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Gut bacteria are critical for optimal muscle function: a potential link with glucose homeostasis

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Running Head: Gut microbiota modulation affects skeletal muscle endurance

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Abstract

Gut microbiota is involved in the development of several chronic diseases, including diabetes, obesity and cancer, through its interactions with the host organs. It has been suggested that the cross-talk between gut microbiota and skeletal muscle plays a role in different pathological conditions, such as intestinal chronic inflammation and cachexia. However, it remains unclear whether gut microbiota directly influences skeletal muscle function. In this work, we studied the impact of gut microbiota modulation on mice skeletal muscle function and investigated the underlying mechanisms. We determined the consequences of gut microbiota depletion after treatment with a mixture of broad spectrum antibiotics for 21 days and after 10-days natural reseeded. We found that in gut microbiota-depleted mice, running endurance was decreased, as well as the extensor digitorum longus muscle fatigue index in an *ex vivo* contractile test. Importantly, the muscle endurance capacity was efficiently normalized by natural reseeded. These endurance changes were not related to variation in muscle mass, fiber typology or mitochondrial function. However, several pertinent glucose metabolism markers such as ileum gene expression of short fatty acid chain and glucose transporters, *Gpr41* and *Sglt1* and muscle glycogen level, paralleled the muscle endurance changes observed after ATB treatment and reseeded. As glycogen is a key energetic substrate for prolonged exercise, modulating its muscle availability via gut microbiota represents one potent mechanism that can contribute to the gut microbiota/skeletal muscle axis. Taken together, our results strongly support the hypothesis that gut bacteria are required for host optimal skeletal muscle function.

Keys words: Dysbiosis – Contractile properties – Mitochondrial biogenesis – Maximal aerobic velocity – muscle fatigue

INTRODUCTION

About 10^{14} bacteria live in our gut in symbiosis. This community is now considered as a central organ due to its tremendous impact on human health (17, 37). Indeed, gut microbiota dysbiosis is involved, directly or indirectly, in numerous chronic diseases, such as obesity, type 1 and type 2 diabetes, allergy and autism (63). The impact of gut microbiota in chronic diseases can be explained by its interactions with the host organs, including brain (21), liver (9) and lung (62).

Recently, the hypothesis of a crosstalk between gut microbiota and skeletal muscle has emerged in the literature (5). For example, in mouse models of intestinal chronic inflammation, the restoration of commensal *E. coli* levels efficiently prevents skeletal muscle atrophy (52). Moreover, the recovery of a "healthy" microbiota by synbiotic treatment prolongs survival and reduces cancer proliferation and cachexia development in mouse models of cancer (4, 69). Gut microbiota may also influence the muscle metabolism and fiber phenotype, and consequently the exercise performance. For instance, after microbiota transplantation from obese or lean pigs, germ-free (GF) mice replicate the fiber characteristics and lipid metabolic profile of the donor's skeletal muscle (70). Interestingly, probiotic supplementation in mice also improves muscle mass, promotes the slow and oxidative muscle phenotype associated with increased muscle endurance, and reduces exercise-induced muscle damage (14, 35). More recently, *in vitro* experiments showed that microbiota-derived phenolic metabolites found in blood promote muscle glucose uptake in differentiated human skeletal muscle myoblast in a dose-dependent manner (33).

Considering microbiota as a regulatory element of skeletal muscle function is an innovative idea with significant scientific and socio-economic consequences. Indeed, loss of muscle function and mass is a risk factor of morbidity and mortality in neuromuscular diseases, and also in many chronic diseases (e.g., cancer cachexia, obesity, diabetes) (24, 40). The identification of the role played by gut microbiota in the skeletal muscle dysfunction related to such chronic diseases could clearly improve the management of these patients. However, the *in vivo* mechanisms underlying the functional link between gut microbiota and skeletal muscle in healthy conditions remain poorly explored. In this context, assessing the effect of microbiota modulation on skeletal muscle function would constitute a significant step forward.

Germ-free mice have been previously used to investigate the functional link between microbiota and different organs (42). However, these mice, have developed compensatory mechanisms to the life-long absence of microbiota (25). On the other hand, antibiotic treatment is a recognized procedure to induce short-term gut microbiota depletion for studying its impact on the host organs (56). Finally, another advantage of antibiotic treatment is that it allows the subsequent recovery of healthy gut microbiota through natural reseeded.

The aim of this study was to determine in healthy mice whether gut microbiota affects skeletal muscle. To this purpose, we modulated gut microbiota in healthy mice by antibiotic treatment and natural reseeded. We found that antibiotic-mediated gut microbiota depletion induced a decrease of skeletal muscle endurance. Interestingly, this deleterious effect was entirely normalized by natural reseeded. We also investigated putative mechanisms and discovered that glucose homeostasis is modified by gut microbiota as we observed that short fatty acid chain and glucose transporters (Gpr41, Sglt1) expression in ileum and glycogen content in muscle were decreased after gut microbiota depletion and normalized after natural reseeded. In summary, our study strongly suggests that the presence of bacteria in the gut influences the skeletal muscle metabolism and function of the host.

MATERIAL AND METHODS

Animal experiments were approved by the Animal Experimentation Ethics Committee (APAFIS#2551-2015110311365663) in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996) and according to the European directives (86/609 / EEC).

C57BL/6 mice. 14-week-old C57BL/6 male mice (Charles River, Saint-Germain-Nuelles) were randomly divided in three experimental groups: control group (CTL, n=10), group treated with antibiotics for 21 days (ATB, n=10), and group treated with antibiotics for 10 days followed by natural reseeded for another 11 days (NAT, n=9). Mice were maintained on a 12:12-h dark–light cycle in a temperature-controlled room in individual cages with filter lids, and ad libitum access to food and water. The diet (3395 Kcal/kg) was standardized and identical for all groups (ref A03, safe diets, Augy, France). It included 69.2% of cereals, 20.2% of vegetal protein, 6.0% of

animal protein and 4.6% of mineral and vitamin cocktail. The following is a general description of the macronutrient composition: protein carbohydrates 61.3%, protein 25.2% and lipids 13.5%. This diet is currently used in our animal care facility since the birth of mice. Except for running tests, mice were housed in individual standard cages (not wheel cages), and thus were not subjected to daily exercise.

The ATB group received an oral cocktail of broad-spectrum antibiotics (1mg/mL ampicillin, 5mg/mL streptomycin, 1mg/mL colistin, and 45µg/mL vancomycin in drinking water).

To investigate the role of microbiota, not antibiotics per se, has impacts on potential muscle function, we included natural seeding groups (NAT) by allowing antibiotics-treated groups (ATB) to restore microbiota through providing soiled litter from control groups (CTL) after cessation of antibiotics treatment. More precisely, the NAT group received the same antibiotic mixture during the first 10 days of the experiment. Then, antibiotic mixture was removed. During the following 10-days reseeding period, NAT group received twice soiled litter from CTL group cages. For all groups, bottles were filled with filtered animal care drinking water. All antibiotics bottles were changed every 2 days. Feces were individually collected at D0, D7 and D21. After 21 days and overnight fasting, mice were weighed and euthanized.

Euthanasia and sampling. Mice were euthanized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). The soleus, quadriceps, extensor digitorum longus (EDL), gastrocnemius muscles, white adipose tissue, brown adipose tissue and caecum were isolated and weighed. Muscles, intestinal sections (duodenum, jejunum, ileum, and colon) and caecum content were collected and immediately frozen in liquid nitrogen.

***In vivo* running performance.** Running performance was assessed in the three groups (CTL, ATB and NAT) at the beginning (D0), after one week of antibiotic treatment (D7-D8) and before the end of the experiment (D17-D18). Maximal aerobic velocity (MAV) was determined with a running test in which the speed was gradually increased by 2 m/min from 10 m/min until exhaustion. Exhaustion was defined as the inability to maintain the normal running position and/or after five consecutive seconds in contact with the shock grid (<0.2mA) at the rear of the treadmill. Endurance capacity was estimated by calculating the endurance limit time (T_{lim}) during a submaximal running test, in which the speed started at 12 m/min for the first 2 min and

was individually set to 70% of MAV until exhaustion. All tests were repeated at the same time of the day.

***Ex-vivo* muscle contractile function** Muscle contractile properties were assessed in *ex vivo* conditions as previously described (54, 60). This technique allows evaluating the intrinsic muscle contractile properties. After 15min equilibration in the bath, EDL samples were connected to a force transducer/length servomotor system (model 305B, Cambridge Instruments, Aurora Scientific Inc, Ontario, Canada) and were stimulated along their entire length with platinum wire electrodes. The optimum muscle length (L₀; i.e., the muscle length producing maximal twitch tension) was determined. All subsequent measurements were made at L₀. The isometric tetanic tension was determined (701B Stimulator, Aurora Scientific Inc, Ontario, Canada) using stimulation trains of 500ms, with pulse duration of 0.5ms at different frequencies, from 1 to 150Hz. Stimulus trains were separated by a 1min interval. The maximum isometric tetanic tension (P₀) was determined from the plateau of the frequency-tension curve. Three minutes after the tension-frequency determination, the resistance to fatigue was evaluated using a low-frequency fatigue protocol of 50Hz trains of 700ms delivered every 2s for 5min (43). 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.

Composition of fecal microbiota.

DNA extraction from feces. Total cellular DNA was extracted from 0.1 g of animal fecal material using the G'NOME® kit (BIO 101, La Jolla, CA) with modifications (26). Fecal samples were homogenized in the supplied cell suspension solution. Cell lysis/denaturing solution was then added and samples incubated at 55 °C for 2h. To improve cell lysis, 0.1mm-diameter silica beads (750 µL) were added, and samples mixed at maximum speed in a Beadbeater (Biospec, Bartlesville, OK) for 10min. Polyvinylpyrrolidone (15mg) was added to ensure removal of polyphenol contamination that could inhibit the qPCR assays. Samples were vortexed and centrifuged at 20 000 g for 3min and supernatants were recovered. The remaining pellets were washed with 400 µL of TENP [50mM Tris (pH 8), 20mM EDTA (pH 8), 100mM NaCl, 1% polyvinylpyrrolidone] and centrifuged at 20 000 g for 3min. The washing step was repeated

once more and the resulting supernatants pooled. Nucleic acids were precipitated by addition of one volume of isopropanol, incubation at -20°C for 20min, and centrifugation at 20 000 g for 10min. Pellets were resuspended in 400 μL of distilled water plus 100 μL of salt-out mixture and incubated at 4°C for 10min. Samples were spun at maximum speed for 10min, 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 5min followed by centrifugation at 16 000 g for 5min. DNA was resuspended in 150 μL of TE buffer and stored at -20°C .

Evaluation of total bacteria by real-time qPCR analysis of bacterial 16s rRNA genes. The total bacteria present in the fecal samples of each mouse were evaluated by real-time quantitative PCR (qPCR) targeting “all bacteria” 16S rRNA genes using the universal primers F-bact1369 CGGTGAATACGTTCCCGG and R-prok1492 TACGGCTACCTTGTTACGACTT (26). Analyses were performed using the StepOne Plus detection system (Applied Biosystems, Courtaboeuf, France) with 10 μL of Mastermix (PowerUpSybrGreen Master Mix, ThermoFisher Scientific, Courtaboeuf, France), 500nM of both forward and reverse primers, 5 μL of diluted cDNA template and water to a final volume of 15 μL . All qPCR assays were performed in duplicate using the following cycling conditions: 50°C for 2min, then 95°C for 2min followed by 40 cycles of 95°C for 3s and 60°C for 30s, with a final melting step to improve the amplification specificity. For the quantification, the *E. coli* DNA standard curve was generated by plotting the threshold cycles (Ct) vs. bacterial quantity. When qPCR assays were performed using fecal DNA samples, this standard curve was used to quantify the bacterial population. The lower limit of detection for bacterial enumeration with good precision is 10^6 bacteria per gram of stool.

Evaluation of microbiota composition. The V3-V4 region of the 16SrRNA genes was amplified using the bacterial primers 343F (5'-CTT TCC CTA CAC GAC GCT CTT CCG ATC TAC GGR AGG CAG CAG-3') and 784R (5'-GGA GTT CAG ACG TGT GCT CTT CCG ATC TTA CCA GGG TAT CTA ATC CT-3') modified to add adaptors during the second PCR amplification. PCR assays were performed 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 (10mM), 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:

94°C for 3min, followed by 40 cycles at 94°C for 15s, 60°C for 30s, 72°C for 60s, and a final extension at 72°C for 5min. Sequencing was performed using the MiSeq technology (Illumina) at the Genopole Toulouse Midi-Pyrenees (GeT) genomics facility (<http://get.genotoul.fr/>).

Metagenomics analysis. Sequencing data were demultiplexed at the GeT platform. The Galaxy-supported pipeline, called FROGS (Find, Rapidly, Otus with Galaxy Solution), is designed to analyze large sets of amplicon sequences and produce abundance tables of Operational Taxonomic Units (OTUs) and their taxonomic affiliation (23). Briefly, this pipeline includes a preprocessing step where reads are merged with FLASH (45), dereplicated, and filtered according to their length, mismatches in primers with cutadapt (47) and N content. This step is followed by Swarm clustering (46) with an agglomeration distance of $d=3$. Chimera detection is then performed using Vsearch (59) before applying an OTU abundance filter (OTUs < 0.005% of the total abundance are discarded (8)). The most abundant sequences of each OTUs were then affiliated with blastn against the Silva v132 database (55). Abundance tables and taxonomy files were manually imported into RStudio (v1.1.419) package Phyloseq 1.23.1 (48). Estimates of bacterial OTU diversity was estimated using the Shannon and Simpson indices, in addition to observed richness and Chao indices. β -diversity analyses were performed on weighted and unweighted Jaccard and Bray-Curtis distances matrix of DNA samples and were then visualized using PCoA (Principal Coordinate Analysis) and the ggplot2 R package.

Bacteroidetes and Firmicutes prevalence in ATB group. We explored the phyla levels in ATB group despite a very low bacteria DNA concentration in these samples. We quantified two main phyla by real-time qPCR analysis using PCR primers specific for the ribosomal gene of two main bacterial phyla (Bacteroidetes and Firmicutes), gathered from the literature: Firm934F, GGAGYATGTGGTTTAATTCGAAGCA, Firm1060R, AGCTGACGACAACCATGCAC for firmicutes (29) and MIBF, GGCGACCGGCGCACGGG, MIBR, GRCCTTCCTCTCAGAACCC for bacteroidetes (49).

Muscle fiber phenotype. The muscle fiber phenotype was determined by assessing the presence of the different myosin heavy chain (MHC) isoforms (slow MHC I and fast MHC IIa, IId/x, I Ib isoforms), as previously described (20, 66). To obtain cytosolic protein extracts, gastrocnemius samples were lysed in cold lysis buffer (10mM Tris-HCl, pH 7.4, 0.5M sucrose, 50mM NaCl, 5mM EDTA, 30mM Na₄P₂O₇, 1% NP-40, 0.25% sodium deoxycholate, 50mM NaF, 100 μ M

sodium orthovanadate and proteases inhibitors cocktail (Sigma P8340, 5 μ L/ml)). Protein concentration was determined using the Lowry assay. Protein samples (6 μ g) were separated on high-glycerol-containing (30%) gels with an acrylamide-to-bis-acrylamide ratio of 50:1 for the separating gel (8% total acrylamide, pH 8.8) and for the stacking gel (4% total acrylamide, pH 6.8) using a Mini-Protean II dual slab cell apparatus (Bio-Rad, Hercules, CA). After electrophoresis (at 140V in a refrigerated room at 4°C for 22h), gels were stained with Coomassie blue (IRDye® Blue Protein Stain, LI-COR Biosciences, Lincoln, NE, USA) and the different MHC isoforms were identified according to their electrophoretic mobility pattern (20, 66).

Cross-sectional muscle fiber by immunohistochemistry. Serial transverse sections (10 μ m-thick) from liquid nitrogen-cooled isopentane frozen quadriceps muscle samples were obtained using a cryostat at -25°C, and mounted onto glass microscope slides. Sections were then washed in phosphate buffered saline (PBS), blocked and permeabilized with 0.1% Triton-X100 and 10% horse serum. For fiber cross-sectional area (CSA) determination, sections were stained with an anti-laminin antibody (1/200 in PBS, L9393 - Sigma) at 37°C for 1h, followed by washes in PBS and incubation with the secondary antibody Alexa Fluor 488 goat anti rabbit IgG (1/1000 in PBS, A11034 – Invitrogen) for 1h. Whole histological sections were imaged with a digital slide scanner Hamamatsu NanoZoomer 2.0-HT and analyzed with the ImageJ software package. On average, 1800 fibers per animal were quantified according to Demangel *et al.* method (19).

Transcript and protein expression profiles in ileum and skeletal muscle. To explore potential mechanisms linking gut microbiota composition and skeletal muscle, we measured markers involved in protein turnover, mitochondrial metabolism (36), inflammation, and related to lipid and glucose metabolism in both ileum and/or muscle, as described below (Table 1 and 2).

Immunoblotting. Frozen gastrocnemius muscle samples were homogenized using an Ultra-Turrax homogenizer in an ice-cold extraction buffer (20mM Tris, pH 7.4, 150mM NaCl, 1mM EDTA, 0.1% SDS, and 1% Triton X-100) with 0.5% Protease inhibitors, 1% NP40, 0.25% sodium deoxycholate, 0.2% NaF and 100 μ M sodium orthovanadate added just before extraction. Samples containing 50 μ g of proteins were resolved on 10–15% SDS-PAGE and transferred onto nitrocellulose membrane. Total proteins were visualized by Ponceau red staining to verify equal

loading. Blots were blocked with 5% bovine serum albumin (BSA) in TBS/0.2% Tween 20 (TBST) for 1h, followed by overnight incubation with antibodies against proteins involved in muscle metabolism and proteolysis/synthesis balance (Table 1). After three 5min washes at room temperature with TBST, membranes were incubated with a secondary antibody conjugated with horseradish peroxidase at room temperature for 1h. Blots were washed (three times for 5min/each) with TBST at room temperature, and antibody-bound proteins were revealed using the ECL reagent (Bio-Rad Life Science, Hercules, CA). Films were scanned and analyzed using the Image Lab software. All blots were corrected for loading based on the Ponceau red staining.

RNA isolation and RT-qPCR. Total RNA was isolated from gastrocnemius and ileum samples using TRIzol Reagent (Invitrogen 15596-018 200 ml, United States of America). RNA concentration was determined by spectrophotometry (Eppendorf AG, Hamburg, Germany) and purity was checked by calculating the OD 260nm/OD 280nm ratio (>1.7). RNA quality was verified by 1% agarose gel electrophoresis. Reverse transcription was performed with 1 µg of total RNA and the TAKARA kit (TAKRR037A, PrimeScript RT reagent Kit, Perfect Real Time) according to the manufacturer's instructions. Quantitative PCR (qPCR) analysis was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) with 10µL of Powerup SYBR Green Master Mix (A25742, ThermoFisher Scientific, Courtaboeuf, France), 10nM of both forward and reverse primers (Table 2), and 5µL of diluted cDNA (final volume of 15µL). All PCR assays were performed in duplicate using the following cycling conditions: 50°C for 2min, then 95°C for 2min followed by 40 cycles of 95°C for 3s and 60°C for 30s. The relative mRNA levels were normalized to α -tubulin (α -tub) and acidic ribosomal phosphoprotein P0 (*Rplp0*), as housekeeping genes unaffected by the experimental conditions. Results were expressed using the comparative cycle threshold (Ct) method to generate $\Delta\Delta C_t$ values with template dilutions ranging from 10^1 to 10^6 copies. The PCR overall efficiency (E) was calculated from the slopes of the standard curves according to the equation $E = [10^{(-1/\text{slope})}] - 1$ and this value was higher than 95% for all assays. The relative abundance of each sample was then normalized according to the equation: Relative Quantity (RQ) = $2^{-\Delta\Delta C_t}$. All the experiments were performed according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (11, 61).

Mitochondrial DNA (mtDNA) isolation and measurement by RT-qPCR.

Total DNA was extracted from the gastrocnemius muscle using a QIAamp DNA kit (Qiagen). Genomic (ATP synthase beta) and mitochondrial DNA (means of NADH dehydrogenase subunit 5 and NADH dehydrogenase subunit 2) were quantified by quantitative real-time polymerase chain reaction (10). Each sample was run in duplicate. Results were expressed using the comparative cycle threshold (Ct) with genomic DNA as the control. The relative changes in the expression level of mitochondrial DNA were calculated by the $\Delta\Delta C_t$ formula.

Muscle glycogen content. The acid-hydrolysis method used for glycogen quantification was adapted from the protocols described by Adamo and Jansson (1, 38). 20-30 mg of frozen gastrocnemius muscle powder was placed in ice-cooled 6% perchloric acid and 1M hydrochloric acid. Samples were then boiled at 100°C for 2h, cooled on ice for 10min and centrifuged (1300 RPM, for 5min). 12 μ L of supernatant was removed from each sample, diluted in 200 μ L of GOD-PAP solution (Biolabo, Maizy, France) and incubated in the dark at 37°C for 20min to determine the glucose concentration. Absorbance was measured at 500nm using a microplate reader. Glycosyl units produced by glycogen hydrolysis were expressed as μ mol/mg of wet muscle weight.

Muscle triglycerides content. Muscle triglycerides were determined by using DiaSys kit (Diagnostic System, Grabels, France) following a preliminary organic phase extraction according to Bligh & Dyer's method. Briefly, 50 μ g of gastrocnemius samples were crushed with 500 μ L of 150mM sodium chloride. Then 150 μ L of muscle homogenates were extracted with 600 μ L of a methanol-chloroform mixture (1:1, v/v). The organic layers were collected after centrifugation (10,000g for 10 min) and dried under nitrogen. Dry samples were reconstituted in 37.5 μ L of isopropanol/acetonitrile/water mixture (2:1:1, v:v:v) and 10 μ L were analyzed according to the manufacturer recommendations (7).

Statistical analysis. All data are presented as the mean \pm SEM. The normality of each distribution and homogeneity of variance were assessed with the Kolmogorov-Smirnov and Fischer test, respectively. Significance between CTL, ATB and NAT data were checked using a one-way analysis of variance (ANOVA). Significant interactions effects were analyzed with the Fischer LSD post-hoc test. For some parameters, the unpaired *t*-test was used to compare directly

CTL vs. NAT or CTL vs ATB. The Mann–Whitney rank sum test was chosen when the normality and/or equal variance tests failed. For running tests, significance between parameters at D0, D7 and D17 were checked using the paired *t*-test, after checking normality. For all statistical analyses, the significance level was set at 0.05. Data were analyzed using the statistical package GraphPad Prism version 6.02 for Windows (GraphPad Software, La Jolla, California).

RESULTS

Treatment tolerance. During the entire study period, neither diarrhea nor visible adverse side effect was recorded. Consequently, no mouse was excluded from the data analysis. Food intake and hydration were checked daily and no significant change was observed during the entire study period (Table 3).

Antibiotic treatment and natural reseeded efficiently modulate gut microbiota. To confirm gut microbiota depletion induced by the antibiotic treatment, we quantified by Q-PCR all-bacteria in feces, as well caecum weight (caecum hypertrophy is a marker of gut microbiota depletion (56)). At D0, microbiota DNA content did not differ in the three mouse groups (CTL, ATB and NAT) (Fig. 1A). After 7 days of antibiotic treatment (D7), bacterial DNA content was strongly reduced in the ATB and NAT groups (Fig. 1B). This effect was still present in the ATB group after 21 days of treatment (D21) (Fig. 1C). Despite a very low bacteria DNA concentration in gut samples in the ATB group at D21, we managed to estimate the phyla prevalence by qPCR analysis and found the following: 83% of firmicutes and 17% of bacteroidetes, the other phyla were not detectable (data not shown).

Conversely, natural reseeded restored the bacterial DNA level in the NAT group (Fig. 1C). At D21, alpha diversity (Fig. 1D) and beta diversity analyses (Fig. 1E-F) showed no statistical differences in microbiota between CTL and NAT groups. Neither phyla abundance (Fig. 1G-H), nor levels of main family from Bacteroides and Firmicutes showed statistical differences between CTL and NAT groups (Fig 1I-J). Moreover, caecum weight at D21 was significantly higher in ATB mice than in the CTL group (+456%, $p < 0.001$, SI supplemental Table 1). Caecum weight in the NAT group was significantly lower than in the ATB group (-76%, $p = 0.003$), and did not significantly differ from that of the CTL group (Table 3). All together, these results

support that NAT group can be used to assess the effects of gut microbiota *per se* on skeletal muscle function together with ATB group.

Effects of gut microbiota depletion on the structure of skeletal muscle mass. At the end of the study, the muscle wet weight (Fig. 2A) and mass index calculated as wet weight normalized to the whole-body weight (Fig. 2B) were significantly lower for gastrocnemius and quadriceps, but not for EDL and soleus, in the ATB group compared with the CTL and NAT groups (on average, between 7-10% of reduction). Normalization to the whole-body weight could be misleading because of the caecum hypertrophy in the ATB group (Table 3). Indeed, when the muscle mass index was normalized to body weight without the caecum weight, the weight of gastrocnemius, quadriceps, EDL and soleus were comparable between CTL and ATB mice (Fig. 2C). Moreover, histological analysis on quadriceps muscle samples showed that the fiber cross-sectional area (CSA) was comparable between CTL and ATB groups (Fig. 2D-E). Similarly, gene/protein activation of several key factors involved in protein synthesis, proteolysis, inflammation and autophagy (*Igf1*, *Murfl*, *Trif*, *Foxo-3a* and *NfκB*, PRAS40, 4EBP1, RPS6 and LC3B) was comparable in the gastrocnemius muscle of CTL and ATB mice (Fig. 2F-G-H).

Gut microbiota depletion induces muscle fatigability that is reversed by natural reseeded.

To determine the impact of gut microbiota depletion/natural reseeded on skeletal muscle function, mice underwent running tests at different time points. Moreover, at the end of the study, we tested *ex vivo* the contractile properties of EDL muscle samples to identify the specific role of skeletal muscle on the running performance (Fig. 3). MAV, which is dependent on the maximal oxygen consumption, was comparable in the three groups (CTL, ATB and NAT) and at all time points (D0, D7 and D17) (Fig. 3A-B-C). Conversely, performance indicators related to endurance were significantly affected by gut microbiota changes (Fig. 3D-E-F). Specifically, the endurance limit time (T_{lim}) during the running endurance test was significantly lower at D9 compared with the D0 values in ATB and NAT mice (-27%, in ATB group $p=0.015$ and -12% in NAT group, $p=0.012$ respectively, Fig. 3E). At D19, T_{lim} was still lower than at D0 in the ATB group (-19.3%, $p=0.037$), whereas it was back to the D0 values in the NAT group (Fig. 3F). *Ex vivo* contractile tests performed at the end of the protocol showed that EDL maximal strength (sP0) was not affected by the experimental conditions (Fig. 3G), whereas EDL muscle fatigue

index was significantly reduced in the ATB group compared with both CTL and NAT groups (-26%, $p=0.036$ and -37%, $p=0.001$, respectively (Fig. 3H). EDL muscle fatigue index in the CTL and NAT groups did not differ significantly.

Potential mechanisms linking gut microbiota and skeletal muscle function: focus on glucose homeostasis. Several cellular adaptations, including muscle fiber phenotype, mitochondrial biogenesis and/or substrate availability, affect skeletal muscle endurance (22). Here, we observed that the expression of the different MHC isoforms in gastrocnemius muscle was comparable in CTL and ATB (Fig. 4A-B). Similarly, the protein level of key markers of mitochondrial metabolism (AMPK, PGC1 α , COX IV; Fig. 4C-D) or the mRNA expression of *Glut4*, *Cd36*, *Cpt1*, as well *Nrf-1* and the mitochondrial DNA content (Fig. 4E-F) did not change after ATB treatment. These results suggest that disruption of gut microbiota composition does not impact the myofiber phenotype or the mitochondrial metabolism.

Gut microbiota depletion leads to major changes in intestinal epithelial cells (71) and the gut microbiome has a profound influence on inflammatory states (65). Here, we reported in ileum that ATB treatment and subsequent natural reseeded did not affect gene expression of myeloid differentiation primary response 88 (*Myd88*) and Toll Like Receptor 4 (*Tlr4*), two canonical proteins involved in inflammatory signaling pathways (Fig. 5A).

As shown by others, the lack of some bacteria-derived metabolites could interfere with the metabolism of energetic substrate, such as lipid and glucose metabolism (12, 33) and this could play important roles in the gut-skeletal muscle axis. In this context, fasting-induced adipocyte factor, *Fiaf* also known as angiopoietin like protein 4, is a key protein reducing fatty acids synthesis, adipogenesis and lipogenesis (13). *Fiaf* expression is known to be negatively regulated by intestinal bacteria (2). In our study, *Fiaf* gene expression in ileum was indeed significantly higher in ATB group compared to CTL and NAT groups (+164%, $p=0.001$ and +135%, $p=0.002$, respectively, Fig. 5A). However, this *Fiaf* increase was not associated with changes in muscle triglycerides levels in all experimental conditions (Fig. 5C). Finally, gut microbiota modulation did not affect *Lat1* expression, suggesting no major effects on branched amino acids transport (Fig. 5A).

Antibiotic treatment and reseeded did not exert any effect on free fatty acid receptor 1 and 4 (*Gpr40* and *Gpr120* respectively), both receptors being activated by medium to long chain fatty acids. However, Short Chain Fatty Acids (SCFAs) are the most abundant microbial metabolites derived from intestine, and SCFAs activated enteroendocrine cells via the free fatty acid receptor 3 (*Gpr41*). Interestingly, we reported in ileum samples that *Gpr41* gene expression was significantly decreased in the ATB group compared to CTL group (-48%, $p=0,007$; Fig.5B). After natural reseeded, NAT group exhibited higher *Gpr41* mRNA levels compared to ATB group (+111%, $p<0.001$; Fig.5B). As the activity of these enteroendocrine receptors are linked to glucose metabolism (30, 44, 68), we measured additional markers both in ileum and skeletal muscle. It's noteworthy that the gene expression of sodium/glucose cotransporter 1 (*Sglt1*) followed the same pattern in ileum than *Gpr41*, i.e significant lower levels in ATB condition (-34%; $p=0.001$ vs CTL group; Fig. 5B) such effect being reversed by natural reseeded (+44%, $p=0.033$ vs ATB group; Fig.5B). However, these gene expression changes were not associated with modification in blood glucose levels (Table. 3). Finally, as glucose stored in glycogen is the one main substrate involved in muscle energy supply during endurance exercise (22), we monitored muscle glycogen content. In agreement with results obtained in ileum on *Gpr41* and *Sglt1*, muscle glycogen content was significantly lower in ATB than in CTL mice (Fig. 5D) and this was normalized after natural reseeded (Fig. 5D).

DISCUSSION

The present study is the first one designed to explore the consequences of gut microbiota depletion and reseeded on skeletal muscle structure and metabolism. Using experimental model of gut microbiota modulation, we demonstrated negative impacts on skeletal muscle endurance, reversed by bacterial reseeded. Our *ex vivo* functional analyses clearly support a role of gut microbiota on muscle intrinsic contractile properties. Gut microbiota appears to play a role in glucose homeostasis, as we observed that short fatty acid chain and glucose transporters (*Gpr41*, *Sglt1*) in ileum and muscle glycogen content paralleled the muscle endurance changes observed after ATB treatment and reseeded. Altogether, our results highlight that gut microbiota depletion impact on muscle endurance can be mediated through the modulation of muscle glycogen availability.

Gut microbiota and muscle mass

Researches from the group of Delzenne suggested a link between gut microbiota profile and skeletal muscle mass in pathological conditions (4, 6). Recently, Varian *et al.* (69) showed that probiotic supplementation partially prevents skeletal muscle atrophy related to cancer cachexia and sarcopenia. Here, in healthy mice, we found lower wet weight and mass index of some muscles in ATB mice. However, the caecum hypertrophy, a widely known phenomenon associated with depleted bacteria condition (41), could mask a body weight reduction in these mice, as indicated by the absence of difference on muscle mass when normalization was done after subtraction of the caecum weight. This suggests that gut microbiota modulation does not directly cause skeletal muscle atrophy in our experimental conditions. This is emphasized by the absence of changes in muscle strength or changes in protein expression of factors involved in muscle protein turnover, in quadriceps fiber CSA, and in the *ex vivo* maximal strength. In view of these data, we suggest that, gut microbiota only slightly influences skeletal muscle mass in healthy conditions.

Gut microbiota and muscle endurance

It has been recently proposed that gut microbiota could affect physical performance (15). For instance, the diversity of gut microorganisms is higher in professional athletes (16). In addition, endurance performance is enhanced in conventionalized and specific pathogen-free (SPF) mice than in GF mice (34). Several skeletal muscle-independent factors can alter the endurance performance, including cardiovascular deficiency, insufficient muscle capillarity, central fatigue or pain (27, 31, 50, 53). To determine the specific impact of gut microbiota modulation on skeletal muscle, we performed in addition to *in vivo* running tests, *ex vivo* muscle contractility tests. We observed in both experimental conditions a negative effect of gut microbiota depletion on muscle endurance, fully reversed by natural reseeded. Altogether, the *ex-vivo* results confirm that gut microbiota specifically and directly affects muscle endurance.

Skeletal muscle endurance can be affected by several muscle alterations, including changes in muscle contractile phenotype, mitochondrial biogenesis and/or reduction in substrate availability (22). Interestingly, after transplantation of gut microbiota from obese or lean pigs to germ-free mice, these mice replicated the skeletal muscle fiber characteristics and lipid metabolic profiles of the donor (70). Here, the absence of significant modifications in the

proportion of the different MHC isoforms in the ATB group excludes major effects of microbiota changes on the muscle contractile phenotype. Bäckhed *et al.* (3) observed that in GF mice, AMPK activity is increased in liver and skeletal muscle, and suggested that this adaptation is a key mechanism by which GF mice are better protected from obesity. In our study however, we did not find any modification in skeletal muscles in the activity of AMPK, the expression of PGC-1 α and mitochondrial DNA content in skeletal muscle of ATB mice. This suggests that mitochondrial biogenesis remained unchanged in ATB mice and therefore that endurance alteration is probably not due to reduction of the maximal muscle oxygen consumption capacity. Such muscle metabolic discrepancies between ATB mice and GF mice could be due to differences in the duration of skeletal muscle exposure to a systemic environment with depleted gut microbiota.

Gut microbiota modulation targets muscle glycogen availability

Muscle endurance also depends on metabolic parameters, including substrate availability in skeletal muscle, such as glycogen and triglycerides, two key energetic substrates that regulate muscle capacity during prolonged strenuous exercise (28, 32). Interestingly, GF mice exhibit higher gut Fiaf expression compared with conventionalized mice, leading among others to a reduction of triglyceride storage in peripheral tissues including liver (2). Fiaf is a glycoprotein recognized to also promote fatty acid oxidation through AMPK activation in skeletal muscle, and to attenuate muscle lipid uptake by inhibiting LPL activity (13, 57). Here, we confirmed the data of Bäckhed and colleagues by showing that gut Fiaf gene expression is modulated by the lack/presence of gut bacteria. However, these results were not associated in skeletal muscle with changes in i) FFA transporters gene expression (*Cd36*, *Cpt1*), ii) AMPK activation and iii) muscle triglyceride content. Altogether, these findings suggest that muscle lipid metabolism is unlikely to play a major role in alterations of muscle endurance observed after gut microbiota modulation.

Our data on glucose homeostasis both in ileum and skeletal muscle open some interesting hypotheses. Indeed, in ileum, *Gpr41* and *Sglt1* gene expression patterns followed the skeletal muscle endurance capacity (i.e decrease in bacteria-depleted conditions reversible upon natural reseeded). Dependent to metabolites-derived from gut bacteria, Gpr41 contributes to the enteroendocrine cells activation and, by its action on glucagon-like peptide 1 release, regulates

glucose-mediated insulin secretion (30, 68). In the same line, we observed the modulation of expression of *Sglt1* gene, encoding a Na⁺/glucose cotransporter located in enterocytes, which could reflect glucose homeostasis dysregulation in the intestinal tract. Overall, these results are in agreement with previous studies using antibiotic-induced microbiome depletion and showing a modulation of gene expression programs required for glucose homeostasis both in intestine and liver (18, 58, 72). Zarrinpar and colleagues also highlighted that antibiotics, by depleting gut bacteria in colon, reduce SCFAs production and deprive colonocytes from butyrate their main fuel source; and this results in lowering serum glucose and improving insulin sensitivity (72). This potential mechanism may contribute to the reduction of muscle glycogen store reported in the present study, since glycogen storage depends of both carbohydrates availability and uptake (39). As reduction of glycogen content affects muscle fatigue in both *in vivo* and *ex vivo* conditions (51), the muscle glycogen depletion observed in ATB mice could be one of the key mechanisms to explain the muscle endurance reduction in these animals. Furthermore, the restoration of glycogen content to values close to CTL mice after natural reseeded reinforced this hypothesis. Interestingly, we demonstrated the same muscle glycogen levels reduction in Germ-Free mice another recognized model of gut microbiota depletion, in comparison with GF-reseeded mice (data not shown). All these findings finally suggest that the reduction of energetic substrate availability, especially glucose, due to intestinal microbiota disruption could induce a reduction of muscle glycogen storage to maintain glucose homeostasis, translating in skeletal muscle endurance impairment.

To conclude, our results indicate for the first time that gut bacteria are critical for optimal muscle function in mice at least in part by altering glucose homeostasis regulation. How could we translate these finding in human? The presence of a gut-muscle axis in the pathogenesis of age-related sarcopenia in humans has been proposed recently based on very promising results (67). The interplay between microbiome and skeletal muscle has been highlighted by Siddhart and colleagues (64) who demonstrated that age-related changes of gut microbiome are associated with the physiological decline of musculoskeletal function. Future studies will be needed to identify the bacterial species and/or the underlying mechanisms and the putative metabolites that contribute to the functional link between gut microbiota and skeletal muscle. Our work opens promising perspectives to develop new therapeutic strategies based on gut microbiota modulation for muscle-related diseases, muscle disuse and sport training.

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TABLES

Marker	Protein	Molecular weight (kDa)	Reference	Source	Dilution
Protein Turnover	4EBP1	17	Cell Signaling, 9644	Rabbit	1:1000
	p-4EBP1 ^{Thr37/46}	17	Cell Signaling, 2855	Rabbit	1:1000
	RPS6	32	Cell Signaling, 3944	Rabbit	1:1000
	p-RPS6 ^{Ser235/236}	32	Cell Signaling, 4856	Rabbit	1:1000
	PRAS40	40	Cell Signaling, 2691S	Rabbit	1:1000
	p-PRAS40 ^{T246}	40	Cell Signaling, 13175S	Rabbit	1:1000
	LC3b	14	Cell Signaling, 3868	Rabbit	1:1000
Energetic sensor	AMPK α	62	Cell Signaling, 5832	Rabbit	1:1000
	p-AMPK α ^{THR172}	62	Cell Signaling, 2535	Rabbit	1:1000
Mitochondrial metabolism	PGC1- α	105	Calbiochem, 516557	Rabbit	1:1000
	COX IV	17	Santa Cruz, sc-69360	Mouse	1:200

Table 1. List of primary antibodies used for Western Blotting

Markers	Gene	Reference	Forward	Reverse
Gene expression in skeletal Muscle				
Protein Turnover	<i>Igf-1</i>	XM_006513261.2	AGCAGCCTTCCAACCTCAATTAT	GAAGACGACATGATGTGTATCTTTATC
	<i>FoxO3a</i>	XM_006512806.1	GGAAATGGGCAAAGCAGA	AAACGGATCACTGTCCACTTG
	<i>Murfl (Trim63)</i>	NM_001039048.2	TCCTGCAGAGTGACCAAGG	GGCGTAGAGGGTGTCAAAC
Inflammation	<i>Trif (Ticam1)</i>	NM_174989.4	CAGCTCAAGACCCTACAGC	CTCCACACAGCCTCGTC
	<i>Nf-κB</i>	NM_008689.2	TGAGGACGGGGTATGCAC	TCACATGAAGTATTCACAGTTT
Lipid transport	<i>CD36</i>	NM_001159557.1	CAGATCCGAACACAGCGTAG	GCGACATGATTAATGGCACA
Mitochondrial metabolism	<i>Cpt1</i>	NM_009948.2	CATCCCAGGCAAAGAGACA	AAGCGACCTTTGTGGTAGACA
	<i>Nrf-1</i>	NM_001164226	GGTGGGGACAGATAGTAGTCT	ATGCTCACAGGGATCTGGAC
Mitochondrial Content	<i>ATP5b</i>	NC_000076	GTTTGTGAAGCCACTCA	CATGATTCTGCCAAGGTCT
	<i>ND2</i>	NC_005089.1	AAGGGATCCCCTGCACATA	AGTCCTCCTCATGCCCTAT
	<i>ND5</i>	NC_005089.1	ATAACCGCATCGGAGACATC	GAGGCCAAATTGTGCTGATT
Gene expression in ileum				
Glucose and lipid metabolism	<i>Fiaf (Angptl4)</i>	NM_020581.2	GGGACCTTAACTGTGCCAAG	GAATGGCTACAGGTACCAAACC
Inflammation	<i>Myd88</i>	NM_010851.2	GCCTTGTTAGACCGTGAGGAT	CTAAGTATTCTGGCAGTCCTCT
	<i>Tlr4</i>	NM_021297.3	GGACTCTGATCATGGCACTG	CTGATCCATGCATTTGGTAGGT
Branched Amino Acids Receptor	<i>Lat-1</i>	NM_011404.3	ATGTGGCTCCGATTCAAGA	GGAGGGCCAGATTCACCT
Short-Chain Fatty Acids Receptor	<i>Gpr40 (Ffar1)</i>	NM_194057.2	AGGCGCTCTCCTCACACTC	CTAGCCACATTGGAGGCATTA
Mean-Chain Fatty Acids Receptor	<i>Gpr41 (Ffar3)</i>	NM_001168509.1	TGCTCTGAAGAAGCCAATCA	TTCTCTCTGGTCCAGTGCT
Long-Chain Fatty Acids Receptor	<i>Gpr120 (Ffar4)</i>	NM_181748.2	TTGGTGTGAGCGTCGTG	CCAGCAGTGAGACGACAAAAG
Glucose transporter	<i>Sglt1</i>	NC_000071.6	CTGGCAGGCCGAAGTATG	TTCCAATGTTACTGGCAAAGAG
Reference Gene	<i>Arp (Rplp0)</i>	NM_007475.5	ACTGGTCTAGGACCCGAGAAG	TCCCACCTTGCTCCAGTCT
	<i>α-Tubulin</i>	NM_011653.2	CTGGAACCCACGGTCATC	GTGGCCACGAGCATAGTTATT

Table 2. Gene expression in skeletal muscle and ileum- Sequences of primers (5'–3')

	CTL	ATB	NAT
Body weight (g)	25.5 ± 0.8	25.4 ± 0.4	25.0 ± 0.7
Glycemia (mg/dL)	101.0 ± 3.8	101.7 ± 5.1	93.4 ± 4.4
Caecum weight (mg)	324.7 ± 35.0	1804.0 ± 154.0***	394.9 ± 25.2††
Liver weight (mg/g)	44.3 ± 1.4	38.3 ± 0.7**	42.3 ± 1.2††
Adipose Tissue weight (mg/g)			
White Adipose Tissue	27.8 ± 4.4	19.8 ± 3.2	26.4 ± 2.1
Brown Adipose Tissue	3.0 ± 0.2	2.7 ± 1.7	3.4 ± 0.2
Food intake (g/week)	29.9 ± 1.2	28.4 ± 1.1	29.0 ± 0.7
Hydration (ml/week)	34.6 ± 1.9	36.1 ± 1.6	36.0 ± 1.1

Table 3. Effects of antibiotic treatment and natural reseeded on body mass, glycemia, caecum liver and adipose tissue weights, as well food intake and hydration. Values are group mean at D21 for weights, and weekly for food intake and hydration ± SEM. Significance was checked using ANOVA followed by LSD Fisher post hoc test *: p<0.05; **: p≤0.01; ***: p<0.001. vs. CTL group; ††<0.01 and ††† p<0.001 vs. ATB group n=9-10 for each group.

Figure Legends

Figure 1. Effect of gut microbiota depletion and natural reseeded on bacterial DNA content, α - and β diversity indices, phyla and families abundance. Real-time qPCR quantification of all-bacteria in feces at D0 (A) and D7 (B) and in the caecum at D21 (C) (n=5). Bacterial α -diversity (D) and β -diversity (E-F) in caecum at D21 (n=5); Sample denotation: red: CTL, blue: NAT. Bacterial phyla (G-H) and families (I-J) abundance in caecum at D21 (n=5). For bacterial DNA content, a one-way ANOVA followed by the LSD Fisher post-hoc test was used. To evaluate the reseeded process, bacterial and families abundance, as well α -diversity was compared in CTL and NAT groups using unpaired non parametric *t*-test. For β -diversity, significance between CTL and ATB was checked using PERMANOVA, as implemented in the 'adonis' function from the R vegan package, with 9999 permutations. ***: $p \leq 0.001$ vs CTL, controls (no treatment); ATB, antibiotic treatment for 21 days; NAT, 10-days antibiotic treatment followed by natural reseeded for 11 days.

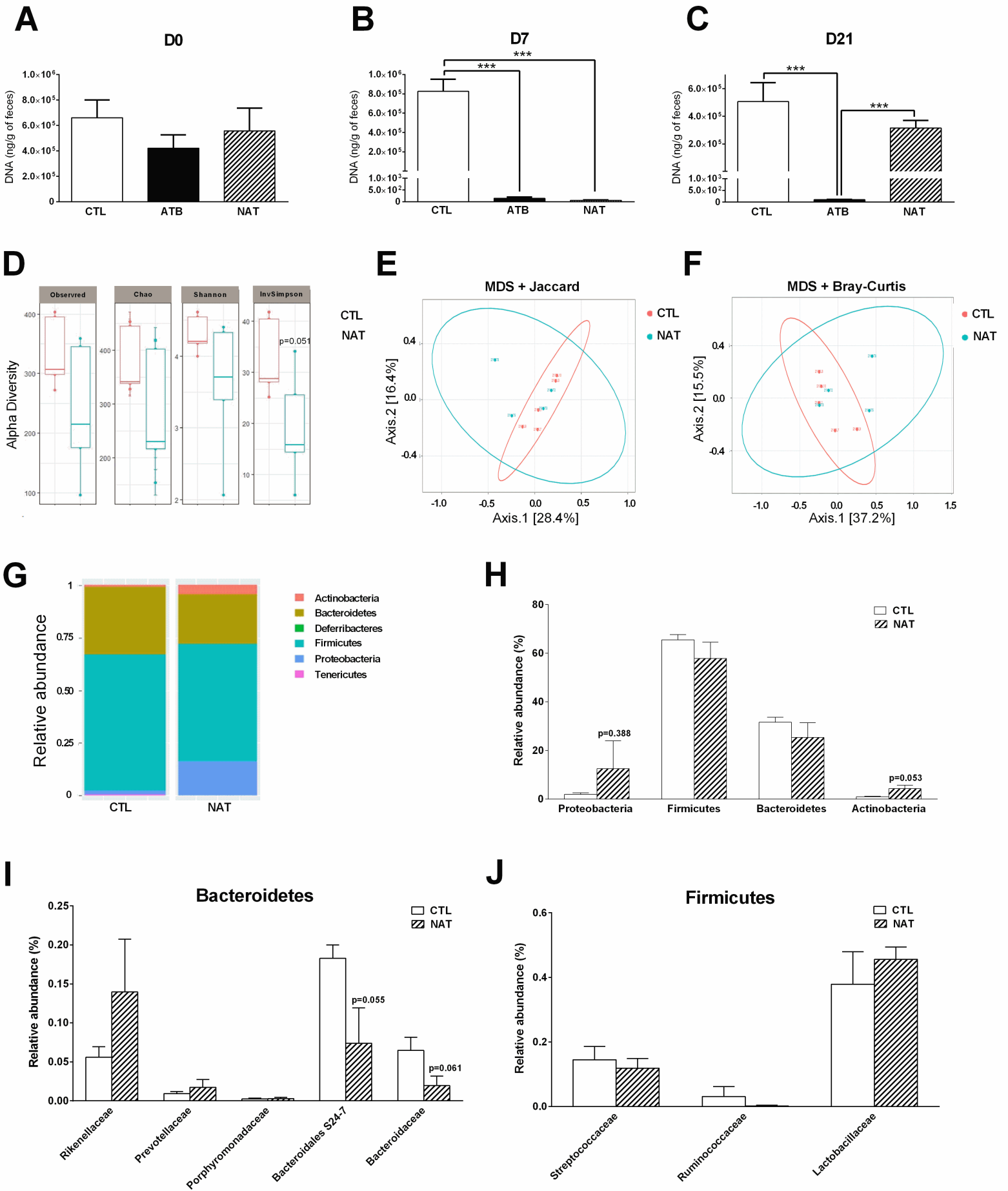
Figure 2. Effects of gut microbiota depletion and natural reseeded on skeletal muscle structure. In CTL, ATB and/or NAT mice gastrocnemius, quadriceps, soleus and EDL (A) wet weight (B) weight relative to body weight at D21. (C) weight relative to body weight after subtraction of caecum weight at D21. (D) Quadriceps fiber CSA distribution at D21. (E) Representative images of quadriceps muscle sections at D21. (F) RT-qPCR analysis of *Igf-1*, *Murf-1*, *Trif*, *Foxo-3a* and *Nf- κ B* expression in gastrocnemius muscle at D21. (G) Activation of PRAS40, 4EBP1, RPS6, LC3B in gastrocnemius muscle at D21. (H) Representative blotting images used for the quantification shown in (G). On the blot for RPS6 (G), a line was added to show an arrangement due to an empty well between the samples on the western blot gel. Values are the mean \pm SEM. Significance between CTL, ATB and NAT was checked using a one-way ANOVA followed by the LSD Fisher post-hoc. Significance was checked using a one-way ANOVA followed by the LSD Fisher post-hoc test (A-B-C-F-G). The mean muscle fiber cross-sectional area was compared in CTL and NAT groups using unpaired *t*-test (D). *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$ (n=8-10 for each group).

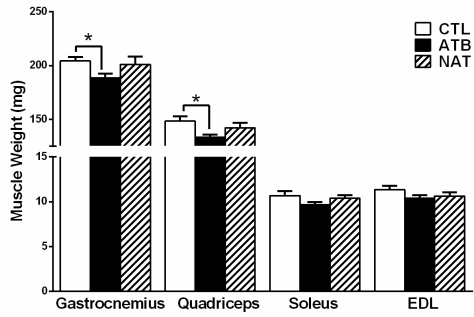
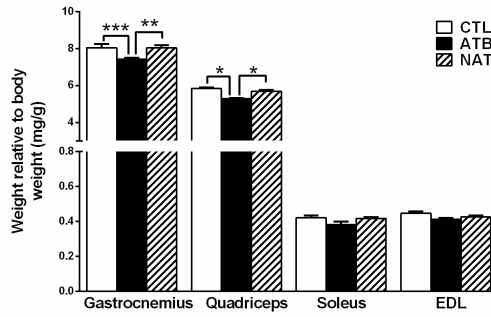
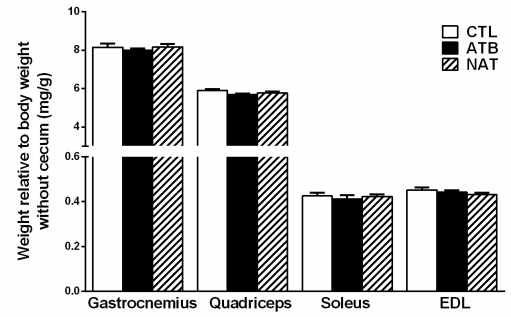
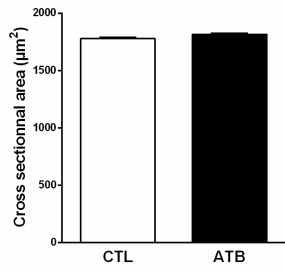
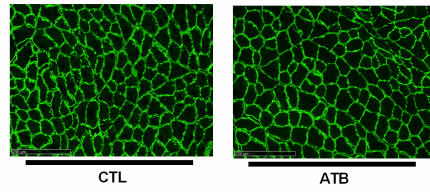
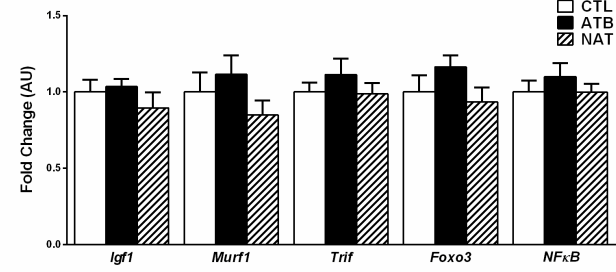
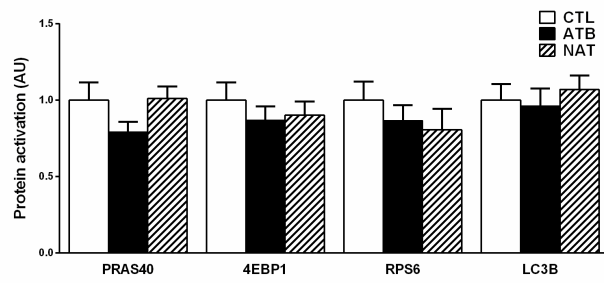
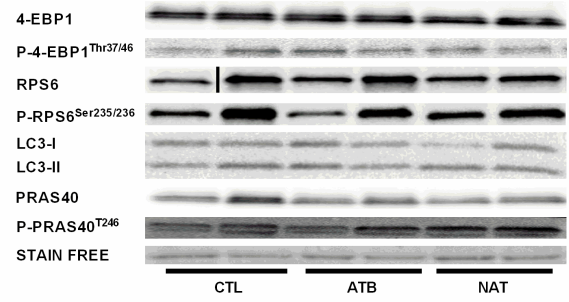
Figure 3. Effects of gut microbiota depletion and natural reseeded on skeletal muscle function. Maximal Aerobic Velocity (MAV) in the CTL (A) ATB (B) NAT groups (C) at D0, D7 and D17. Limit time to exhaustion during a submaximal running test in the CTL (D), ATB (E) and NAT groups (F) at D0, D9 and D19. (G) *Ex vivo* EDL maximal strength relative to its weight at D21. (H) *Ex vivo* EDL muscle fatigue index at D21. Values are the mean \pm SEM. Significance was checked using the paired *t*-test (A-B-C-D-E-F) and a one-way ANOVA followed by the LSD Fischer post-hoc test (G-H). *: $p \leq 0.05$; ** vs ATB vs CTL and NAT; $p \leq 0.01$ ATB vs CTL and NAT: (n=9-10 for each group).

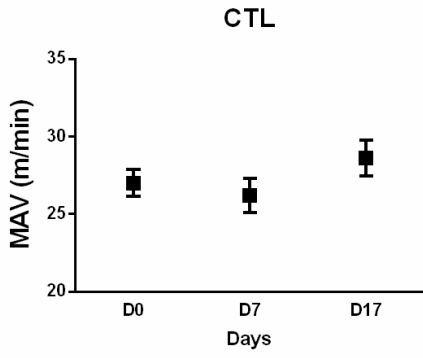
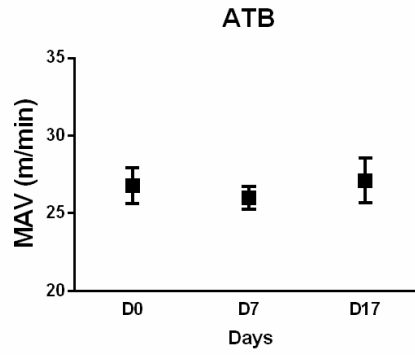
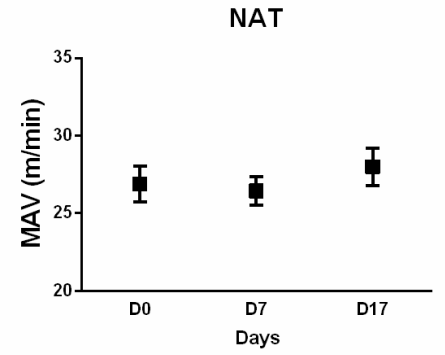
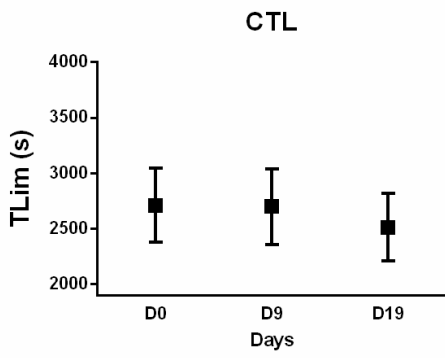
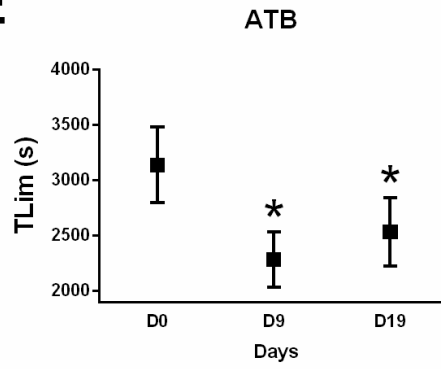
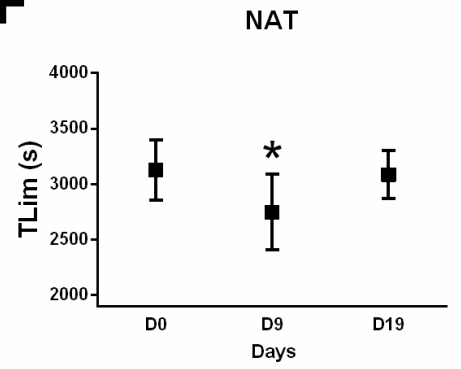
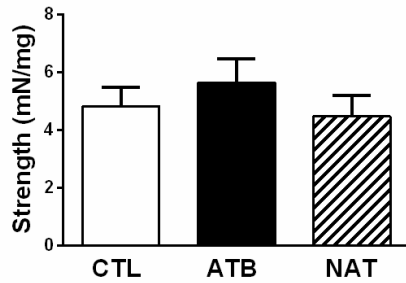
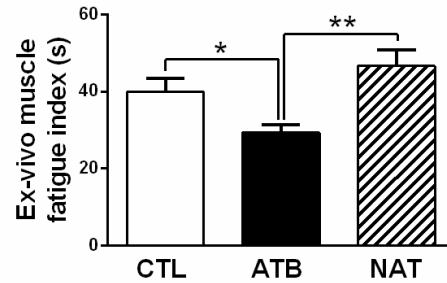
Figure 4. Effects of gut microbiota depletion and natural reseeded on skeletal muscle metabolism. (A) Gastrocnemius MHC distribution at D21. (B) Representative images of protein electrophoresis shown in (A). (C) *Cd36* and *Cpt1* mRNA expression in gastrocnemius samples at D21. (D) Mitochondrial DNA content and *Nrf-1* mRNA expression in gastrocnemius samples at D21. (E) AMPK phosphorylation, PGC-1 α and COX IV protein content in gastrocnemius muscles at D21. (F) Representative blotting images used for the quantification shown in (E). Values are the mean \pm SEM. Significance between groups was checked using a one-way ANOVA followed by the LSD Fisher post-hoc test, except for mt-DNA, a Mann-Whitney were used to compare CTL and ATB groups (n=8-10 for each group).

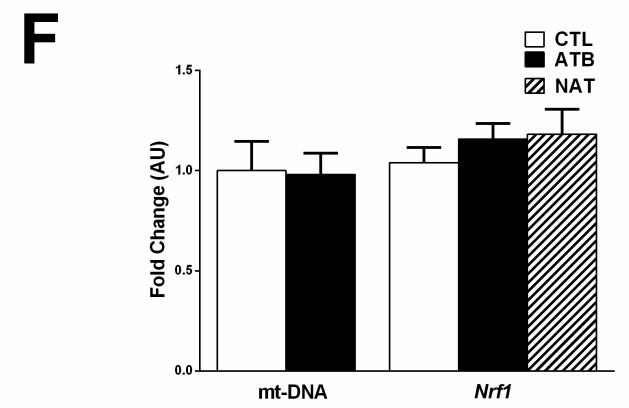
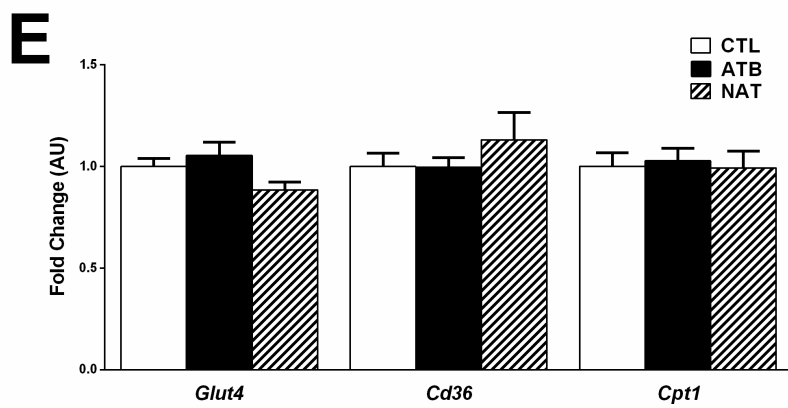
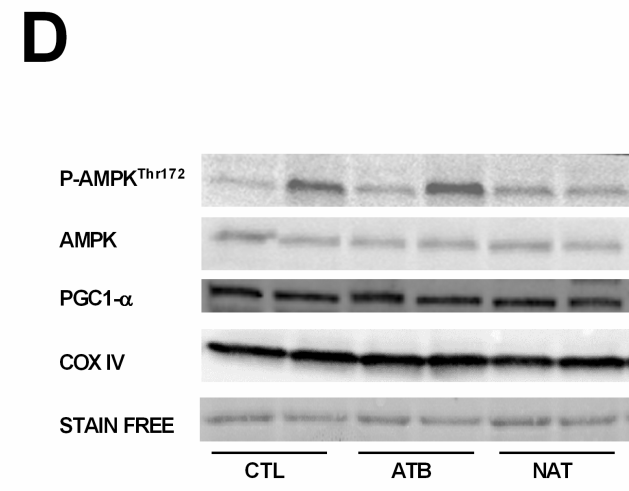
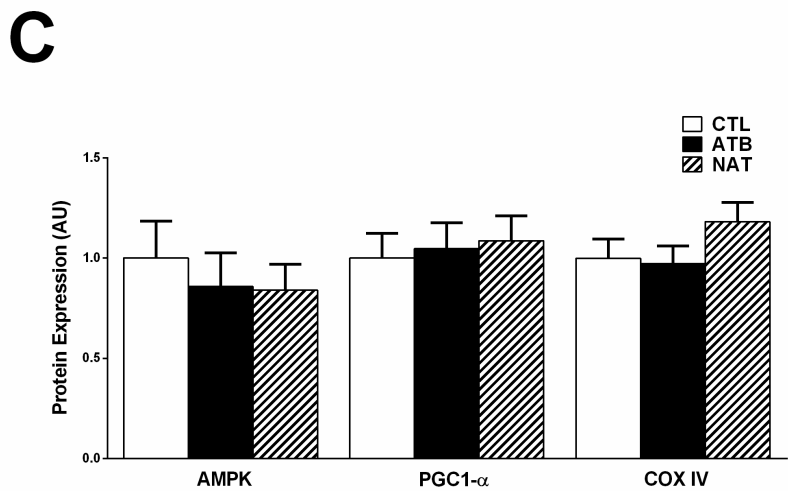
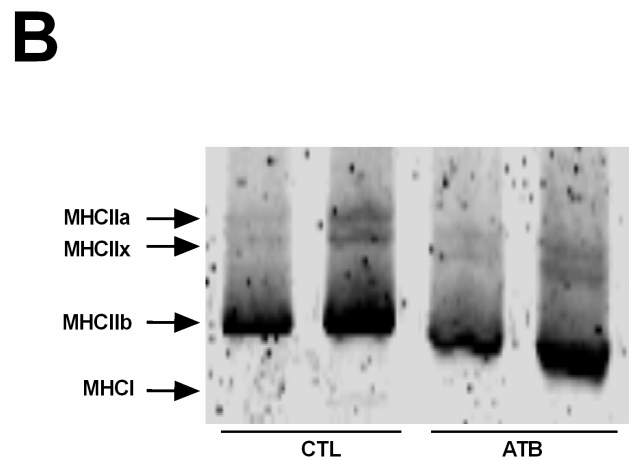
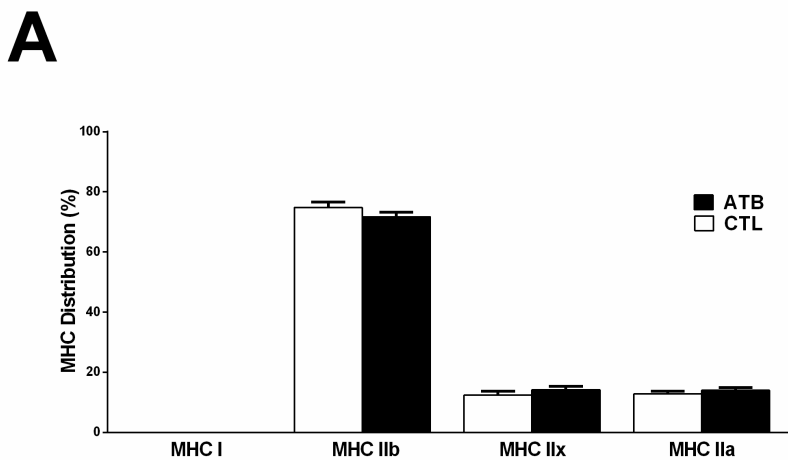
Figure 5. Effects of gut microbiota depletion and natural reseeded on energetic substrates metabolism. (A) RT-qPCR expression of *Fiaf*, *Myd88*, *Tlr4* and *Lat1* in the ileum at D21 (n=8). (B) RT-qPCR expression of *Gpr41*, *Gpr40*, *Gpr120* and *Sglt1* in the ileum at D21 (n=8). (C) Gastrocnemius triglyceride content at D21 (n=9-10). (D) Gastrocnemius glycogen content at D21 (n=9-10). Values are the mean \pm SEM. Significance between the 3 groups was checked using a one-way ANOVA followed by

the LSD Fisher post-hoc test *: $p \leq 0.05$ ATB vs CTL and NAT; **: $p \leq 0.01$ ATB vs CTL and NAT; ***: $p \leq 0.001$ ATB vs CTL and NAT.

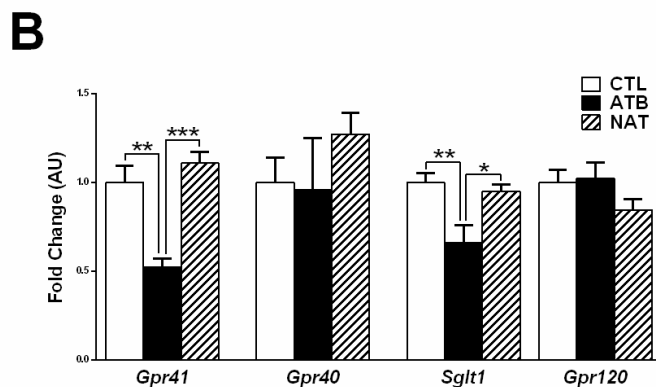
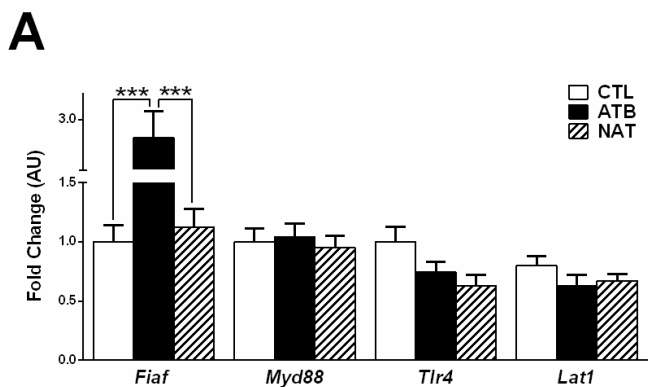


A**B****C****D****E****F****G****H**

A**B****C****D****E****F****G****H**



Ileum



Skeletal Muscle

