mounted on glass microscope slides. Sections were then washed in phosphate-buffered saline, blocked, and permeabilized with 0.1% Triton X-100 and 10% horse serum. Staining was performed with anti-laminin \((\text{L9393}; 1/200;\text{ MilliporeSigma, Burlington, MA}),\) anti-dystrophin \((\text{ab15277}; 1/200;\text{ Abcam, Cambridge, United Kingdom}),\) and Hoechst. This was followed by secondary antibody incubation with 546 donkey anti-rabbit \((1/500;\text{ Fluoroprobes, Interchim, Montluçon, France})\) for anti-laminin and Alexa 488 goat anti-rabbit \((1/1000;\text{ Thermo Fisher Scientific, Waltham, MA})\) for anti-dystrophin. Whole histologic sections were imaged with an automated imaging device (MRI-INM platform, Institute of Neuroscience, Montpellier, France; Axioscan, Zeiss, Oberkochen, Germany) and analyzed with ImageJ software version 1.53p (NIH, Bethesda, MD; \url{http://imagej.nih.gov/ij}).

\textit{Ex Vivo} Assessment of Contractility in EDL

Muscle contractile properties were assessed in \textit{ex vivo} conditions as previously described.\textsuperscript{32} This technique allows evaluation of the intrinsic muscle contractile properties. After 15 minutes’ equilibration in the Krebs solution bath continually bubbled with 95% oxygen to 5% carbon dioxide \((pH~7.4)\) and thermostatically maintained at 37°C, EDL samples were connected to a force transducer/length servomotor system (model 305B; Cambridge Instruments, Aurora Scientific Inc., Aurora, ON, Canada) and were stimulated along their entire length with platinum wire electrodes. The optimum muscle length \((i.e.,\) the muscle length producing maximal twitch tension) was determined. All subsequent measurements were made at the optimum muscle length. The isometric tetanic tension was determined \((701B\text{ Stimulator, Aurora Scientific Inc.})\) using stimulation trains of 500 milliseconds, with pulse duration of 0.5 millisecond at different frequencies, from 1 to 150 Hz. Stimulus trains were separated by a 1-minute interval. The maximum isometric tetanic tension was determined from the plateau of the frequency-tension curve. Three minutes after the tension-frequency determination, the resistance to fatigue was evaluated by using a low-frequency fatigue protocol of 50 Hz trains of 700 milliseconds delivered every 2 seconds for 5 minutes. The muscle fatigue index was defined as the time taken to produce a 50% reduction from the initial maximum power output. After all measurements, EDL samples were removed from the bath, trimmed of the connective tissue, blotted dry, and weighed. For each animal, the second EDL was placed in oxygenated Krebs solution throughout the procedure and was used in case of emergency.

Detection and Quantification of Plasma Proteins

One-quarter of the diluted plasma samples were analyzed by using the Meso Scale Discovery U-PLEX Metabolic Combo 1 (mouse) 11-Plex assays (MSD, Rockville, MD).
antibody set contains biotinylated capture antibodies and corresponding detection antibodies for 11 proteins: IL-1β, IL-4, IL-6, tumor necrosis factor, MCP-1, ghrelin (active and total), glucagon-like peptide-1 (active and total), leptin, and peptide YY (total). Assays were performed following manufacturer’s instructions using Meso Scale Discovery 96-well, 10-spot plates and recommended diluents, with all plasma samples being vortexed thoroughly before use. For experimental measurements with below detection limits, concentrations were considered as “0.” Levels of circulating adiponectin were measured by using ELISA. Adiponectin doses were determined by using commercial ELISA kits (#DY1065; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Ex Vivo Jejunum Basal Contractile Function

Jejunum basal contractile properties were assessed ex vivo at 1 year of age. Specifically, 1 cm of the first part of the jejunum was connected to a force transducer/length servo-motor system (model 305B; Cambridge Instruments, Aurora Scientific Inc.). The optimum smooth muscle length (ie, the muscle length producing maximal twitch tension) was determined. All subsequent measurements were made at optimum smooth muscle length. After 15 minutes’ equilibration in Krebs solution continually bubbled with 95% oxygen to 5% carbon dioxide (pH 7.4) and thermostatically maintained at 37°C, the basal peristalsis was recorded at 200 Hz for 15 minutes. This method is adapted from Alves et al.33 To determine the period of peristalsis, the signal obtained with DMC version 3.500 software (Aurora Scientific Inc.) was extracted and imported to MATLAB 1.8.0_202 (MathWorks, Portola Valley, CA), in which a fast Fourier transform was used to convert the signal to a frequency domain. The peak frequency was then obtained as the frequency corresponding to the peak of the power spectrum. The period P was then estimated as the inverse of the peak frequency.

Histologic Analyses of the Small Intestine

The small intestine was removed, divided into two parts with the mucosal layer outward using a long wooden stick and immersed in a solution of 4% paraformaldehyde in phosphate buffer (0.1 mol/L, pH 7.4) for 4 hours at room temperature.34 They were then rinsed in phosphate buffer and immersed in 20% sucrose in phosphate buffer (0.1 M, pH 7.4) for 24 hours at 4°C. Two parts of the intestine were opened longitudinally and coiled with the mucosal layer outward using a wooden stick, embedded in optimal cutting temperature medium, and frozen in isopentane cooled in liquid nitrogen. Swiss Rolls of intestine were cut in longitudinal sections into serial 10 μm thick slices and stained with hematoxylin and eosin and Alcian blue (stain goblets) for histologic analysis. Goblet cell density was determined by counting blue-stained cells per millimeter of intestine. Whole histologic sections were imaged with a digital slide scanner (MRI-INM platform; Nano-Zoomer 2.0-HT; Hamamatsu, Montpellier, France). The analysis was performed on NDP.view 2 software (Hamamatsu). Five fields of the same surface (7 mm²) were captured, and all cells marked were counted and analyzed with ImageJ software version 1.53p.

For structural analysis, Swiss Rolls of intestine were cut in longitudinal sections into serial 10 μm thick slices and stained with anti-laminin (L9393; 1/200; MilliporeSigma, Burlington, MA), anti-dystrophin (ab15277; 1/200; Abcam), and Hoechst. This was followed by secondary antibody incubation with 546 donkey anti-rabbit (1/500, Fluoroprobes, Interchim) for anti-laminin and Alexa 488 goat anti-rabbit (1/1000; Thermo Fisher Scientific) for anti-dystrophin. The images were acquired with an automated imaging device (Axioscan; Zeiss) provided by the facility imaging MRI-INM (Institute of Neuroscience).

mRNA Expression Analysis by Quantitative RT-PCR

Total RNA was isolated from ileum, TA, and soleus samples using TRIzol (15596-018, Invitrogen, Carlsbad, CA). RNA concentration was determined by spectrophotometric analysis (BioDrop DUO; BioDrop, Cambridge, UK), and purity was checked by calculating the OD260nm/OD280nm absorption ratio (>1.8). RNA quality was verified by using 1% agarose gel electrophoresis. Reverse transcription was performed with 2 μg of total RNA and the high-capacity cDNA Reverse Transcription Kit (catalog no. 4368813; Applied Biosystems, Waltham, MA) according to the manufacturer’s instructions. One-tenth of the obtained cDNA was used in each PCR assay. real-time quantitative PCR analysis was performed using a Step One Plus detection system (Applied Biosystems) with 10 μL of Mastermix (PowerUp SYBR Green Master Mix, Scientific A25742; Thermo Fisher Scientific), 10 nmol/L of forward and reverse primers, 5 μL of diluted cDNA template, and water to a final volume of 15 μL. Forward and reverse primers are listed in Table 1.

All PCR assays were performed in duplicate using the following cycling parameters: 50°C for 2 minutes, then 95°C for 2 minutes followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Relative mRNA levels were normalized to the levels of the housekeeping genes Arp, Tuba1a for ileum samples, and Arp and Rps9 for TA and soleus samples. Results are expressed using the comparative cycle threshold method to generate ΔΔCₜ values with template dilutions ranging from 10¹ to 10⁶ copies. The PCR overall efficiency (E) was calculated from the standard curve slopes according to the equation E = [10⁷⁻¹/(slope)] − 1, and this value was >95% for all assays. The relative abundance of each sample was normalized according to the equation: Relative Quantity = 2⁻ΔΔCₜ.
DNA Extraction from Feces

Total cell DNA was extracted from 0.1 g of animal fecal material using the GNOME Kit (BIO 10; MP Biomedicals, Irvine, CA) with modifications. Fecal samples were homogenized in the supplied cell suspension solution. Cell lysis/denaturing solution was added, and samples were incubated at 55°C for 2 hours. To improve cell lysis, 0.1-mm diameter silica beads (750 μL) were added, and samples were mixed at the maximum speed in a Fast-Prep (Bio10). The washing step was repeated once, and the remaining pellets were recovered. T he remaining pellets were resuspended in 400 μL of salt-out mixture and incubated at 4°C for 10 minutes. Samples were spun at maximum speed for 10 minutes, and DNA-containing supernatants were transferred to clean 1.5-mL microcentrifuge tubes. DNA was precipitated with two volumes of 100% ethanol at room temperature for 5 minutes, followed by centrifugation at 16,000 × g for 5 minutes. DNA was resuspended in 150 μL of TE buffer and stored at −20°C.

Evaluation of Microbiota Composition by Sequencing

The V3-V4 region of the 16S rRNA genes was amplified using the bacterial primers 343F (forward: 5′-CCTACAGGAGGCAGCAG-3′) and 784R (reverse: 5′-GGAGGTACCTTGGTATCTGAG-3′) modified to add adapters during the second PCR amplification. PCR assays were performed by using the MolTaq 16S DNA polymerase and the corresponding master mix (Molzym GmbH & Co. KG, Bremen, Germany). The PCR mix contained 10 ng of DNA, 1 μL of dNTPs (10 mmol/L), 1.25 μL each of forward and reverse primer (20 μM), and 0.5 μL of Taq polymerase in a total volume of 50 μL. The cycling program was as follows: 94°C for 3 minutes, followed by 40 cycles at 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 3 minutes.

Table 1 Primers Used for qPCR in Ileum, Soleus, and TA

<table>
<thead>
<tr>
<th>Function</th>
<th>Sample</th>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Housekeeping genes</td>
<td>Ileum, TA</td>
<td>Arp</td>
<td>5′-TCCACACCTGGTTGTCACGCTCT-3′</td>
<td>5′-ACTGCTAGTGGCATGACGACAGG-3′</td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>Tubalα</td>
<td>5′-GTGGCCACAGCATAGTTAT-3′</td>
<td>5′-CTGGAACCCACCGTCATC-3′</td>
</tr>
<tr>
<td>Tight junction proteins and gut permeability</td>
<td>Ileum</td>
<td>Tjp1 (alias Z0-1)</td>
<td>5′-ATCGCCAGTACGACCCATATA-3′</td>
<td>5′-TCTTACGTCGCTGGAC-3′</td>
</tr>
<tr>
<td>BCAAR receptor</td>
<td>Ileum</td>
<td>Ffar1</td>
<td>5′-AAATTACATCGCGACTCTCTGTC-3′</td>
<td>5′-CAAGTGGATCCAAACAGGACTTA-3′</td>
</tr>
<tr>
<td>BCAAR receptor</td>
<td>Ileum, Soleus</td>
<td>Ffar3</td>
<td>5′-CAAGGAGGAAGGAGGTGAGA-3′</td>
<td>5′-CCAGGATCCAAACAGGACTTA-3′</td>
</tr>
<tr>
<td>Adiponectin receptors</td>
<td>Ileum, TA</td>
<td>Adipor1</td>
<td>5′-AGAGAAACAGCAAGGAGGCAC-3′</td>
<td>5′-ACCAACAGCAGGAGCTT-3′</td>
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<tr>
<td>Lipid metabolism</td>
<td>Ileum, TA</td>
<td>Angpt4</td>
<td>5′-CCAGAACTTAACTGTCAGCAA-3′</td>
<td>5′-GAATGACTAGTCACTACAA-3′</td>
</tr>
<tr>
<td>Glucose transporter</td>
<td>Soleus</td>
<td>Slc2a4</td>
<td>5′-GCAACAGCTCCATCTCAGTG-3′</td>
<td>5′-GCCACAGTGAGGATCAG-3′</td>
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<tr>
<td>Inflammation</td>
<td>Ileum, Soleus</td>
<td>Myd88</td>
<td>5′-GGGTGGTGGCGGCAGAAGGAC-3′</td>
<td>5′-CTTGAATTTTTGTCACAGCT-3′</td>
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<tr>
<td></td>
<td>Ileum, Soleus</td>
<td>Tol4</td>
<td>5′-GGAGGCCTGTCAAGATGAC-3′</td>
<td>5′-CTTATCCGCAAGGATTCA-3′</td>
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<tr>
<td></td>
<td>Ileum, Soleus</td>
<td>Nfkβ1</td>
<td>5′-TGAGCAGGGTATGGCAGAC-3′</td>
<td>5′-TCACATGAAAGTTGCCAGTG-3′</td>
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<tr>
<td></td>
<td>Soleus</td>
<td>Rps9</td>
<td>5′-GGTGGGATGACGACAAAGGAC-3′</td>
<td>5′-GCCACAGTGAGGATCAG-3′</td>
</tr>
</tbody>
</table>

BCAA, branched-chain amino acids; LCFA, long-chain fatty acids; qPCR, real-time quantitative PCR; SCFA, short-chain fatty acids; TA, tibialis anterior.
60 seconds, and a final extension at 72°C for 5 minutes. Sequencing was performed by using MiSeq technology (Illumina) at the Genopole Toulouse Midi-Pyrenees genomics facility (Toulouse, France).

Metagenomic Analysis

Sequencing data were demultiplexed at the Genopole Toulouse Midi-Pyrenees platform. Version 3.2.3 was used to produce abundance tables of operational taxonomic units (OTUs) and their taxonomic affiliation following author guidelines. The most abundant sequences of each OTU were then matched with blastn to the Silva version 132 database. Abundance tables and taxonomy matrices were imported into RStudio (version 1.2.1335), and phyloseq 1.28.0, ggplot2 3.4.0, and custom scripts were used for data analysis. Samples were rarefied to even sampling depths before computing within-sample compositional diversities (Observed richness, Chao1, Shannon, and InvSimpson) and between-samples compositional diversity (UniFrac). Principal coordinates analysis was also performed on dissimilarity matrices to obtain a two-dimensional representation of the samples. Alpha diversity data were analyzed by using repeated measures analysis of variance. Permutational multivariate analysis of variance tests were performed on UniFrac matrices using 9999 random permutations and a significance level of 0.01. The relative abundances of phyla were compared by using repeated measures analysis of variance and GraphPad Prism version 10.0.0 for Windows (GraphPad Software, La Jolla, CA). As published by Segata et al, the linear discriminant effect size (LEfSe) method was performed by combining the Kruskal-Wallis test or Wilcoxon rank-sum test with the linear discriminant analysis scores to estimate the effect size of differentially abundant features with biologic consistency and statistical significance (the \( \alpha \) value was set at 0.05, and the linear discriminant analysis score threshold for discriminative features was \( >2.0 \)) (The Huttenhower Lab, [http://huttenhower.sph.harvard.edu/galaxy](http://huttenhower.sph.harvard.edu/galaxy), last accessed December 5, 2022).

The data sets analyzed in this article are publicly available [https://doi.org/10.57745/ZSX5M4; last accessed November 27, 2023 (login required)].

Statistical Analysis

All data are presented as means \( \pm \) SEM. Statistical significance was checked by using two-way analysis of variance to compare the \( mdx \) and B10 groups at different time points; when not applicable, a \( t \)-test was used. For all statistical analyses, the significance level was set at 0.05. Data were analyzed by using GraphPad Prism version 10.0.0 for Windows.

Results

Reduced Gut Microbiota \( \alpha \)- and \( \beta \)-Diversities in \( mdx \) Genotype

Comparison of the observed, Chao1, and InvSimpson indices obtained at different ages showed that \( \alpha \)-diversity varied between genotypes. Specifically, the observed \( (P < 0.01) \) and Chao1 \( (P < 0.05) \) indices were significantly reduced, whereas the InvSimpson index was increased, in \( mdx \) mice compared with wild-type B10 littermates (Figure 1A). The Shannon index was comparable between genotypes (Figure 1A). The graphical representation of \( \beta \)-diversity using principal coordinates analysis plots for the UniFrac distances showed a strong and significant effect of the genotype \( (P < 0.001) \) (Figure 1B). Separation analysis at each time point confirmed that the separation of the genotype is significant at all time points \( (P < 0.001) \). The ordination plot showed that the first axis of the principal coordinates analysis corresponds to the genotype and accounts for almost 20% of the diversity \( (P < 0.0001) \) (Figure 1B).

Specific Taxonomic Modification of Main Phyla and Included Genera in \( mdx \) Genotype

Comparison of the abundance of the six main phyla during the first year of life revealed significant differences in gut microbiota composition in \( mdx \) and B10 mice. The abundance of Actinobacteria, Proteobacteria, and Tenericutes was significantly increased in \( mdx \) mice compared with that in B10 mice (Figure 2). Moreover, the Deferrribacteres phylum was only present in \( mdx \) mice (all ages tested) (Figure 2).

Differential abundances using LEfSe highlighted 30 up-regulated OTUs (including eight unknown genera and species) were analyzed in the gut microbiota of \( mdx \) mice compared with B10 mice. Conversely, nine OTUs (including eight unknown genera and species) were characteristic of B10 mice based on their linear discriminant analysis score compared with \( mdx \) mice (Figure 3A). Bacteroidaceae, Bacteroides, Alistipes, Rikenellaceae, Rhodospirillales, Rhodospirillaceae, Deferrribacterales, Deferrribacteraceae, and Deferrribacteres were overexpressed by at least 3.6-fold in \( mdx \) mice. Conversely, the Lachnospiraceae NK4A136 group and Bacteroidales S24_7 group were decreased by almost 4.8-fold in B10 mice.

The cladogram representation of the LEfSe results according to the taxonomic rank (Figure 3B) allowed easy identification of the taxonomic branches (from phyla to OTUs) that were modified in \( mdx \) mice, as well as the extent of the modification. The abundance of the phylum Deferrribacteres was increased in \( mdx \) mice, as was that of class Deferrribacteres, the order Deferrribacterales, the family Deferrribacteraceae, and the genus Mucispirillum. Similarly, two other main continuous
taxonomic changes were identified in *mdx* mice: the phylum Proteobacteria, and the class (Alphaproteobacteria), order (Rhodospirillales), family (*Rhodospirillaceae*), and genus (unknown species); and the phylum Actinobacteria, then the class (Coriobacteriia), order (Coriobacteriales), family (*Coriobacteriaceae*), and genus (*Enterorhabdus*) (Figure 3B). For other phyla, some taxonomic ranks were altered; for instance, the prevalence of *Bacteroides* and *Bacteroidaceae* belonging to the Bacteriodetes phylum was increased. Moreover, the Lachnospiraceae NK4A136 group from the Firmicutes phylum was reduced in B10 mice. However, no impact of dystrophin deficiency in the phylum Tenericutes was observed.

**Figure 1**  Dystrophin deficiency is associated with gut microbiota α- and β-diversity altered. **A**: α-Diversity indices in *mdx* (C57BL10SnSc-DM0/DM0/J) mice and B10 (wild-type C57BL10SnSc) mice from 8 weeks to 1 year of age. **B**: Principal coordinates analysis plots of β-diversity between genotypes using the UniFrac distances. *N* = 10 per group per age. Green: *mdx* mice; Black: B10 mice. *P* < 0.05 versus B10 mice.
Low-Grade Circulating Inflammation and Reduced Adipokines Levels in Mdx Genotype

Plasma biomarker analysis highlighted a low-grade inflammation in mdx mice characterized by higher levels of IL-6 (Figure 4A), tumor necrosis factor (Figure 4B), and MCP-1 (Figure 4C) compared with B10 mice. IL-1β involved in the inflammatory process was not modulated (Supplemental Table S1), and IL-4 was undetectable in both groups.

In addition, a strong down-regulation of circulating adipokines such as adiponectin (Figure 4D) and leptin (Figure 4E) was observed in mdx mice compared with their B10 littermates. Interestingly, as shown in Table 2, adiponectin and leptin explain 10% and 11.9%, respectively, of the gut microbiota signature of dystrophin-deficient mice.

The entero-endocrine hormone ghrelin differed between groups, with a normalization with age of circulating level in mdx mice compared with B10 mice (Supplemental Table S1). Nevertheless, glucagon-like peptide-1 and peptide YY measurements did not reveal differences between groups (Supplemental Table S1).

Figure 2 Four major gut microbiota phyla are increased in mdx (C57BL10SnSc-DMDmdx/J) mice. Relative abundance of Bacteriodetes phylum (A), Firmicutes phylum (B), Actinobacteria phylum (C), Proteobacteria phylum (D), Tenericutes phylum (E), and Deferribacteres phylum (F) at 8 weeks, 12 weeks, 6 months, and 1 year of age. n = 10 per group per age. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 versus B10 (wild-type C57BL10SnSc) mice.

Slowed Down Gut Peristalsis in Mdx Genotype, Not Likely Linked with the Dystrophin Protein Deficiency

In mdx mice, the limited amount of dystrophin protein synthesis is characterized by altered structure and function of skeletal muscle (Supplemental Figure S1A). Indeed, even with hypertrophy during the first 6 months of life (Supplemental Figure S1, B–D), hindlimb muscles revealed a force weakness from 8 weeks old worsening with aging
Because gut microbiota is involved in intestinal smooth muscle motility, intestine morphology and function were investigated. Dystrophin staining by immunofluorescence revealed an absence of dystrophin in the intestine independently of the phenotype (Figure 5A). In addition, small intestine goblet cell density was assessed, as mucus production by the goblet cells is important for intestinal integrity. No difference was found in labeling and, consequently, in small intestine goblet cell density between the two phenotypes of mice (B10 mice, 52.1 ± 0.2 cells/mm²; mdx mice, 54.1 ± 4.3 cells/mm²) (Figure 5, B and C). The intestinal smooth muscle properties were assessed with the ex vivo contractility test, and the mean period of spontaneous basal contraction of jejunum was calculated. The period of peristalsis was significantly longer in 1-year-old mdx mice than in B10 littermates, suggesting a slowing of peristalsis (P < 0.01) (Figure 5D).

Reduction of Ileum Gene Expressions Linked with Intestinal Functionality/Permeability in Mdx Genotype

Analysis of the expression in ileum samples of various genes linked to intestinal functionality/permeability showed that Tjp1 and Tjp2 were significantly down-regulated in mdx mice compared with that in B10 mice (P < 0.05) (Figure 6, A and B). Similarly, the gene encoding the SCFA receptor Ffar2 was down-regulated in mdx mice (P < 0.05) (Figure 6C). Conversely, Angptl4 was up-regulated in mdx mice (P < 0.005) (Figure 6D). Expression of genes encoding inflammatory markers, amino acid receptors, and lipid transporters were comparable between genotypes (data not shown).

Dysregulation of Muscle-Specific Receptors of Gut Bacterial Metabolites and Activation of the Muscle Tlr4/MyD88 Pathway in Mdx Genotype

In TA samples, gene expression of Bcat2 (the skeletal muscle-specific isoform of transaminase 2) was strongly up-regulated in mdx mice compared with that in B10 mice at both ages (Figure 7A). In contrast, in oxidative soleus muscle, Bcat2 expression was down-regulated at both ages in mdx mice (12 weeks, 0.42 ± 0.2-fold change versus B10 mice; 1 year, 0.56 ± 0.07-fold change versus B10 mice; P < 0.01).

The adiponectin receptor 1 expression was also up-regulated at both ages in mdx TA muscle (Figure 7B), whereas the nonmuscle-specific adiponectin receptor 2 was not significantly different (Figure 7C). Interestingly, in soleus muscle, Adipor1 (12 weeks, 0.73 ± 0.16-fold change versus B10 mice; 1 year, 0.79 ± 0.08-fold change versus B10 mice; P < 0.01) and Adipor2 (12 weeks, 0.64 ± 0.19-
fold change versus B10 mice; 1 year, 0.76 ± 0.06-fold change versus B10 mice; P < 0.001) were significantly down-regulated in mdx mice compared with that in B10 mice from 12 weeks old to 1 year old.

The mRNA level of Tlr4/Myd88 inflammation pathway showed high activation in mdx mice muscles compared with those in B10 mice (Figure 8). The expression of the gene encoding the Toll-like receptor 4, which recognizes pathogen-associated molecular patterns and specifically bacterial lipopolysaccharides, was up-regulated in TA at both ages (Figure 8A) and soleus at 1 year old (B10 mice, 0.68 ± 0.09; mdx mice, 1.08 ± 0.08; interaction, P < 0.001) in mdx mice compared with B10 mice. In mdx mice, Myd88 also was significantly up-regulated in TA (Figure 8B) and soleus (12 weeks, 1.60 ± 0.9-fold change versus B10 mice; 1 year, 1.05 ± 0.2-fold change versus B10 mice; P = 0.02) muscles. Angptl4, known to be up-regulated during inflammation, was highly up-regulated only in TA muscle (Figure 8C). The expression of other genes linked to gut microbiota metabolites (Table 1) was not different between genotypes (data not shown).

Discussion

This is the first study designed to explore gut microbiota in mdx mice to investigate the gut microbiota-skeletal muscle crosstalk. Monitoring mdx mice and B10 wild-type littermates at 8 weeks, 12 weeks, 6 months, and 1 year of age highlighted a unique intestinal bacterial composition in mdx mice associated with an impairment of specific plasma and muscle inflammatory biomarker levels.
Additionally, a slowing of gut peristalsis independently of dystrophin deficiency was confirmed in smooth muscle combined with the intestinal structure impairment in mdx mice. Taken together, these alterations might contribute to worsen the physiopathology of the skeletal muscle in mdx mice.

This research shows the impact of dystrophin deficiency on gut microbiota of the host. The lower Observed and Chao indices in mdx mice revealed reduced richness, and the increased InvSimpson index highlighted fewer dominant species and greater evenness. Thus, in mdx mice, the overall number of different OTUs and their abundance were significantly reduced. The nonsignificant difference of the Shannon index suggests that this overall lower diversity does not concern rare OTUs. Principal coordinates analysis plots for β-diversity clearly clustered the two genotypes.
independently of age. Indeed, \textit{mdx} genotype predicted 20% of \(\beta\)-diversity divergence, validating the theory that dystrophin deficiency affects bacterial composition from birth.

Besides the gut microbiota diversity, the metagenomic analysis revealed strong taxonomic modifications. The abundances of four main phyla (Actinobacteria, Proteobacteria, Tenericutes, and Deferribacteres) were increased in \textit{mdx} mice. For three of them, this concerned the phyla and also the included genera (LEfSe analysis): the \textit{Mucispirillum} genus and \textit{Deferribacteraceae} family in the Deferribacteres phylum; the \textit{Enterorhabdus} genus and \textit{Coriobacteriaceae} family in the Actinobacteria phylum; and the \textit{Rhodospirillaceae} family in the Proteobacteria phylum were the most concerned. Interestingly, the Deferribacteres phylum and related taxa were only detected for three of them, this concerned the phyla and also the included genera (LEfSe analysis): the \textit{Mucispirillum} genus and \textit{Deferribacteraceae} family in the Deferribacteres phylum; the \textit{Enterorhabdus} genus and \textit{Coriobacteriaceae} family in the Actinobacteria phylum; and the \textit{Rhodospirillaceae} family in the Proteobacteria phylum were the most concerned. Interestingly, the Deferribacteres phylum and related taxa were only detected

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6}
\caption{In \textit{mdx} (C57BL10SnSc-DMD\textsuperscript{mdx}/J) mice, alteration of gut microbiota composition is associated with gene expression dysregulation in ileum. A: \textit{Tjp1}. B: \textit{Tjp2}. C: \textit{Ffar2}. D: \textit{Angptl4}. \(n = 8\) per group per age. *\(P < 0.05\), **\(P < 0.01\), ****\(P < 0.0001\) versus B10 (wild-type C57BL10SnSc) mice.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure7}
\caption{The gene expressions of receptors linked to gut microbiota metabolites are modulated in tibialis anterior muscle of \textit{mdx} (C57BL10SnSc-DMD\textsuperscript{mdx}/J) mice. A: \textit{Bcat2}. B: \textit{Adipor1}. C: \textit{Adipor2}. \(n = 8\) per group per age. **\(P < 0.01\), ****\(P < 0.0001\) versus B10 (wild-type C57BL10SnSc) mice.}
\end{figure}
in stools from mdx mice and not in wild-type littermates, thus constituting a specific phylum and taxa related to dystrophin deficiency. This phylum includes six genera that are all Gram-negative bacteria.42

To the best of our knowledge, these are original results, and no similar data have been published previously on this DMD model. Nevertheless, some hypotheses on the potential links between these microbiota abundance modifications and the dystrophic phenotype could be proposed based on findings of other pathologic models. For instance, in a mouse model of colorectal cancer, an increase in *Mucispirillum schaedleri* drives lipopolysaccharide production that is associated with an inflammatory response.43 In a model of ulcerative colitis (inflammatory bowel disease family), the active period of the disease is characterized by a larger abundance of Rhodospirillales.44 Clostridial are in close relationship with intestinal cells, to possibly modulating gut cells’ immune processes.45 Some Enterorhabdus-related species are known to degrade mucus and could expose the intestinal barrier to assault.46 The combination of metagenomic and metatranscriptomic analyses might reveal the functional activity and implication in the dystrophic phenotype development of the specific commensal microbes identified in the current study.

The current study also found that in mdx mice, gut microbiota composition changes were associated with slowed intestinal motility, emphasized by the increased jejunal basal contraction wave period. This result is in accordance with a recent study by Singh et al47 showing fewer full peristaltic waves in mdx mice associated with reduced contraction-stimulated force and mRNA expression of contractile proteins. Furthermore, dystrophin protein was not revealed by intestine staining, either in the control B10 group or in the mdx mice. Thus, the difference of intestinal microbiota between mdx and B10 is unlikely due to the absence of the dystrophin protein. In addition, the down-regulation of genes encoding the tight junction proteins (*Tjp1* and *Tjp2*), crucial for the epithelial barrier integrity maintenance48,49 observed in mdx ileum samples, indicates a potential impact on the epithelial barrier permeability. Furthermore, results on the gut microbiota suggest that intestinal bacteria could play a role in the intestinal dysfunction observed in mdx mice. The unique gut microbiota signature in mdx mice with the over-representation of Gram-negative bacteria could promote inflammation in the lumen and disrupt the intestinal contractile properties, comforting the hypothesis of the low-grade circulating inflammation (discussed in the following paragraph). Moreover, *Ffar2* (SCFA receptor) down-regulation in mdx ileum highlights lower SCFA production that could alter the smooth muscle metabolism and contractile function.50–52 Interestingly, *Bcat2* profiles suggest a modulation of bioavailability of branched-chain amino acids. Because branched-chain amino acid production is partially regulated by the gut microbiota, the alteration of the gut microbiota in mdx mice could contribute to impairment in this production. Nevertheless, *Bcat2* up-regulation in TA and the opposite down-regulation in soleus indicate a more complicated and intricate response between muscle phenotypes, warranting further investigations.

The reduced *Tjp1* and *Tjp2* expression suggest an increased gut permeability that might lead to a leak of bacterial components in the circulatory system, thus contributing to low-grade inflammation, as confirmed by higher plasmatic levels of IL-6, tumor necrosis factor, and MCP-1 observed in mdx mice and/or bacterial infections in distant organs.53 Furthermore, the LEfSe analysis showed an increase in lipopolysaccharide-producing Gram-negative bacteria (*Deferribacteres phylum, Bacteroides genus*) in mdx mice gut. Concomitantly, the gene expression of *Tlr4* (receptor of lipopolysaccharide), *myd88*, and *Angptl4* important proteins of the inflammation pathway were up-regulated in skeletal muscles of mdx mice, especially in the tibialis anterior. Thus, alteration of gut microbiota associated with disruption of the intestinal barrier could worsen inflammation in dystrophin-deficient skeletal muscle. Finally, circulating adipokine dysregulation such as adiponectin, ghrelin, and leptin may indicate an adipose tissue disruption in mdx mice.

![Figure 8](image-url)
Interestingly, the expression of Adipor1, the suspected main driver of adiponectin cascade in skeletal muscle, was altered in TA and soleus muscles from mdx mice (TA, up-regulation; soleus, down-regulation). The various patterns highlighted in mixed TA and oxidative soleus muscles might be explained by a different metabolism associated with the typology. Indeed, adiponectin electrophoresis in skeletal muscle has been shown to modulate myosin heavy chain genes toward oxidative phenotype. Thus, adiponectin stimulation might be linked to muscle typology. Interestingly, plasma biomarker analysis also revealed a massive release of leptin in the blood flow at 1 year of age in B10 mice compared with mdx mice. Although this satiety hormone has not been studied intensively in the dystrophin-deficiency field, a lower level of leptin could be explained by the mdx fat metabolism impairment. These findings raise questions on a third contributor as adipose tissue in the interorgan crosstalk.

To summarize, this study observed for 1 year the gut microbiota signature in relation with the intestinal structure and function as well as blood biomarkers and skeletal muscle function in a context of dystrophin deficiency. The results showed a strong gut microbiota clustering between genotype, independently of age, with a modulation of four main phyla and genera related to inflammation with overall less diversity in mdx mice. Twenty percent of the β-diversity divergence was explained by the genotype, confirming the relationship between dystrophin deficiency and gut bacterial composition from birth. An over-representation of lipopolysaccharide-producing Gram-negative bacteria was shown in mdx mice, with reduced intestinal motility as well as gene expressions of ileum tight junction proteins, which suggested an increased intestinal porosity contributing to the low-grade inflammation. This is supported by the systemic inflammation, the up-regulation of bacterial pro-inflammatory receptor Tlr4/Myd88 in mdx muscles, and the adipose tissue secretion profile. Finally, the decrease of Fjam2 in the ileum might reveal a dysregulation of the gut microbiota SCFA production and bioavailability for skeletal muscles. This study highlights gut microbiota as a potential central metabolic organ in mdx physiopathology. Additional studies would be required to better understand gut microbiota involvement in dystrophy development/progression through the intestine–skeletal muscle crosstalk. This finding also encourages studies into developing novel approaches to address the gastrointestinal and muscle dysfunction in patients with DMD to improve the global therapeutic management of muscular dystrophies.

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Author Contributions


Disclosure Statement

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Supplemental Data

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