Original research

Roseburia inulinivorans increases muscle strength

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ABSTRACT

Background Gut bacteria have been implicated in a wide range of health conditions, yet their potential role in preventing and treating muscle-wasting disorders remains largely unexplored.

Objective We aimed to investigate whether specific gut microbial species are associated with muscle strength and to explore underlying mechanisms linking the gut microbiota to muscle health.

Design We conducted metagenomic analyses in cohorts of younger and older adults extensively phenotyped for muscle strength. Associations were tested between bacterial taxa and performance measures. Causality was assessed by oral supplementation of candidate species in antibiotic-treated mice. Metabolomic profiling and muscle phenotyping were performed to elucidate mechanisms.

Results The relative abundance of *Roseburia inulinivorans*, but not other *Roseburia* species, was positively associated with multiple strength measures including handgrip, leg press and bench press in humans. Supplementation of *R. inulinivorans* in mice significantly enhanced forelimb grip strength, whereas other *Roseburia* species had no effect. Metabolomic analyses revealed that *R. inulinivorans* reduced amino acid concentrations in the caecum and plasma, while activating the purine and pentose phosphate pathway in muscle. These changes coincided with increased muscle fibre size and a shift from type I to type II fibres. Accordingly, we observed that the relative abundance of *R. inulinivorans* is lower in older adults compared with young adults.

Conclusion *R. inulinivorans* emerges as a species-specific modulator of muscle strength, linking gut microbiota to muscle metabolism and function. These findings support its potential as a probiotic candidate for nutraceutical interventions targeting age-related muscle-wasting diseases.

Trial registration number NCT02365129.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The gut microbiota influences host metabolism and systemic health, with links to metabolic, neurodegenerative and cardiovascular diseases. However, its role in muscle strength and muscle-wasting conditions has remained unclear, and no specific bacterial species have been identified as causal modulators of muscle function.

WHAT THIS STUDY ADDS

⇒ We identify *Roseburia inulinivorans* as a species specifically associated with muscle strength in humans and causally linked to improved muscle performance in mice. Mechanistically, *R. inulinivorans* alters amino acid metabolism, activates the purine and pentose phosphate pathway in muscle and promotes fibre hypertrophy with a shift towards type II fibres. Additionally, we observed a lower abundance in older adults compared with young adults.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These findings highlight *R. inulinivorans* as a potential probiotic candidate for nutraceutical interventions against muscle-wasting diseases. They also support the gut-muscle axis as a therapeutic target and open new avenues for microbiome-based strategies in healthy ageing and sarcopenia management.

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INTRODUCTION

Loss of muscle mass and strength, seen in conditions like sarcopenia and cachexia, is a major contributor to frailty, reduced mobility, functionality and poor clinical outcomes, particularly in ageing and chronic disease.[1] While lifestyle factors such as exercise and nutrition are key modulators of muscle health, growing evidence points to the gut microbiota as a crucial regulator of host metabolism, inflammation and tissue function.[2]

Recent studies suggest that the gut microbiome is a key player in the ageing process, with far-reaching effects on host metabolism, inflammation and systemic physiology.[3] Notably, older adults whose gut microbiota composition resembles that of younger individuals exhibit a lower risk of age-related diseases, highlighting the potential of the microbiome to influence healthy ageing and longevity.[4] While much attention has been paid to its role in cardiometabolic health, growing interest has turned to the possible involvement of the gut in musculoskeletal decline. In particular, the concept of a gut–muscle axis has emerged, describing a bidirectional communication pathway through which

gut microbes, their metabolites and immune signalling may influence muscle mass and function.[5–7] However, direct evidence linking specific microbial species to muscle health in humans is scarce, and mechanistic insights remain limited. Addressing this knowledge gap is crucial for understanding how the gut microbiome might be leveraged to counteract muscle wasting during ageing and chronic disease.

In this study, we integrated human metagenomic analyses, causal inference in mice and metabolomic profiling to identify that the bacterial species *Roseburia inulinivorans* is functionally linked to muscle health in humans. Oral administration of *R. inulinivorans*, but not other *Roseburia* species, to antibiotics-treated mice significantly increased muscle strength and fibre size. These physiological benefits were accompanied by alterations in amino acid metabolism and activation of the pentose phosphate pathway (PPP) in skeletal muscle, suggesting a previously unrecognised mechanism by which gut microbes may influence muscle function. Collectively, our findings identify *R. inulinivorans* as a specific modulator of the gut-muscle axis and as a promising probiotic in the search for microbiome-based strategies to prevent or treat muscle-wasting diseases.

RESULTS

R. inulinivorans positively correlates with muscular strength in older and young adults

To identify whether gut bacteria play a role in muscle strength, we investigated the relationship between faecal bacteria and muscle strength and mass outcomes in cohorts of older[8] and young adults.[9] Faecal DNA was extracted using the QIAamp DNA Stool Mini Kit, and the V3–V4 regions of the bacterial 16S rRNA gene were sequenced on an Illumina MiSeq platform (2×300 bp). Sequence data were processed using the DADA2 pipeline in R, and taxonomy was assigned using the Ribosomal Database Project classifier with an 80% bootstrap confidence threshold. Phylogenetic assignment at the species level was based on ≥97% sequence identity, following established criteria, ensuring robust taxonomic resolution and data reliability (all samples exceeded 10 000 reads and were rarefied to 30 982 reads). Among all bacteria, we found that the relative abundance of *Roseburia* genus was positively related to muscle-related outcomes (online supplemental figure S1A,B). In the cohort of older adults, the relative abundance of *R. faecis* ranged from 0 to 2.2% (mean±SD: 0.3±0.4%), *R. intestinalis* ranged from 0% to 0.7% (0.2±0.2%), and *R. inulini*vorans ranged from 0% to 1.3% (0.1±0.2%). In the cohort of young adults, the relative abundance of *R. faecis* ranged from 0% to 3.3% (0.3±0.6%), *R. intestinalis* ranged from 0% to 5.5% (0.4±0.8%) and *R. inulinivorans* ranged from 0% to 6.6% (1.1±1.6%). In publicly available metagenomic data from the Human Gut Microbiota Repository (GMrepo),[10] a curated database comprising 71 642 human gut metagenomes of healthy individuals, the mean relative abundances of *R. faecis* (0.6%), *R. intestinalis* (1.6%) and *R. inulinivorans* (1.4%) are comparable to those observed in our healthy cohort. In relation to muscle strength, we found that older adults (age: 68.4±2.8 years; body mass index (BMI): 29.6±5.4 kg/m^2; sex: 52% male; n=33) with detectable *R. inulinivorans* (relative abundance 0.3±0.3%) exhibited markedly higher handgrip strength (+29%; p<0.01) compared with those without detectable *R. inulinivorans* (0.0%), without a concomitant increase in peak oxygen uptake (VO_2) (figure 1A). The presence of *R. faecis* and *R. intestinalis* was not significantly associated with handgrip strength or VO_2 peak (figure 1B, C). In young adults (age: 21.9±2.3; BMI: 24.9±4.7 kg/m^2; sex: 30% male; n=90), we

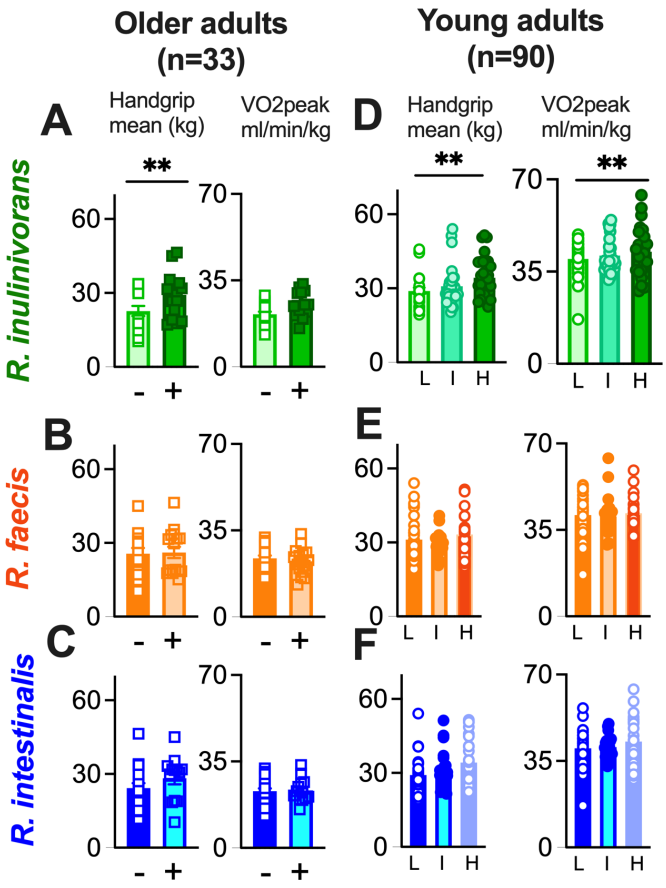

Figure 1 Association between *Roseburia* species abundance, handgrip strength and VO_2 peak in older and young adults. (A, B, C) Associations between *R. inulinivorans*-DSM16841 (A), *R. faecis*-DSM16840 (B) and *R. intestinalis*-DSM14610 (C) abundance and parameters of physical performance (ie, handgrip strength and VO_2 peak) in older adults (n=33). Older adults were grouped based on the presence (symbol '+') or absence (symbol '–') of *Roseburia* species in their faeces for older adults. (D, E, F) The absence category showed a relative abundance of *R. inulinivorans* of 0%, whereas the presence category showed 0.3±0.3%. Associations between *R. inulinivorans*-DSM16841 (D), *R. faecis*-DSM16840 (E) and *R. intestinalis*-DSM14610 (F) abundance and parameters of physical performance (ie, handgrip strength and VO_2 peak) in young adults (n=90). Individuals were stratified into tertiles based on Roseburia relative abundance (L: low, I: intermediate, H: high). The relative abundance for R. inulinivorans was (L: 0.05±0.05%, I: 0.5±0.2%, H: 2.7±1.7%). Handgrip strength (kg) and VO_2 peak relative to body weight (mL/min/kg) were compared across groups. Significant differences are indicated (**p<0.01). To ensure data robustness, all samples had more than 10 000 reads and were rarefied to a uniform sequencing depth (30 982 reads per sample). VO_2, oxygen volume.

stratified participants into tertiles based on relative abundance of *R. inulinivorans* (low: 0.05±0.05%, intermediate: 0.5±0.2%, high: 2.7±1.7%; figure 1D), *R. faecis* (figure 1E) and *R. intestinalis* (figure 1F) and evaluated their handgrip strength and VO_2 peak. This approach was feasible and statistically appropriate given the sample size (n=90), which provided sufficient power to detect potential trends across distribution-based subgroups. In this cohort, we found that higher *R. inulinivorans* abundance was positively associated with both handgrip strength (p<0.01) and with VO_2 peak (p<0.01; figure 1D), while *R. faecis* and *R. intestinalis* were not (figure 1E, F). In this cohort of young adults, the relative abundance of *R. inulinivorans* and

R. intestinalis was positively correlated with leg press and bench press strength (all r≥0.26, all p<0.05, online supplemental figure S1C). In contrast, the relative abundance of *R. faecis* and *R. hominis* showed no significant associations with any of these measures (online supplemental figure S1C), indicating that *Roseburia* species may differentially contribute to muscle-related traits. For the young healthy cohort, we did not find significant correlations between the relative abundance of *Roseburia* strains and energy, carbohydrate, fat, protein or fibre intake (data not shown).

R. inulinivorans increases muscular strength and muscle fibre size in non-exercising mice

To determine causality between *R. inulinivorans* and muscular strength, we conducted an experimental study in mice. To this end, we cultivated and administered *Roseburia* strains (ie, *R. inulinivorans*-DSM16841, *R. faecis*-DSM16840 and *R. intestinalis*-DSM14610). While these were not directly isolated from our participants, they do correspond to the same strains identified in the human faecal microbiota reported above, reinforcing the translational link between humans and mice. 32 male C57BL/6J mice (6 weeks old) were treated with a cocktail of antibiotics for 2 weeks to deplete their gut microbiota[11] (figure 2A). Following this treatment, mice were randomly assigned to one of four groups (n=8 per group), receiving (1) vehicle (200 µL; 15% glycerol in phosphate buffered saline (PBS); control), (2) *R. faecis*-DSM16840 (200 µL; 2×10^8 colony-forming units (CFU)), (3) *R. intestinalis*-DSM14610 (200 µL; 2×10^8 CFU) or (4) *R. inulinivorans*-DSM16841 (200 µL; 2×10^8 CFU). Bacterial suspensions (vs vehicle) were transplanted into mice via oral gavage three times per week for a total period of 8 weeks. While mice receiving *R. inulinivorans* or *R. intestinalis* gained body weight similarly to controls, mice receiving *R. faecis* gained more body weight and lean mass compared with the other groups (figure 2B, C). Fat mass (figure 2D) and food intake (figure 2E) remained unchanged across groups. None of the *Roseburia* species altered plasma glucose levels or the clearance of radiolabelled glucose or fatty acids in these healthy mice (online supplemental figure S2A–C). *Roseburia* species did not affect weights of organs, including the liver, spleen, white and brown adipose tissue depots or skeletal muscle (online supplemental figure S2C–E). After 8 weeks of intervention, we performed 16S rRNA gene sequencing on the caecal content of mice. Overall, alpha diversity was not altered (online supplemental figureS3A), but beta diversity was significantly affected in the mice treated with *R. inulinivorans* (online supplemental figure S3B), with a slight increase observed in the relative abundance of *Muribaculum*, *Blautia* and *Ruminococcus* genera compared with controls (online supplemental figure S3C). Caecum samples collected 72 hours after oral gavage showed low relative abundance of the *Roseburia* genus, indicating that the human-derived *Roseburia* species may not have colonised in the caecum under these conditions (relative abundances from 0.0 to 0.006: online supplemental figure S3D). None of the *Roseburia* strains enhanced running time to exhaustion compared with control (figure 2F). Nonetheless, *R. inulinivorans* induced a remarkable increase in forelimb grip strength, a proxy for muscle function, after 4 weeks, 6 weeks and 8 weeks of treatment by approximately 30% compared with control treatment (all p<0.001), while *R. faecis* and *R. intestinalis* did not (figure 2G). The effect of *R. inulinivorans* persisted after correcting forelimb grip strength for lean body mass (data not shown). As the experiment was designed to last only 8 weeks, grip strength could not be measured beyond this period, and its long-term evolution remains unknown.

To evaluate the impact of *Roseburia* species on skeletal muscle morphology, we assessed muscle fibre cross-sectional area (CSA). We found that mice treated with *R. inulinivorans* exhibited larger muscle fibre size compared with the control group, while *R. faecis* displayed similar fibre size to control (figure 2I). In line with these findings, fibre typing based on nicotinamide adenine dinucleotide (NADH) staining revealed that *R. inulinivorans* treatment led to a significantly higher proportion of type II fibres in the soleus muscle compared with other groups, although not when compared with *R. intestinalis* (figure 2H). Further analysis of fibre size distribution revealed that the control group presented a relatively even distribution across fibre size ranges, whereas mice treated with *R. inulinivorans* supplementation resulted in a higher frequency of larger fibres (>5000 µm²) compared with mice treated with the other *Roseburia* species or control (figure 2J).

R. inulinivorans modifies intestinal amino acids rather than short-chain fatty acids

To investigate how *R. inulinivorans* enhances muscle strength in mice, we conducted targeted metabolomics analysis in the caecum content. Given that *Roseburia* species are well-known butyrate producers,[12 13] we first focused on short-chain fatty acids (SCFAs). However, caecal concentrations of SCFAs were comparable across all groups, showing no significant differences from the control group (online supplemental figure S4). We next assessed amino acids and intermediates of the tricarboxylic acid cycle. Mice treated with *R. faecis* exhibited significantly lower levels of aspartate, 3-methyl-2-oxovalerate, malate and valerate in caecum compared with controls (figure 2K). Administration of *R. intestinalis* resulted in significantly lower levels of lactate and aspartate relative to controls (figure 2L). Importantly, the most pronounced metabolic shifts were observed in mice treated with *R. inulinivorans*, where caecum levels of methionine (Met), leucine, isoleucine, alanine, valine, lysine (Lys) and 5-aminopentanoate were markedly lower relative to the control group (figure 2M). We calculated the (Met+Lys)/(Aspartate (Asp)+Threonine (Thr)) ratio as a proxy for amino acid balance (ie, the relative availability of essential amino acids required for protein synthesis) and methylation potential (ie, amino acids that contribute to one-carbon metabolism, which supports DNA and histone methylation). *R. inulinivorans* treatment induced a marked decrease in the (Met+Lys)/(Asp+Thr) ratio compared with controls, indicating a shift in amino acid metabolism in pathways related to protein turnover, methyl donor availability and metabolic regulation (figure 2M). Consistently, enrichment analyses in young human adults revealed lower amino acid-related pathways, such as valine and glutathione metabolism, in individuals with high compared with low abundance of *R. inulinivorans* (figure 2N), but not with other *Roseburia* species (data not shown), mirroring the metabolic signature observed in mice.

R. inulinivorans decreases metabolites involved in amino acid biosynthesis and enhances muscle purine metabolism

To further understand why *R. inulinivorans* increases muscular strength, we conducted untargeted metabolomics analysis on plasma and muscle samples collected from mice at week 8 (figure 3). Plasma and muscle were obtained 72 hours after the last oral gavage. Mice treated with *R. inulinivorans* exhibited marked alterations in plasma metabolites, with differences

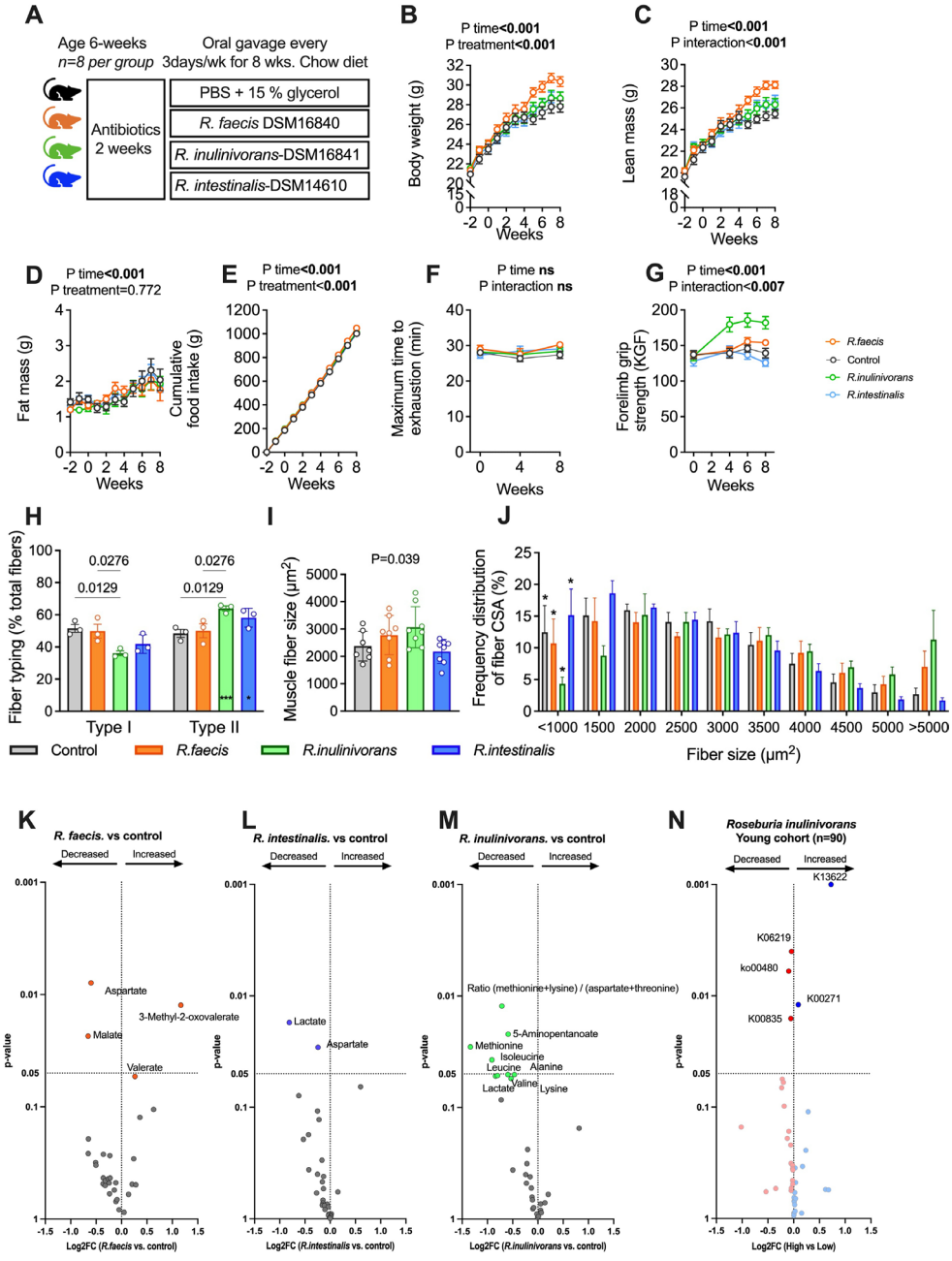

Figure 2 Effects of *Roseburia* species supplementation on body composition, food intake, muscle strength, time to exhaustion, muscle fibre typing, muscle cross-sectional area (CSA) and caecal metabolite concentrations in mice, along with pathway analysis in young adults. (A) Experimental design: 6-week-old male C57BL/6 J mice (n=8 per group) were treated with antibiotics for 2 weeks before being randomised into four groups: control (PBS+15% glycerol), *R. faecis*-DSM16840, *R. intestinalis*-DSM14610 and *R. inulinivorans*-DSM16841. Mice received oral gavage three times per week for 8 weeks while maintained on a chow diet. (B–D) Weekly progression of body weight, lean mass and fat mass. (E) Cumulative food intake. (F) Maximum time to exhaustion was assessed on a treadmill with incremental speed at baseline, week 4 and week 8. (G) Forelimb grip strength was measured after antibiotic treatment at weeks 4, 6 and 8 in kilogram-force (KGF) . Data are presented as mean±SD, and statistical significance was determined using repeated measures Analyses of variance (ANOVA). (H) Soleus muscle fibre type distribution based on nicotinamide adenine dinucleotide (NADH) staining, expressed as percentage of total fibres. *** p<0.0001, * p<0.05 for intragroup comparison between type I and type II fibres. (I) Average soleus muscle fibre CSA (µm²) in different experimental groups. Data are presented as mean±SD, with individual data points shown. P value =0.039 indicates a statistically significant difference among groups (ANOVA). (J) Frequency distribution of soleus muscle fibre CSA across fibre size categories (µm²). * Indicates significant intra-group differences between CSA bins. (K–N) Volcano plots illustrating the log2 fold change (Log2FC) of short-chain fatty acids (SCFAs), amino acids and tricarboxylic acid cycle intermediates in the caecum of mice treated with different *Roseburia* species compared with controls. (K) *R. faecis* versus control, (L) *R. intestinalis* versus control, (M) *R. inulinivorans* versus control. (N) Pathway enrichment analysis in faecal samples from young adults, comparing individuals in the highest tertile of *R. inulinivorans* abundance versus those in the lowest tertile. Functional pathways were inferred from 16S rRNA gene sequencing (V3–V4 regions, Illumina MiSeq) using predictive metagenomic analysis in R, based on taxonomic profiles generated with the DADA2 pipeline and Ribosomal Database Project classifier. Significant metabolic pathways altered include valine metabolism (K00271, K00835), S-adenosylmethionine-dependent methyltransferase activity (K06219, K13622) and glutathione metabolism (ko00480). PBS, phosphate buffered saline.

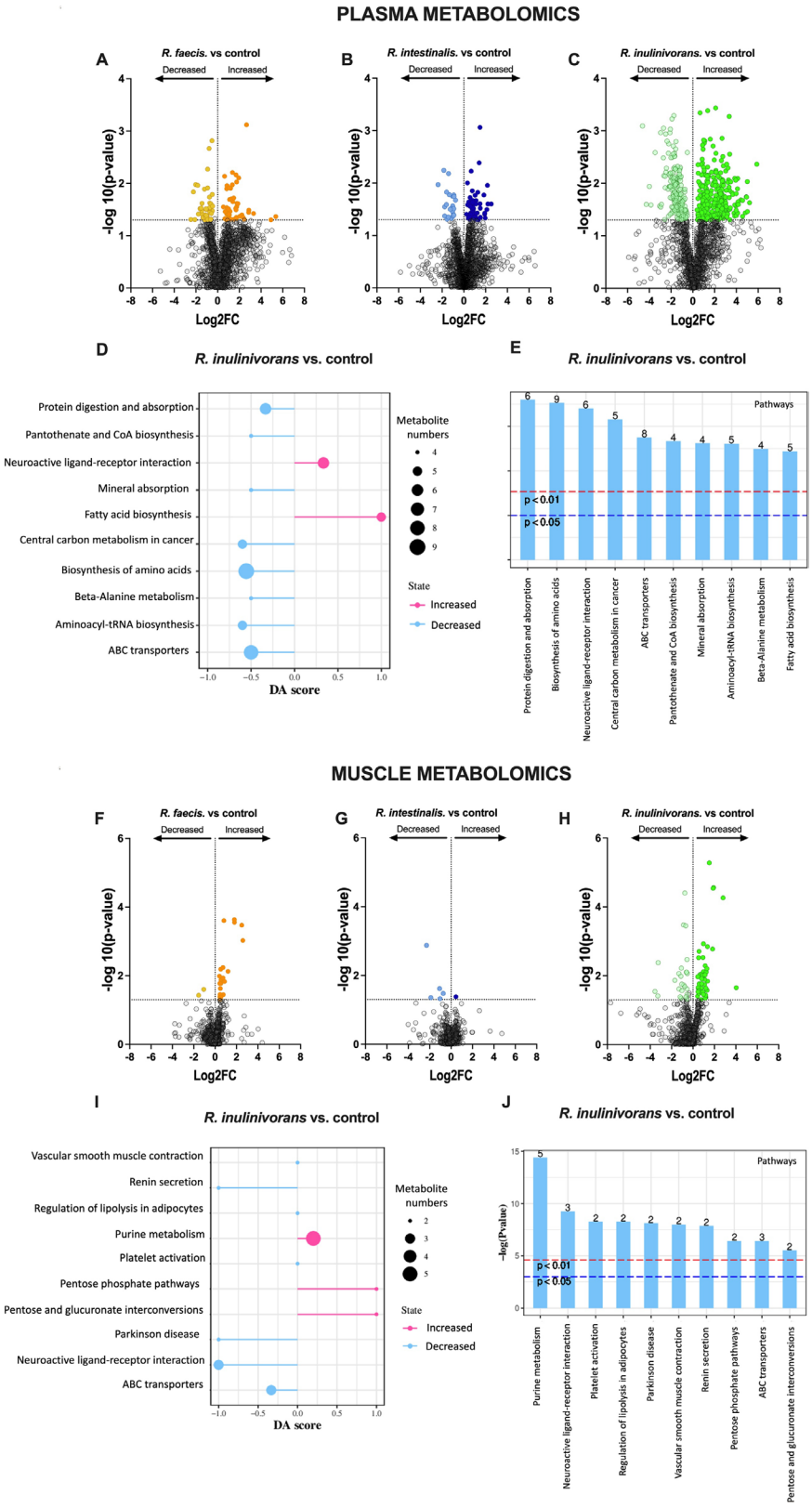

Figure 3 Effects of *Roseburia* species supplementation on untargeted metabolomics in plasma and skeletal muscle in mice. (A, B, C, F, G and H) Volcano plots depicting differentially abundant metabolites in plasma and muscle for the comparisons: *R. faecis* versus control (A, F), *R. intestinalis* versus control (B, G) and *R. inulinivorans* versus control (C, H). (D, I) Metabolic pathway enrichment analysis (DA score plot) for plasma (D) and muscle (I). The Y-axis represents metabolic pathways, while the X-axis (DA score) indicates the overall trend of all metabolites within a pathway. A DA score of +1 suggests an upregulation of all annotated metabolites in the pathway, whereas a DA score of −1 indicates down-regulation. The line length represents the absolute DA score, and the dot size corresponds to the number of metabolites in the pathway. (E, J) Pathway enrichment analysis bar charts comparing *R. inulinivorans* versus control in plasma (E) and muscle (J), highlighting key metabolic pathways affected by *R. inulinivorans* supplementation. CoA, coenzyme A. DA, differential abundance.

observed for 492 metabolites compared with the control group (figure 3A–C). In contrast, *R. faecis* and *R. intestinalis* modified only 88 and 75 metabolites, respectively, compared with the control group (online supplemental figure S5A-D). To further explore these metabolic shifts, we performed metabolic pathway enrichment analysis using the differential abundance (DA) score to identify pathway-level changes. Compared with the control group, *R. inulinivorans* decreased the concentrations of metabolites involved in amino acid biosynthesis, beta-alanine metabolism, ATP-Binding Cassette (ABC) transporters and aminoacyl-tRNA biosynthesis, while increasing the concentrations of metabolites associated with fatty acid biosynthesis and neuroactive ligand-receptor interactions (DA score >0, figure 3D, E).

Using the same approach in the skeletal muscle of the mice (figure 3), we found that mice treated with *R. inulinivorans* exhibited significant differences in 65 muscle metabolites compared with controls (figure 3F–H). In contrast, *R. faecis* and *R. intestinalis* modified only 22 and six metabolites in the muscle, respectively, compared with the control group (online supplemental figure S6A–D). Metabolic pathway enrichment analysis showed that *R. inulinivorans* increased metabolites associated with purine metabolism (ie, adenosine, D-ribose-1-phosphate, ADP, xanthine and uric acid) and the PPP (D-ribose-1-phosphate and D-ribulose-5-phosphate), as well as glucuronate interconversions (figure 3I, J). Although the PPP enrichment was driven by only two metabolites, both are central intermediates that link carbohydrate metabolism to purine biosynthesis through the production of ribose-5-phosphate. Conversely, several metabolites were decreased (DA score <0), including those involved in neuroactive ligand-receptor interactions and ABC transporters (figure 3J).

R. inulinivorans tends to decline with age

Using the data from our cohorts, described in figure 1, we found that older adults (+65 years old) had significantly lower levels of *R. inulinivorans* compared with young adults (from 18 years to 25 years old), while no significant differences were observed for *R. faecis* and *R. intestinalis* (figure 4A). We next used the publicly available resource "curatedMetagenomicData"[14] to obtain metagenomic profiles of 3512 human faecal samples to investigate whether the relative abundance of *Roseburia* species differed between adults (from 18 years to 65 years old) and older adults (+65 years old). We observed that adults had a slightly higher relative abundance of *R. inulinivorans* than older adults (p=0.016), whereas no significant differences were found for *R. faecis* (p=0.26) or *R. intestinalis* (p=0.063) (figure 4B). We

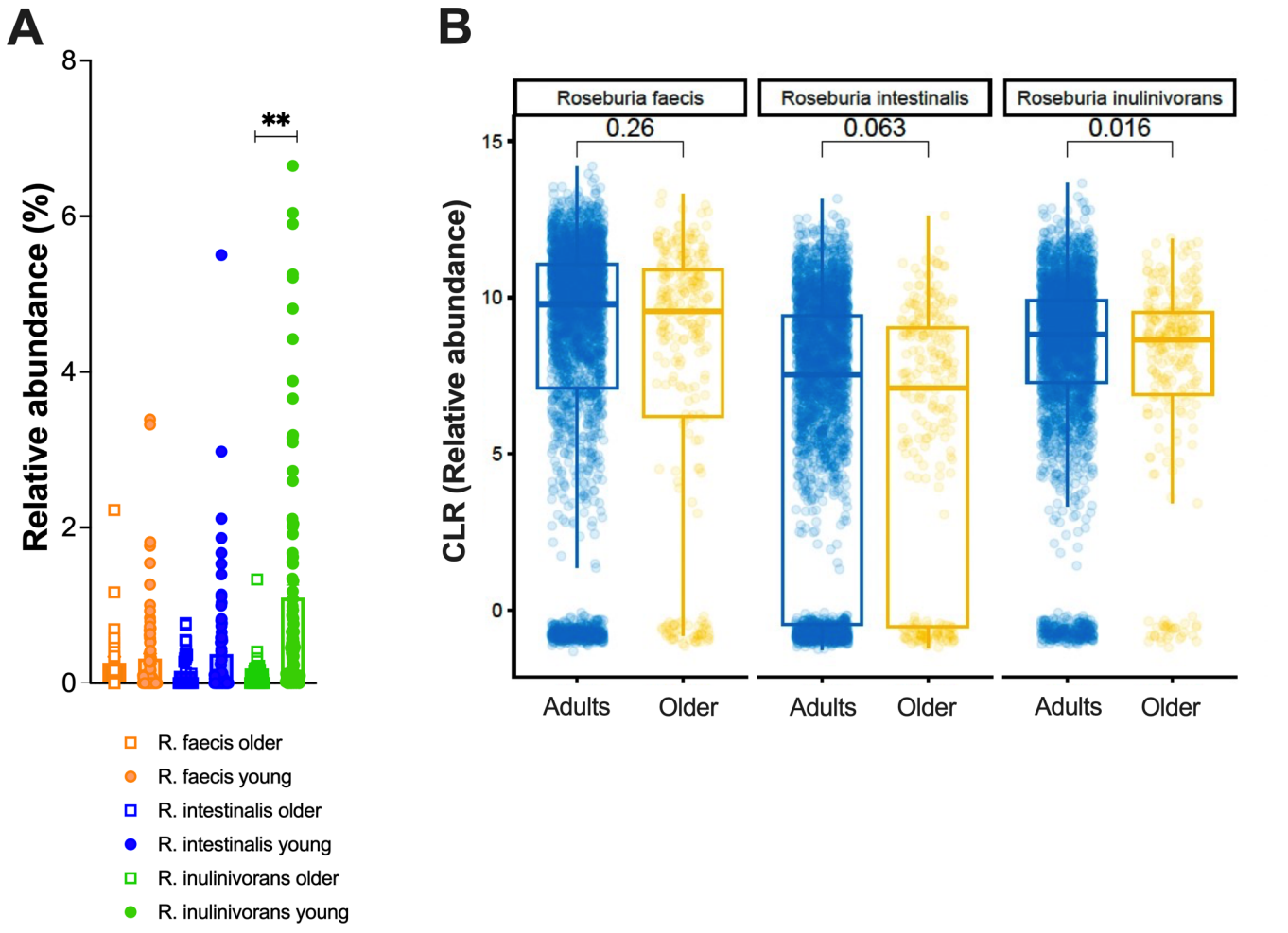

Figure 4 Differences in the relative abundance of *Roseburia* species between young (18–25 years and older adults (≥65 years) using data from our cohorts, and between adults (18–65 years) and older adults (≥65 years) using publicly available datasets. (A) Relative abundance of *Roseburia* species (ie, *R. faecis*-DSM16840, *R. intestinalis* DSM14610 and *R. inulinivorans*-DSM16841) compared between older (n=33) and young adults (n=90). (B) Boxplots show median and IQR, and p values correspond to Wilcoxon rank-sum tests comparing age groups. CLR, centred-log-ratio.

considered the possibility that these differences might be driven by the large number of participants with zero abundance for these species. After repeating the analyses excluding participants with zero relative abundance, the results did not change (data not shown). Using the data from figure 4B, we conducted a meta-analysis incorporating all publicly available cohorts and the LifeLines cohort[15] (online supplemental figure S7). Although some cohorts contributed more heavily to the overall effect size, the meta-analysis showed no statistically significant differences in the relative abundance of any *Roseburia* species between adults and older adults (online supplemental figure S7). Notably, the overall effect size for *R. inulinivorans* was slightly negative, indicating a trend toward lower abundance in older adults within this meta-analysis, although this did not reach statistical significance.

DISCUSSION

Here, we showed that the relative abundance of *R. inulinivorans* correlates positively with muscle strength in young and older humans, and that supplementation of mice with *R. inulinivorans* causally increases muscle strength and fibre size. These beneficial effects were accompanied by plasma alterations in amino acid and carbohydrate metabolism, including enrichment of the muscle purine and PPPs, which are central to energy production, redox balance and nucleotide biosynthesis. Collectively, our findings provide robust evidence supporting a gut-muscle axis in which *R. inulinivorans* positively modulates muscle metabolism and muscle strength. Additionally, we observed that the relative abundance of *R. inulinivorans* is lower in older adults than in young adults. Its abundance appears to decline with advancing age, a period during which the prevalence of sarcopenia increases, suggesting a potential role for *R. inulinivorans* as a probiotic candidate for preserving muscle strength.

Mechanistic insights into *R. inulinivorans*-mediated muscle strength enhancement

Although *R. inulinivorans* is known to produce butyrate,[13] its muscle-strengthening effects do not appear to be driven by changes in SCFA metabolism, but rather by targeted alterations in amino acid handling and activation of the muscle purine and PPPs. A recent study characterised both the core and pangenome of the *Roseburia* genus, revealing a broader functional repertoire beyond SCFA production.[16] They observed that, although all Roseburia species can synthesise amino acids, *R. inulinivorans* lacks the capacity to produce tryptophan and several vitamins (B2, B5, B6 and B7) and shows two distinctive metabolic traits that may underlie the unique effects observed in this study. Specifically, *R. inulinivorans* relies on a succinylation-dependent lysine biosynthesis pathway and lacks the ability to metabolise urea as a nitrogen source, making it more dependent on amino acids available in the gut environment.[16] This unique dependency may increase microbial uptake of luminal amino acids, thereby lowering their availability to the host. In response, the host may adapt by prioritising the allocation of available amino acids to key metabolic tissues such as skeletal muscle. Supporting this idea, *R. inulinivorans* treatment of mice enhanced activation of the purine and PPPs in skeletal muscle, a metabolic pathway essential for nucleotide biosynthesis and redox balance. This likely serves as a compensatory mechanism to support muscle protein synthesis, repair and growth under conditions of limited amino acid availability. In parallel, purine and PPP activity generates nicotinamide adenine dinucleotide phosphate (NADPH, which supports lipid synthesis and may contribute to sarcoplasmic expansion and increased muscle fibre size, potentially explaining the observed increase in muscle fibre size in mice treated with *R. inulinivorans*. Additionally, we observed upregulation of purine metabolism in muscle, a process that can be stimulated by activation of mTORC1, a key regulator of anabolic signalling and muscle growth.[17] Notably, purine biosynthesis is tightly coupled to the PPP through the generation of ribose-5-phosphate, linking nucleotide turnover with carbohydrate and energy metabolism. Interestingly, we previously found that genetic overexpression of glucose-6-phosphate dehydrogenase, the rate-limiting enzyme of the PPP, improves health span and delays the onset of frailty in aged mice.[18 19] The protective mechanism involves enhanced ROS detoxification and reduced age-associated muscle damage, suggesting a possible link between PPP activity, purine metabolism and muscle integrity.

Notably, although these metabolic adaptations might suggest the need for sustained microbial presence in the gut, prior studies have shown that human gut anaerobic bacteria administered to mice are only detectable in faeces for up to 16 hours post gavage, with no trace beyond 24 hours.[11] In line with this, we did not detect *R. inulinivorans* in the colon or faeces of treated mice, likely because samples were collected more than 24 hours after administration. Additionally, we observed that mice treated with *R. inulinivorans* showed an increased relative abundance of the genera *Muribaculum, Blautia* and *Ruminococcus,* suggesting that the beneficial effects observed in this study may be mediated, at least in part, by interactions between *R. inulinivorans* and these gut microbiota. These observations support the idea that the muscle-strengthening effects of *R. inulinivorans* are mediated by transient microbial signals or metabolites rather than stable colonisation. Similarly, oral administration of pasteurised *Akkermansia muciniphila* has been shown to reduce body weight, as well as concentration of total cholesterol, insulin, gamma-glutamyl transferase and lipopolysaccharide levels in mice and humans, even though the bacterium did not stably engraft in the colon. These beneficial effects have been shown to be mediated by the ability of *A. muciniphila* to produce SCFAs[20–22] and a purified membrane protein (Amuc_1100).[21 23]

Roseburia as a key mediator of the bidirectional gut-muscle axis

In this study, we demonstrate that the gut-muscle axis has a distinct directional component: *R. inulinivorans* can beneficially impact muscle health. Whether the gut-muscle axis has a bidirectional relationship remains an open question. In theory, activation of skeletal muscle may release secretory factors capable of altering gut microbiota composition, creating a feedback loop between these systems.[23–25] The most impactful non-pharmacological intervention for enhancing muscle function is exercise training. Indeed, we[26–28] and others[6 29] have reported that exercise can modulate gut microbiota composition. However, we noted that exercise-induced changes in the gut microbiota depend significantly on the initial composition of the gut ecosystem; if the microbiota does not initially exhibit reduced diversity or an over-representation of potentially pathogenic taxa, the impact of exercise on further improving microbial profiles may be limited.[26] Importantly, changes in gut microbiota composition observed following exercise do not necessarily indicate that muscle is directly mediating these changes. Exercise is a powerful physiological stimulus that alters the concentration of over 9000 plasma metabolites, many of which can be released by organs beyond skeletal muscle after a single session in humans.[30] Additionally, vigorous exercise has been demonstrated to transiently

increase gut permeability, often referred to as *gut leakiness*, likely due to elevated internal temperature and reduced mesenteric blood flow, potentially creating localised anaerobic conditions within the gut.[25] This transient gut permeability should not necessarily be viewed as detrimental, as it may serve as a physiological adaptation that facilitates nutrient mobilisation and the release of pro-inflammatory mediators essential for muscle repair and adaptation after exercise.[31] In line with this, a previous study randomised 32 sedentary young adults living with obesity (aged 18–35 years, BMI 25–45 kg/m^2) to a 6-week strength training intervention.[32] Remarkably, despite minimal overall changes in gut microbiota composition, strength training intervention selectively increased the relative abundance of the genus *Roseburia* by approximately 2%. While this alone does not definitively establish a muscle-to-gut communication pathway, it strongly supports the existence of a bidirectional link between muscle strength and *Roseburia* abundance.

These exercise-induced shifts in *Roseburia* abundance raise important questions about the clinical relevance of this genus in muscle-related conditions. Given that *R. inulinivorans* has been reported to be absent in individuals with sarcopenia,[23] the *Roseburia* genus is consistently depleted in conditions associated with low muscle mass, such as cerebral palsy,[33] anorexia nervosa[34] and breast cancer, where cachexia may occur.[35] Cachexia is a complex metabolic syndrome marked by severe skeletal muscle loss and is commonly observed in chronic illnesses such as cancer. Together, these observations suggest a potential link between *Roseburia* and muscle health that warrants further investigation, highlighting *R. inulinivorans* as a promising probiotic candidate for maintaining or improving muscle strength.

Limitations

This study has several limitations that warrant consideration. First, although our human data show consistent associations between *R. inulinivorans* and muscle-related outcomes in both older and young adults, longitudinal studies are necessary to determine whether changes in *R. inulinivorans* abundance are a cause or consequence of muscle function alterations. Second, while we used a well-controlled mouse model to test causality, none of the human *Roseburia* strains achieved long-term colonisation in the mouse gut. Consequently, the observed effects are likely mediated by transient microbial exposure or metabolites rather than stable gut colonisation. Third, the use of broad-spectrum antibiotics to reduce the native gut microbiota constitutes a limitation, as it may alter host physiology and gut ecology in ways that influence the effects of the administered strains. Finally, although untargeted metabolomics allowed broad metabolic profiling, specific pathways such as those related to inflammation or neuromuscular signalling were not directly assessed and warrant further targeted investigation. The next step involves rigorous human intervention studies to determine the therapeutic potential of oral supplementation with *R. inulinivorans* in promoting muscle health, facilitating healthy ageing and combating muscle-wasting conditions.

CONCLUSIONS

Our study provides compelling evidence that *R. inulinivorans* plays an important role in muscle strength and metabolism, opening new avenues for understanding and potentially combating age-related muscle-wasting. We demonstrate that *R. inulinivorans* abundance is positively associated with muscle strength across age groups, is reduced in older compared with young adults, and that its supplementation in mice enhances grip strength and increases muscle fibre size. These effects are accompanied by a shift from type I to type II muscle fibres, independent of exercise. These effects appear to be accompanied by shifts in amino acid metabolism and activation of the purine and PPP in muscle, rather than SCFA production. Our findings highlight *R. inulinivorans* as a key player in the gut-muscle axis and suggest its potential as a novel nutraceutical intervention for preserving muscle function and mitigating sarcopenia.

MATERIAL AND METHODS

Human data

Patient and public involvement

Since the human data used in this study are secondary measurements derived from patients enrolled in two different randomised controlled trials, patients were not involved in the design of the study or in recruitment. The results will be disseminated through public news outlets at both national and international levels. In addition, some co-authors may participate in podcasts or interviews to further disseminate these findings.

Cohorts of humans where we measured muscle strength

124 sedentary adults from two independent cohorts were included here. A total of 90 young adults (64 women), aged 18–25 years, were included in the study. This study used baseline data from the ACTIBATE study,[36] an exercise-based randomised controlled trial (ClinicalTrials.gov ID: NCT02365129). All assessments were conducted in Granada, Spain, between October and November 2016. Participants reported engaging in less than 20 min of moderate-to-vigorous physical activity on fewer than 3 days per week, maintaining a stable body weight over the past 3 months (with a change of less than 3 kg) and being non-smokers. Additionally, they were not taking any medication (including antibiotics in the past 3 months), had no acute or chronic illnesses and were not pregnant. Moreover, a total of 33 older adults (17 males, aged 68.5±2.9 years) from the EFICCOM study (Clinicaltrial.gov. ID: NCT03923712) was included in the analyses. It was undertaken in Cadiz (Spain) between January and February 2020. Their inclusion and exclusion criteria mirrored those of the ACTIBATE cohort, except the age criterion, which required participants to be between 65 years and 75 years old. Participants were informed about the study procedures, and if they met the inclusion criteria and agreed to participate, they signed the informed consent. The study protocol and the written informed consent were performed following the Declaration of Helsinki, as revised in 2013. They were approved by the Human Research Ethics Committee of the University of Granada (n°924) and that of the Servicio Andaluz de Salud (Centro de Granada, CEI-Granada), as well as the Human Ethics and Research Committee of the research in Cádiz and the Andalusian Coordinating Committee on Biomedical Research Ethics (codes: 0667-M1-17 and 04/2018, respectively).

Muscular strength measurements

Muscular strength capacity was assessed using the handgrip strength test and the one-repetition maximum (1-RM) leg and bench press tests in the young cohort. Handgrip strength was measured with a Takei 5401 digital Grip-D hand dynamometer (Takei, Tokyo, Japan), with results expressed in kilograms similarly in both cohorts. Participants were instructed to squeeze the grip gradually and continuously, exerting maximal effort during each test. The test was performed twice, alternating between both hands, with a 1 min rest between attempts. For men, the grip span of the dynamometer was fixed at 5.5 cm, whereas for

women, it was adjusted based on individual hand size using a validated equation.[37] To assess lower and upper body strength, 1-repetition maximum (RM) leg and bench press tests were performed using a KEISER Air 300 pneumatic resistance machine and a KEISER Power Rack (Keiser, Fresno, California, USA), respectively. Given that participants were sedentary, the Wathen equation[38] was used to estimate 1-RM values: [*1-RM=weight lifted per repetition (kg)/((48.8+53.8e$^{-0.075 \times \text{number of repetitions}}$)/100)*]. They had three attempts to provide the needed lift data; moreover, if they failed to do it, they could return another day to repeat the test. At the beginning, several lifts with no weight were conducted for participants to familiarise themselves with the exercise. The participants were able to perform more than 10 repetitions with a particular weight. They stopped and rested for 5 min and next they followed with another try with a heavier weight. The test was finished when participants made <10 repetitions at their maximum strength capacity. Data for all muscular strength tests were saved in absolute terms. Participants were categorised into tertiles according to their handgrip strength levels (ie, low 19.25–27.50 kg; medium 27.55–32.20 kg and high 32.55–54.05 kg).

Cardiorespiratory fitness

Before carrying out a cardiorespiratory fitness test, the participants had to fulfil specific requirements: fasting for 3–5 hours, not to perform vigorous exercise in the previous 48 hours nor moderate exercise in the previous 24 hours and not to consume coffee or tea in the latter period. The cardiorespiratory fitness test was performed using a maximum treadmill exercise (H/P/Cosmos Pulsar treadmill, H/P/Cosmos Sports & Medical GmbH, Nussdorf-Traunstein, Germany), following the modified Balke protocol. The warm-up consisted of walking for 1 min at 3 km/hour, followed by 2 min at 4 km/hour. The incremental protocol started at the fourth minute, at a speed of 5.3 km/hour (0% slope). The speed was kept constant while every minute the treadmill slope was increased by 1% until the participants became exhausted. Finally, they went through a cooling-down period at 4 km/hour (0% slope) for 5 min. During the test, respiratory gas exchange (VO_2 and carbon dioxide volume (VCO_2)) was monitored by indirect calorimetry using a CPX Ultima CardioO_2 gas exchange analysis system (Medical Graphics, St Paul, Minnesota, USA), with a facemask, model 7400 plastic (Hans Rudolph, Kansas City, Missouri, USA) and equipped with a preVent metabolic flow sensor (Medical graphics, St Paul, Minnesota, USA).[39] VO_2 was measured using a galvanic fuel cell, whereas VCO_2 was measured using a non-dispersive infrared sensor.[39] The criteria for defining maximum VO_2 (VO_2max) were a respiratory exchange ratio of ≥1.1, a VO_2 plateau (changes of<100 mL/min over three consecutive 10 s intervals) and a heart rate within 10 beats per minute of the age-predicted maximum (209–0.73×age).[40] Time to exhaustion was measured in seconds. VO2 max was represented in absolute terms. Participants were categorised into tertiles according to their VO_2max capacity (ie, low: 794–2528 mL/min, intermediate: 2544–3077 mL/min and high: 3102–4811 mL/min).

Body composition assessment

Participants' weight and height were measured, without shoes and wearing standard clothes, using SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany). BMI was calculated as body weight (kg)/square of body height (m^2). Body composition was evaluated by Dual Energy X-ray Absorptiometry (HOLOGIC, Discovery Wi, Marlborough, Massachusetts, USA).

Stool collection and DNA extraction

The participants collected approximately 50 g of faecal sample in plastic sterile containers, which were transported in portable coolers to the research centre. Faecal samples were stored at −80°C until extraction of DNA. QIAamp DNA Stool Mini Kit (QIAGEN, Barcelona, Spain) was used for extraction of DNA, following manufacturer's instructions. The samples were incubated at 95°C to ensure lysis of both Gram-positive and Gram-negative bacteria. Then, DNA was quantified with a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA). Finally, DNA purity was determined by measuring the ratio of absorbance at A260/280 nm (protein contamination[41]) and A260/230 nm (salt and phenol contamination[42]).

Sequencing analysis

DNA extracted was amplified by PCR by primer pairs, 16S Amplicon PCR Forward Primer: 5'CCTACGGGNGGCWGCAG 3' (SEQ ID NO: 1) and 16S Amplicon PCR Reverse Primer: 5' GACTACHVGGGTATCTAATCC 3' (SEQ ID NO: 2) targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene.[43] All PCRs were executed in 25 µL reaction volumes incorporating 12.5 µL 2X KAPA HiFi Hotstart ready mix (KAPA Biosystems, Woburn, Massachusetts, USA), 5 µL of each forward and reverse primers (1 µM) and 2.5 µL of extracted DNA (10 ng) under the following cycling circumstances: (a) denaturation at 95°C for 3 min, (b) cycles of denaturation at 95°C for 30 s, (c) annealing at 55°C for 30 s, (d) elongation at 72°C for 30 s and (e) a final extension at 72°C for 5 min. To purify the 16S V3 and V4 amplicon away from free primers and primer dimer, AMPure XP beads (Beckman Coulter, Indianapolis, Indiana, USA) were used. Next, the index PCR attached dual indices and Illumina sequencing adapters using the Nextera XT Index Kit (Illumina, San Diego, California, USA), on a thermal cycler using the requirements previously mentioned. After AMPure XP beads (Beckman Coulter, Indianapolis, Indiana, USA) were used for purifying the pooled PCR products. The resultant amplicons were sequenced at MiSeq (Illumina, California, USA), using paired-end (2×300 nt) Illumina MiSeq sequencing system (Illumina, San Diego, California, USA).

Bioinformatics analysis

The FastQ files were analysed with the 'DADA2'[44] package in R software[45] and got 11 659 014 paired-ends with an average of 126 728±33 395 reads per sample. Cut-off 10 000 reads was surpassed for all samples. Samples were resampled to an equal sequencing depth of 30 982 reads using the 'phyloseq'[46] package in R,[45] coming back 11 158 phylotypes. The 'classifier' function from the Ribosomal Database Project (RDP) was used for assigning the taxonomic affiliation of phylotypes, based on the naïve Bayesian classification[47] with a pseudobootstrap threshold of 80%. A total of 209 genera were obtained that belong to 16 different phyla. 'seqmatch'[48] function from RDP was performed to define the discriminatory power of each sequence read with the purpose of annotating species assignments. Annotation was executed according to criteria published previously, and[49] the annotation was chosen with a threshold of ≥97% of coincidence. Microbial communities were analysed at different taxonomic levels (phylum to species), calculating relative abundances, expressed as percentages. In the faeces of these participants, it was possible to identify the *R. faecis* M72/1 (DSM 16840),

Roseburia intestinalis L1-82 (DSM 14610) and *R. inulinivorans* A2-194 (DSM 16841) species.

Access to publicly available data sets and data analyses

To validate findings on a larger scale, the inventors leveraged the curated MetagenomicData (CMD) resource (V.3.6.2).[14] This valuable resource provides taxonomic and functional profiles of more than 21 000 faecal metagenomes, processed using BioBakery tools. To ensure the robustness of our analysis, several filtering steps were applied to the metagenomic samples. Samples from individuals in the 'newborn' age category (<1 year old) and those taken while under antibiotic treatment were excluded. Additionally, samples lacking exact age information in years were removed, and in cases where multiple samples were available from the same individual, only one sample was randomly selected. Furthermore, only samples from individuals classified as 'healthy' in the CMD resource were retained. As a result, a total of 3809 stool metagenomes from healthy individuals were obtained. Focusing on the adult (19–65 years of age) and older adults (65+ years) age categories, which were of primary interest, the data were further filtered to include only samples from these specific age groups. This yielded 3257 metagenomes from the adult category and 255 metagenomes from the senior category. To address the issue of zero values in log space, a pseudocount of 1e-6 to all relative abundances was added.

Lifelines cohort

We analysed the association between age and the relative abundance of *Roseburia* species using gut microbiome data from 8229 participants in the Lifelines Dutch Microbiome Project (DMP).[50 51] Lifelines is a multidisciplinary, prospective, population-based cohort including 167 729 residents of the northern Netherlands, with a three-generation design focusing on health, behaviour and multimorbidity.[15] During the first Lifelines visit, all participants were invited to join DMP, and stool metagenomes were obtained from 8229 individuals. DMP microbiome data and phenotypes used in this study are available on European Genome-Phenome Archive (EGA, https://ega-archive.org/) as EGA study EGAS00001005027. Raw reads were processed using KneadData V.0.5.1, including adapter trimming, removal of low-quality reads (Phred ≤30) and filtering of human reads (GRCh37/hg19). Taxonomic profiles were generated using MetaPhlAn3[52] and the relative abundances *of R. intestinalis*, *R. faecis* and *R. inulinivorans* were extracted. Relative abundances were centred-log-ratio (CLR) transformed prior to analysis. Relative abundances of gut bacteria were transformed using a CLR transformation under two approaches: (a) CLR applied to all taxa and (b) CLR applied after excluding taxa with a prevalence <10%. Group differences were assessed using the Mann-Whitney U test. The meta-analysis was performed in R using the meta package (V.8.2–1). We applied the metacont() function to pool effect estimates for continuous outcomes comparing seniors (≥65 years) with adults (19–65 years) across cohorts. For each study, we calculated group-specific means, SD and sample sizes for gut bacterial measures in the senior and adult groups. The mean difference between groups was used as the effect size for pooling.

Culture and preparation of *Roseburia* species for the mouse experiment

R. faecis M72/1 (DSM 16840), *R. intestinalis* L1-82 (DSM 14610) and *R. inulinivorans* A2-194 (DSM 16841) were obtained from the Leibniz Institute DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH, Wolfenbüttel, Germany). All *Roseburia* species were cultured in a Baker Ruskinn Concept 1000 anaerobic cabinet on YCFA medium (pH6.8)[53] solidified with 1.5% bacteriological agar or in liquid YCFAC medium (Anaerobe Systems) as described.[12 13] For the preparation of inocula, a single colony from YCFA agar was inoculated into liquid medium and incubated for 16 hours at 37°C. The resulting overnight culture was diluted into fresh prereduced YCFAC medium and incubated until the culture reached mid-exponential growth phase. Then, 50 mL aliquots of culture were centrifuged in parafilm sealed tubes at 5000×g for 20 min and the culture supernatant was removed. Pellets were resuspended in 2.8 mL prereduced PBS-carbonate buffer, pooled and glycerol was added to 15% (vol/vol). 1 mL aliquots of bacterial suspension were made in 3 mL syringes (two times per day) that were sealed with a Luer lock combi lock and parafilm and stored immediately at −80°C. With the exception of the centrifugation and storage steps, the procedure was performed inside the anaerobic cabinet. The procedure was designed to minimise loss of viability due to exposure to oxygen. The number of viable bacteria administered to mice was calculated by plating the bacterial culture before, during and after preparation for administration to mice. The identity of the isolates was confirmed using gene sequencing and MALDI Biotyper (Bruker) at multiple points during the preparation.

Animal experiments

A total of 32 6-week-old male mice (C57BL/6J background, Charles River Laboratories) were group-housed at room temperature (22°C) and 12:12 hour light-dark cycle with ad libitum access to water and standard laboratory chow diet (Rat and Mouse No. 3 Breeding, SDS, Horley, UK). During the first 2 weeks all mice received a cocktail of antibiotics (200 µL; vancomycin 0.25 mg/mL, metronidazole 0.5 mg/mL, neomycin 0.5 mg/mL and ampicillin 0.5 mg/mL) diluted in sterile water by oral gavage every day to deplete their original gut bacteria. After these 2 weeks, mice were randomised to one of the four treatment groups (n=8; each group): (a) mice treated with 200 µL of PBS+15% glycerol (control mice), (b) mice treated with *R. faecis*-DSM16840 (200 µL; 2×10^8 CFU/mL), (c) mice treated with *R. intestinalis*-DSM14610 (200 µL; 2×10^8 CFU/mL) and (d) mice treated with *R. inulinivorans*-DSM16841 (200 µL; 2×10^8 CFU/mL). Vehicle control and bacterial suspensions were transplanted into mice via oral gavage three times per week for a total period of 8 weeks. For this purpose, previously produced aliquots were allowed to thaw and were kept sealed on ice until the moment of gavage. The animal experiments were carried out according to the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and were approved by the National Committee for Animal Experiments (Protocol No. 11600202010187) and by the Ethics Committee on Animal Care and Experimentation of the Leiden University Medical Centre (Protocol No. PE.18.063.005). All animal procedures conformed to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Body weight and body composition

Body weight was measured with a scale, and body composition was measured in conscious mice using an EchoMRI-100 analyser weekly (EchoMRI, Houston, Texas, USA).

Muscular strength measurement

Forelimb grip strength was measured at baseline (0 week, after the treatment with antibiotics) and at weeks 4, 6 and 8 after the treatment with different bacteria. Forelimb grip strength was assessed by means of a grip strength meter (Columbus Instruments, Ohio, USA). Mice were tested five times, with three consecutive measurements per trial (15 in total), and a 2 min interval between trials. The three highest measured values were averaged to calculate the absolute strength (in g), which was divided by the body weight in grams.

Determination of muscle fibre size and type

Transverse serial cross-sections (7 µm thick) of the soleus muscles were obtained using a cryostat (Microm HM 505 n Cryostat Microtome) maintained at −25°C and were mounted onto glass microscope slides. Sections were stained with H&E to measure fibre CSA, and with NADH for fibre typing using established histological techniques. Morphometry of musculoskeletal fibres was performed on a Leica DMI8 microscope using a Leica MC170 HD camera, controlled by the LAS-X imaging software. Images were taken at 20× magnification, and mosaics were generated to capture the entire CSA of each muscle. All muscle fibres of the sections were quantified (an average of 850 fibres per sample) and their CSA was calculated in μm^2 using FIJI (ImageJ) software. Fibre typing was based on NADH staining intensity: dark blue fibres were classified as type I (high NADH activity), while the remaining fibres were considered type II. Quantification of fibre type areas was also performed using FIJI.

Quantification of SCFAs and amino acid levels in the caecum

The method for the nuclear magnetic resonance (NMR) analysis of caecum samples was adapted from the protocol developed by Kim *et al* for faecal samples with a few changes.[54] The mass of each caecum sample (about 200 mg) was carefully measured prior to the sample preparation. To each sample tube, 50 µL of 0.5 mm zirconium oxide beads (Next Advance) ceramic beads and 500 µL of pH 7.4 potassium phosphate buffer (0.15 M) containing 0.2 mM NaN_3 were added. Then, the tubes were subjected to bead beating for 30 s. The tubes were subsequently centrifuged at 18 000 g at 4°C for 15 min. 300 µL of supernatant was transferred to new 1.5 mL Eppendorf tubes. These tubes were centrifuged at 18 000 g at 4°C for 1 hour. 225 µL of supernatant was added to 25 µL of 100% D_2O containing 4 mM TSP-d_4 and 6 mM dimethylsulfone. A customised Gilson 215 liquid handler was used to transfer the samples to a 3.0 mm Bruker NMR tube rack. ^1H NMR data were collected using a Bruker 600 MHz Avance Neo/IVDr spectrometer equipped with a 5 mm TCI cryogenic probe head and a z-gradient system. A Bruker SampleJet sample changer was used for sample insertion and removal. All experiments were recorded at 300 K. A standard sample 99.8% methanol-d4 was used for temperature calibration before each batch of measurements.[55] One-dimensional (1D) ^1H NMR spectra were recorded using the first increment of a NOESY pulse sequence[56] with presaturation (γB_1=50 Hz) during a relaxation delay of 4 s and a mixing time of 10 ms for efficient water suppression.[57] Initial shimming was performed using the TopShim tool on a sample pool from the study, and subsequently the axial shims were optimised automatically before every measurement. Duration of 90° pulses was automatically calibrated for each individual sample using a homonuclear-gated mutation experiment[58] on the locked and shimmed samples after automatic tuning and matching of the probe head. 128 scans of 65 536 points covering 12 335 Hz were recorded. The Free Induction Decay of the 1D experiment was zero-filled to 65 536 complex points prior to Fourier transformation. An exponential window function was applied with a line-broadening factor of 0.3 Hz. The spectra were automatically phase and baseline corrected and automatically referenced to an internal standard (TSP=0.0 ppm). Metabolites were quantified in a select number of spectra using the Chenomx, PO Box 86, Edmonton Main, Edmonton AB, T5J 2G9, Canada, and by fitting the remaining spectra automatically in the KIMBLE environment.[59] The areas were converted to concentrations using the dimethylsulfone internal standard.

Untargeted metabolomics in plasma and muscle tissue

Plasma and gastrocnemius muscle were shipped to Beijing Genomic Institute (BGI, Beijing, China) for performing untargeted metabolomics. In short, BGI preserved the samples and performed metabolite extraction and bioinformatic analyses. First, samples that had been stored at −20°C were thawed in a 4°C refrigerator until no visible ice remained. 100 µL of each sample, including the quality control (QC) samples, was added to individual Eppendorf tubes, and the remaining samples were kept frozen. A solution containing the internal standard (methanol: acetonitrile: water=4:2:1, v/v/v) was added (700 µL) to each sample (including QC samples), followed by shaking for 1 min and placement in a −20°C freezer for 2 hours. The samples were centrifuged at 25 000 g and 4°C for 15 min. The supernatants (600 µL) were transferred to new Eppendorf tubes. Drying was performed using a drying machine. A mixture of acetonitrile and pure water (7:3 v/v) was added (100 µL) to reconstitute the samples, which were vortexed for 10 min until pellets were fully dissolved. The reconstituted samples were centrifuged again at 25 000 g and 4°C for 15 min, and supernatants were again transferred to new Eppendorf tubes. Second, after importing the off-line data of mass spectrometry (MS) into Compound Discoverer 3.2 (Thermo Fisher Scientific, Delaware, USA) software and analysing the MS data in combination with bmdb (BGI metabolome database), mzcloud database and chemspider online database, a data matrix containing information such as metabolite peak area and identification results was obtained. Third, in the data postprocessing step, probabilistic quotient normalisation (PQN) was used to normalise the data and obtain relative peak areas. Quality control-based robust Locally Estimated Scatterplot Smoothing (LOESS) signal correction (Quality Control–based Robust LOESS Signal Correction, (QC-RLSC)) was employed to correct batch effects and remove metabolites with high variability in their relative peak areas. PQN involved obtaining an overall reference vector and correcting the actual sample based on the reference vector, while QC-RLSC used local polynomial regression fitting to correct experimental sample signals using QC samples. Quality control was assessed using principal component analysis (PCA), a statistical method for analysing multidimensional data. Log transformation and Pareto scaling were applied to compute principal components, and the resulting PCA plot was used to observe sample group separation and identify abnormal samples. The variability within and between groups was also examined. The overall analysis involved the functional annotation of metabolites using databases such as the Human Metabolome Database and KEGG PATHWAY. These annotations helped to understand the properties and metabolic pathways associated with the identified metabolites. Partial least squares-discriminant analysis (PLS-DA) was used to screen for differences between groups. This supervised method established a model between metabolite expression and sample categories to predict sample categories. A PLS-DA model was established between the

comparative analysis groups, and log2 transformation was applied with Pareto scaling and cross-validation for model validation.

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Ethics approval This study involves human participants and was approved by the Human Research Ethics Committee of the University of Granada (n°924) and that of the Servicio Andaluz de Salud (Centro de Granada, CEI-Granada), as well as the Human Ethics and Research Committee of the research in Cádiz and the Andalusian Coordinating Committee on Biomedical Research Ethics (codes: 0667-M1-17 and 04/2018, respectively). Participants gave informed consent to participate in the study before taking part.

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