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ARTICLE

The associations of butyrate-producing bacteria of the gut microbiome with diet quality and muscle health

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ABSTRACT

This study aimed to investigate the relationships between diet quality, the relative abundance of butyrate-producing bacteria of the gut microbiome and muscle mass, strength and function. In this cross-sectional study, $n = 490$ men (64.4 ± 13.5 years) from the Geelong Osteoporosis Study provided food frequency questionnaire data, from which the Australian Recommended Food Score (ARFS) and Dietary Inflammatory Index (DII) score were calculated. Muscle mass (skeletal muscle index from DXA-derived lean mass), muscle strength (handgrip strength) and muscle function (Timed Up-and-Go test) were measured. Participants provided stool samples for 16S rRNA gene sequencing. There was no evidence of associations between alpha or beta diversity and muscle health measures. A healthier ARFS score was positively associated with the relative abundance of butyrate-producing bacteria (β 0.09, 95%CI 0.03, 0.15) and a higher (pro-inflammatory) DII score was associated with lower relative abundance of butyrate-producing bacteria (β −0.60, 95%CI −1.06, −0.15). The relative abundance of butyrate-producing bacteria was positively associated with healthier muscle mass, strength and function; however, these relationships were attenuated in multivariable models. These findings support the role of diet quality in achieving a healthier gut microbiome, however, further evidence is required for a gut-muscle axis in humans.

Keywords: Microbiome; diet quality; muscle health; Sarcopenia; butyrate

Introduction

Alterations in the gut microbiome have been implicated in systemic conditions related to poor muscle health, such as frailty and obesity (Ticinesi et al., 2017). Based on findings in animal and human studies a gut-muscle-axis has been proposed, suggesting that bacteria in the gut and/or their associated metabolites play a role in regulating muscle health (Grosicki et al., 2018). Given the impact that diet has on the gut microbiome and muscle health, diet is a likely contributor to the gut-muscle-axis (Donovan, 2017).

Evidence for a role of the gut microbiome in muscle health has emerged from both human and animal studies. Germ-free mice demonstrate reduced skeletal muscle mass and muscle function, with muscle mass returning to normal levels following inoculation with faecal microbiome from wild-type mice (Lahiri et al., 2019). Prebiotic and probiotic supplementation in wild-type mice has also resulted in increased muscle mass, strength and changes in muscle fibre composition (Chen et al., 2016; Everard et al., 2011). In humans, a prebiotic fibre trial in older participants reported increased muscle strength (Buigues et al., 2016). An observational study also reports relationships between frailty and a greater abundance of *Bacteroides* and lower levels of beneficial metabolites (Claesson et al., 2012) and men with high and low muscle mass appear to differ in the abundance of several bacterial species in the gut microbiome (Barger et al., 2020). However, evidence of alpha-diversity (the bacterial diversity within individual participants) associating with muscle health is lacking (Barger et al., 2020).

While a dearth of evidence demonstrates mechanisms by which this gut-muscle-axis may function, one potential pathway is via the actions of short-chain fatty acids (SCFA), which are produced by certain bacteria of the gut microbiome (Morrison and Preston, 2016). Bacteria in the gut ferment non-digestible dietary fibres and the SCFAs, primarily acetate, propionate, and butyrate, are produced as byproducts (Ríos-Covián et al., 2016). Microbiome-derived butyrate has several distinct roles for the intestinal epithelium, being an energy source for colonocytes, regulating mucosal homeostasis and exerting anti-inflammatory effects on the intestinal environment (Canani et al., 2011), functions which may have protective properties for gut conditions such as colon cancer and inflammatory bowel disease (Bach Knudsen et al., 2018; Couto et al., 2020). The anti-inflammatory role of butyrate is of interest in the gut-muscle-axis as peripheral inflammatory markers appear to play a causal role in age-related declines in muscle health (Michaud et al., 2013). A growing body of research suggests that systemic inflammation impacts muscle health at the level of both neural and muscle tissue, with pro-inflammatory cytokines interfering in processes including muscle protein synthesis and disposal of dysfunctional mitochondria (Salminen et al., 2012). Direct evidence for butyrate's role in regulation of muscle health is emerging. An observational study reported that men with higher muscle mass and fibre intake exhibited greater abundance of butyrate-producing bacteria compared to participants with the lowest muscle mass and fibre intake (Barger et al., 2020). An exercise trial reported increases in butyrate-producing bacteria, faecal butyrate levels, and lean mass in participants who were lean but not those with obesity (Allen et al., 2018). More compellingly, the recent NU-AGE trial, comprising a large-scale trial of a Mediterranean-style diet in older adults over one year, reported that the intervention changed microbiota composition, with an increased relative abundance of butyrate-producing bacteria. These microbiota changes were in turn associated with reduced frailty, including better muscle strength, as well as lower inflammatory markers (Ghosh et al., 2020).

Colonic butyrate levels can be modified by the diet (Flint, 2012), with fibre the preferred substrate for butyrate-producing bacteria. In indirect evidence for butyrate's role in muscle health, fibre supplementation has increased muscle mass in mice (Cani et al., 2009), and increased glucose metabolism in human muscle tissue (Robertson et al., 2012). Rather than measuring fibre intake, higher scores on various diet quality indices also have associated with increased SCFAs and muscle health improvements (Davis et al., 2021; Ruiz-Saavedra et al., 2020), potentially due to cross-feeding by members of the gut microbiome (Rivière et al., 2016). Diet quality indices can provide a measure of adherence to dietary guidelines, dietary diversity, nutritional recommendations for management of health conditions, or functional outcomes.

The role of diet quality in the abundance of butyrate-producing bacteria is yet to be investigated. Although there is evidence from a pre-clinical trial of butyrate attenuating declining muscle health

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through its anti-inflammatory properties (Walsh et al., 2015), and evidence from human trials suggesting that improvements in diet quality can positively influence strength via changes to the gut microbiota (Ghosh et al., 2020), population studies assessing the association between butyrate-producing bacteria and muscle health are lacking. The current study aims to assess the relationships between diet quality, butyrate-producing bacteria, and muscle health in a large cohort of randomly selected Australian men. We hypothesised that (1) diet quality increases would be accompanied by increases in the relative abundance of butyrate-producing bacteria, and (2) bacterial diversity and the relative abundance of butyrate-producing bacteria would be positively associated with measures of muscle health.

Methods

Study design and participants

This cross-sectional study utilised data from the Geelong Osteoporosis Study (GOS), a prospective cohort study that has been collecting men's data since 2001. Details regarding participant recruitment, data collection and participation rates have been published previously (Pasco et al., 2012). In brief, participants were randomly identified from the electoral roll for the Barwon Statistical Division in South-Eastern Australia and invited to participate. The current study is based on data from the GOS men's 15-year follow-up, conducted from 2016 to 2020. Inclusion criteria for the entire GOS cohort (men and women) was a listing on the electoral roll for the Barwon Statistical Division, and exclusion criteria were residence in the region for <6 months and inability to provide written informed consent. Inclusion criteria for the current study included the supply a stool samples and at least one concurrent measure of muscle health including handgrip strength (HGS), dual-energy X-ray absorptiometry (DXA) lean mass and/or Timed Up-and-Go (TUG) test. Of the $n = 625$ men who completed the 15-year follow-up assessments, $n = 490$ (78 %) fulfilled these criteria. Of these, five were excluded due to use of a colostomy bag ($n = 1$), prior colon surgery ($n = 1$), unidentifiable samples ($n = 2$) and one identified as a second sample submitted by a participant. Written, informed consent was provided by all participants and approval was provided by the Human Research Ethics Committee at Barwon Health.

Muscle health measures

Muscle mass

Body composition data were collected from whole-body DXA using a densitometer (Lunar Prodigy-Pro, LUNAR Corporation, Madison, WI). From these, appendicular lean mass was used as a surrogate measure for skeletal muscle mass, which provided the muscle data to calculate skeletal muscle index (SMI) (kg/m^2). The cut-off value for low SMI was <7.0 kg/m^2, aligning with the 2019 European Working Group on Sarcopenia in Older People (EWGSOP2) guidelines for low muscle quantity for men (Cruz-Jentoft et al., 2019).

Muscle strength

HGS was employed as an indicator of muscle strength (Roberts et al., 2011). A hand-held dynamometer (Vernier, LoggerPro3, Beaverton, OR) was used to measure handgrip, with participants seated and holding the dynamometer with the elbow flexed at 90°. Participants squeezed the dynamometer for several seconds on both sides, and the maximum reading for each hand was recorded. The values used herein are the mean of these two maximal values. The cut-off value for low muscle strength was identified as <27 kg, as per EWGSOP2 guidelines for low muscle strength in men (Cruz-Jentoft et al., 2019).

Muscle function

Muscle function was measured with the TUG test (Richardson, 1991), which times participants in seconds as they rise from a seated to a standing position without the use of arm rests, walk 3 m, turn

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around and walk back to their original standing position, before sitting down. This test is performed without the use of the upper body, unless the participant requires a walking aid. The cut-off value for poor muscle function was identified as ≥20 seconds, as per EWGSOP2 guidelines for low muscle performance (Cruz-Jentoft et al., 2019).

Microbiome analysis

At GOS appointments, participants were invited to provide a stool sample using the Omnigene®•gut stool home collection kit (DNA Genotek, Ottawa, ON). The kit contained a nucleic acid stabilisation liquid and, after the sample was collected and mixed by the participant, it was returned to the research centre via reply-paid post. Upon receipt, samples were aliquoted and stored at -80^0 within 24 hours. Microbial genomic DNA was extracted from the stool aliquot using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Manchester, UK). Extractions were conducted according to manufacturer's guidelines, with an additional mechanical lysis step using PowerBead tubes (Cat No 13123, QIAGEN). DNA quality and quantity were assessed using the Nanodrop 1000 (Thermo Scientific). Samples were diluted to concentrations of 10 ng/μL and freighted to the Australian Genomic Research Facility for 16S rRNA gene sequencing and taxonomic assignment.

Sequence read processing

The V3–V4 region of the 16S rRNA gene was amplified by polymerase chain reaction using 341-Forward and 806-Reverse primers. Sequencing was conducted using the Illumina MiSeq platform. Bioinformatic processing was performed with QIIME 2 2019.7 (Bolyen et al., 2019). The demultiplexed raw reads were primer trimmed and quality filtered using the cutadapt plugin followed by denoising with DADA2 (Callahan et al., 2016) (via q2-dada2). Taxonomy was assigned to amplicon sequence variants (ASV) using the classify-sklean method from the feature-classifier plugin (Bokulich et al., 2018) using naïve Bayes classifier. Sequence reads were 12,572–378,206 reads per sample, and a mean read count of 67,429. ASVs were then matched to the SILVA database to identify the phylum, class, order, family, genus and species (where available). A preliminary batch of $n = 161$ samples from this cohort was originally sequenced and analysed in 2017 (Davis et al., 2020). In the current study the sequence reads from these $n = 161$ samples were re-analysed for ASV taxonomic distribution and matched to the SILVA database, along with the remaining $n = 329$ samples. Quality control samples from the original and second batches were included in final sequencing to ensure good reproducibility.

Microbiome data pre-processing was conducted in line with recommendations from Callahan et al. (2016). In brief, zero count ASVs, uncharacterised taxa, and non-bacterial taxa such as chloroplast and mitochondria were removed. For beta diversity and butyrate analyses, low prevalence taxa present in less than 1 % of samples were removed.

Alpha and beta diversity

Alpha-diversity was assessed by the Shannon Index and Observed taxa metrics. The Shannon Index is a measure of both richness and evenness of taxa, while Observed taxa is a count of unique ASVs in each sample. Beta diversity analyses compared the microbial community structure between groups dichotomised as high/low for SMI, TUG and HGS. Beta diversity was quantified with both weighted and unweighted UniFrac distances, and a permutational analysis of variance (PERMANOVA) was performed utilising 999 permutations. Clustering was visually inspected using principal coordinates analysis.

Relative abundance of butyrate-producing bacteria

Due to limitations in database species identification following 16S sequencing, butyrate-producing bacteria were identified *a priori* from details of SCFA measures in *in vitro* bacterial cultures (Alessi et al., 2020; Barcenilla et al., 2000; Duncan et al., 2002; Holmstrøm et al., 2004; Louis et al., 2004;

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Reichardt et al., 2014; Schwiertz et al., 2002; Shetty et al., 2018), and then confirmed through details provided by Bergey's Manual of Systematics of Archaea and Bacteria (Whitman and John Wiley & Sons, 2015). The bacteria identified as butyrate-producing were *Faecalibacterium prausnitzii, Subdoligranulum variabile, Anaerobutyricum hallii, Anaerostipes caccae, Anaerostipes hadrus, Coprococcus catus, Clostridium* sp., *Coprococcus* sp., *Eubacterium rectale, Roseburia faecis, Roseburia intestinalis* and *Roseburia inulinivorans*. These species were agglomerated to the genus level and subsequently combined to form a composite variable comprising the relative abundance of all genera combined for a total relative abundance of butyrate-producing bacteria. These values were centre log-ratio (CLR) transformed to account for the compositional nature of microbiome data (Gloor et al., 2017).

Dietary data

The Australian Cancer Council's Dietary Questionnaire for Epidemiological Studies (DQES) was used to collect dietary data; this questionnaire has been validated in a similar population (Giles and Ireland, 1996). The DQES collects information on quantity and frequency of consumption of 74 foods and six alcoholic beverages in the preceding 12 months.

A priori diet quality scores

The Australian Recommended Food Score (ARFS) and the Dietary Inflammatory Index (DII©) were calculated from the DQES to measure diet quality. The ARFS provides a diet quality score based on components of the 2008 Australian Dietary Guidelines, and therefore quantifies adherence to these guidelines. Points are awarded for consumption of (1) vegetables, nuts, and beans, (2) fruit, (3) protein containing foods, (4) grains, (5) dairy, (6) fats, and (7) alcohol, the total of which provides the participant's ARFS, with scores ranging from a theoretical 0 to 74 (Collins et al., 2015). The DII, in contrast, measures the inflammatory potential of the diet and includes up to 45 food and nutrition components (Shivappa et al., 2013). The DQES provides only 22 of these components; so, the DII score used herein has been modified by the original authors to reflect the effect of these 22 components. The DII is based on an exhaustive literature search resulting in 45 components directly associated with changes in inflammatory biomarkers. DII scores range from a theoretical −8.87 to +7.98, with a positive score reflecting a pro-inflammatory diet and a negative score reflecting an anti-inflammatory diet. The ARFS and DII have both been validated in similar populations (Collins et al., 2015; Tabung et al., 2015).

Potential confounding variables

Extensive demographic, lifestyle and medical data were collected for consideration as potential confounders. Area-based socio-economic status was measured with the Index of Relative Socio-economic Advantage and Disadvantage, which accounts for social and economic circumstances for both participants and their households and is considered a measure of both relative advantage and disadvantage. Other variables considered included DXA whole-body fat mass (g), self-reported age, ethnicity, education (never attended school, primary school, some secondary school, completed secondary school, TAFE/trade/apprenticeship, university or other post-secondary qualification) and current smoking status. Self-reported physical activity (PA) level was quantified through administration of Baecke's Physical Activity Questionnaire (BPAQ) (Baecke et al., 1982). The BPAQ is a Likert scale of PA during work, sport, and leisure time, and these three scores were combined for a total PA score. A Likert scale was also used for self-reported presence of stomach symptoms (nausea, gas or indigestion), and intestinal symptoms (constipation, loose bowels or diarrhoea). These responses were then collated to create variables for presence of stomach symptoms and/or intestinal symptoms.

A composite, binary variable was developed for self-reported current medical conditions that may affect muscle health, including osteoarthritis, rheumatoid arthritis, hypoglycaemia, Parkinson's disease, multiple sclerosis, emphysema, chronic bronchitis, other lung disease, chronic fatigue syndrome,

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anaemia and pernicious anaemia. A similar composite variable was also developed based on self-reported medical conditions that may affect the gut, including chronic gastritis, hiatus hernia/oesophageal reflux, peptic ulcer disease, gastric surgery, anorexia nervosa, bulimia, bowel surgery, malabsorption, chronic diarrhoea, irritable bowel syndrome, inflammatory bowel disease and coeliac disease.

A composite, binary variable was developed for current use of medications that may affect muscle, which included anti-rheumatoid agents, muscle relaxants, neuromuscular agents, adrenal steroid, gonadal, and pituitary hormones, insulin preparations, hypoglycaemic agents and anabolic agents. A composite variable of medications that may affect the gut included reflux medications, laxatives, antidiarrheals, digestive supplements, hypoglycaemic agents, antibiotics, narcotic analgesics and antipsychotic and antidepressant medications in light of their well-documented effect on the human gut (Maier and Typas, 2017).

Important contributing factors were identified using causal directed acyclic graphs, and minimum adjustment sets were generated for each hypothesis (Supplementary Figures S1 and S2). Potential confounders included in the diet quality and butyrate-producing bacteria model included age, smoking, PA, medications that may affect the gut, and intestinal symptoms. The models investigating (1) alpha- and beta-diversity -muscle health and (2) butyrate producing bacteria-muscle health relationships included adjustment for age, smoking, the ARFS, DII, PA, medical conditions that may affect the gut and medical conditions that may affect muscle health. Potential confounding variables were then verified using correlation matrices (Supplementary Table S2). Batch effects were also added to both models to account for any impact of the two sequencing batches.

Statistical analyses

Linear regression was used to assess the relationships between (1) diet quality and butyrate producers, (2) alpha-diversity and muscle health outcomes, and (3) butyrate producers and muscle health outcomes. Collinearity between independent and dependent variables and demographics, health, and lifestyle factors were further investigated using a correlation matrix (Supplementary Table S2). Correlations between variables were tested using Pearson's test for continuous variables, Spearman's for categorical variables and point-biserial for a combination of continuous, categorical or binary variables. Deviation from model assumptions, including normal distribution of residuals, was visually inspected including residuals versus fits plot to detect non-linearity, unequal error variances, and outliers, and normal Q–Q plot, and correlation coefficients were used to assess potential collinearity. Significance was set at $p < 0.05$.

Exploratory analyses were conducted with data-driven definitions for muscle health categories. SMI was categorised into tertiles due to its normal distribution. HGS and TUG scores were skewed; thus high/low HGS was dichotomised at 45 kg, and high/low TUG dichotomised at 10 seconds, which were the values at which the distribution visually dropped (Supplementary Figure S3). These data-driven cut-offs were applied in exploratory analyses for beta diversity. Levene's test for homogeneity of variance was conducted following any significant findings for beta-diversity. Further exploratory age-stratified analyses also were conducted, with age dichotomised at the mean (<64.4 years and ≥64.4 years).

Analyses were conducted using R (version 3.6.3; R Core Team, 2013), and microbiome analyses were done using the *phyloseq* (version 1.30.0) package (McMurdie and Holmes, 2013). The analysis plan was preregistered at Open Science Framework (https://osf.io/6wsfe). Deviations from this preregistration included the use of Observed instead of Chao1 taxa metrics for alpha-diversity. This was due to filtering of singletons during bioinformatic processing, which rendered the Chao1 index estimate unreliable. A composite measure of butyrate-producing bacteria identified at genus level was used, since they were not consistently identified to species level. Additional exploratory analyses included beta-diversity using data-driven cut-off scores for muscle health outcomes, and investigation of age interaction in regression models.

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Results

Participant characteristics

Participant characteristics are summarised in Table 1. Participants were men and had an age range of 33–96 years with a mean 64.4 ± 13.6 years, and were all educated at least to primary school level, with 51.3% currently working and 42.5% retired. The majority were current non-smokers, approximately half were taking medications that may impact the gut microbiome, and 37 and 44 % of participants had medical conditions that may affect the gut microbiome or muscle health, respectively. Very few had low muscle mass (SMI < 7.0 kg/m^2, 6.0%), strength (HGS <27 kg, 2.1%) or function (TUG ≥20 seconds, 1.2%) as defined by the pre-defined clinical cut-offs. The ARFS ranged from 7 to 55, with the median slightly below the mid-point (31 from a potential total of 71), and the DII scores ranged from −2.92 to +2.53 with a median of 0.18.

Compared to the current study sample, participants who completed the GOS 15-year follow-up assessment, but did not provide stool samples, were younger, with a median age of 54 years. A similar proportion had low SMI (6.3%), but a marginally greater proportion had low HGS (7.4%) and slow TUG time (3.2%) compared to the current sample. Median ARFS scores were similar (30, IQR 25, 35) and DII scores were marginally more pro-inflammatory (0.25, IQR −0.74, 1.20) when compared to the current sample.

Butyrate-producing bacteria

All genera of butyrate-producing bacteria included in the composite variable were members of the *Clostridiales* order, with two from the *Ruminococcaceae* family, four from *Lachnospiraceae*, and the genus with the lowest counts was from the *Eubacteriaceae* family. The taxonomy and median relative abundance for each of the individual genera are summarised in Table 2.

Associations between diet quality indices and butyrate-producing bacteria

Both a healthy ARFS and an anti-inflammatory diet (as defined by a negative DII score) were associated with increased relative abundance of butyrate-producing bacteria (Table 3 and Figure 1). An increase of one point on the ARFS was associated with a 0.12 increase in the CLR transformed relative abundance of butyrate-producing bacteria (95%CI 0.07, 0.18), and the overall model fit was $R^2 = 0.03$. A one-point decrease on the DII (ie less inflammatory diet), was associated with a 0.80 increase in the CLR transformed relative abundance of butyrate-producing bacteria (95% CI −1.27, −0.34), with an overall model fit of $R^2 = 0.02$. These associations were retained following adjustment for age, smoking, PA, medications that may affect the gut, intestinal symptoms, and potential batch effects [ARFS (β 0.09, 95% CI 0.03, 0.15), DII (β 0.60, 95% CI −1.06, −0.15)]. The overall model fit for both the ARFS and DII adjusted models was $R^2 = 0.11$.

16S rRNA bacterial diversity

The most common phylum present in the gut microbiome was Firmicutes (relative abundance 54.5% ± SD 0.2%), followed by Bacteroidetes (37.4% ± 0.2%), Proteobacteria (5.3% ± 0.1%), Verrucomicrobia 1.3% (0.0%), and Actinobacteria 0.7% (0.0%). No differences in alpha-diversity were observed (Shannon Index or Observed taxa) for continuous measures of SMI, HGS or TUG (Supplementary Figure S4 and Supplementary Table S1). Similarly, there was no distinct clustering for beta diversity in the low versus high categories for SMI or TUG. However, there was weak evidence of a difference between low and high HGS groups in unadjusted models for unweighted UniFrac distance ($p = 0.081$) (Supplementary Figure S5). There was no evidence of differences in multivariate dispersion (variance) between the groups [$F(1,482) = 0.73$, $p = 0.40$].

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Table 1. Study sample characteristics.

	Participants ($n = 485$)
Age, years, mean (±SD)	64.4 (13.6)
Education[a]	
Primary or some secondary school, *n* (%)	118 (24.5)
Completed secondary or vocational training, *n* (%)	200 (41.0)
Tertiary education, *n* (%)	166 (34.5)
Employment	
Working, *n* (%)	249 (51.3)
Not working, *n* (%)	9 (1.9)
Home duties, *n* (%)	4 (0.8)
Student, *n* (%)	3 (0.6)
Retired, *n* (%)	206 (42.5)
Unable to work, *n* (%)	6 (1.2)
Not applicable, *n* (%)	8 (1.7)
PA score, median (IQR)	6.0 (5.0, 8.0)
Current smoker, *n* (%)	30 (6.2)
Medications that may affect the gut, *n* (%)	222 (45.8)
Medications that may affect muscle, *n* (%)	54 (11.1)
Medical conditions that may affect the gut, *n* (%)	179 (36.9)
Medical conditions that may affect muscle, *n* (%)	211 (43.5)
Body fat (kg), median (IQR)	24.4 (18.8, 31.5)
SMI (kg/m^2), mean (±SD)	8.5 (0.9)
Low SMI <7.0 kg/m^2, *n* (%)	29 (6.0)
HGS (kg), median (IQR)	38.4 (32.9, 43.1)
Low HGS <27 kg, *n* (%)	10 (2.1)
TUG (seconds), median (IQR)	8.6 (7.6, 9.9)
Slow TUG ≥20 seconds, *n* (%)	6 (1.2)
ARFS, mean (±SD)	31.2 (9.4)
DII, median (IQR)	0.18 (−0.77, 1.10)
Energy intake (kJ/d), median (IQR)	7,652 (6,075, 9,501)
Daily protein (g/day), median (IQR)	88.5 (68.0, 110.4)

Note: n = 485.
Abbreviations: ARFS, Australian Recommended Food Score; DII, Dietary Inflammatory Index; HGS, handgrip strength; PA, physical activity; SMI, Skeletal Muscle Index; TUG, Timed Up-and-Go.
[a]$n = 1$ missing data.

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Table 2. Relative abundance of butyrate producing bacteria.

Taxonomy	Relative abundance (IQR)
Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *Faecalibacterium*	9.6% (4.8, 18.1)
Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *Subdoligranulum*	1.3% (0.6, 2.7)
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Roseburia*	2.2% (1.1, 3.6)
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Coprococcus 2*	0.2% (0.0, 1.1)
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Anaerostipes*	0.2% (0.1, 0.4)
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Coprococcus 3*	0.1% (0.1, 0.2)
Firmicutes; Clostridia; Clostridiales; Eubacteriaceae; *Eubacterium*	0% (0.0, 0.0)

Table 3. Linear regression results for the Australian Recommended Food Score and the Dietary Inflammatory Index and associations with butyrate producing bacteria.

	β	95%CI	R^2
Australian Recommended Food Score			
Model 1	0.12**	0.07, 0.18	0.03
Model 2	0.09*	0.03, 0.15	0.11
Dietary Inflammatory Index			
Model 1	−0.80**	−1.27, −0.34	0.02
Model 2	−0.60*	−1.06, −0.15	0.11

Note: Model 1 unadjusted, Model 2 adjusted for age, gut medications, intestinal symptoms, PA, smoking and batch effects.
*$p < 0.05$;
**$p < 0.001$.

Due to the very small numbers in the low-muscle-health grouping, additional exploratory analyses were performed utilising data-driven cut-off points for muscle health. These showed no evidence of beta diversity clustering between SMI or HGS categories. Furthermore, the weak difference observed between high/low HGS groups with the original cut-offs was not strengthened with data-derived cut-offs. However, while no clustering was evident visually in principal coordinates analysis, a significant difference was found between high- and low-TUG participants in unadjusted models [unweighted UniFrac distance, PERMANOVA, Sum of squares (SS) = 0.42, $R^2 = 0.005$, $p = 0.003$, weighted UniFrac distance, PERMANOVA, SS = 0.22, $R^2 = 0.005$, $p = 0.011$]. Multivariate dispersion did not differ between groups [$F(1,479) = 0.20$, $p = 0.65$].

Associations between butyrate-producing bacteria and muscle mass, strength and function

In unadjusted models, higher relative abundance of butyrate-producing bacteria was positively associated with better measures of SMI and HGS, and shorter times for TUG. For every unit increase in CLR transformed butyrate-producing bacteria, SMI increased by 0.02 kg/m^2 (overall model fit $R^2 = 0.03$), HGS increased by 0.003 kg m^2 (overall model fit $R^2 = 0.05$), and TUG time reduced by −0.003 seconds m^2 with an overall model fit $R^2 = 0.03$ (all $p < 0.001$). All associations were attenuated slightly following adjustment for covariates (Table 4).

A potential age interaction for HGS, or TUG, was further explored with age-stratified at <64.4 years and ≥64.4 years for HGS and TUG. However, no age interaction was evident, with similar coefficients between the age groups for each of the muscle outcomes (Supplementary Figure S6).

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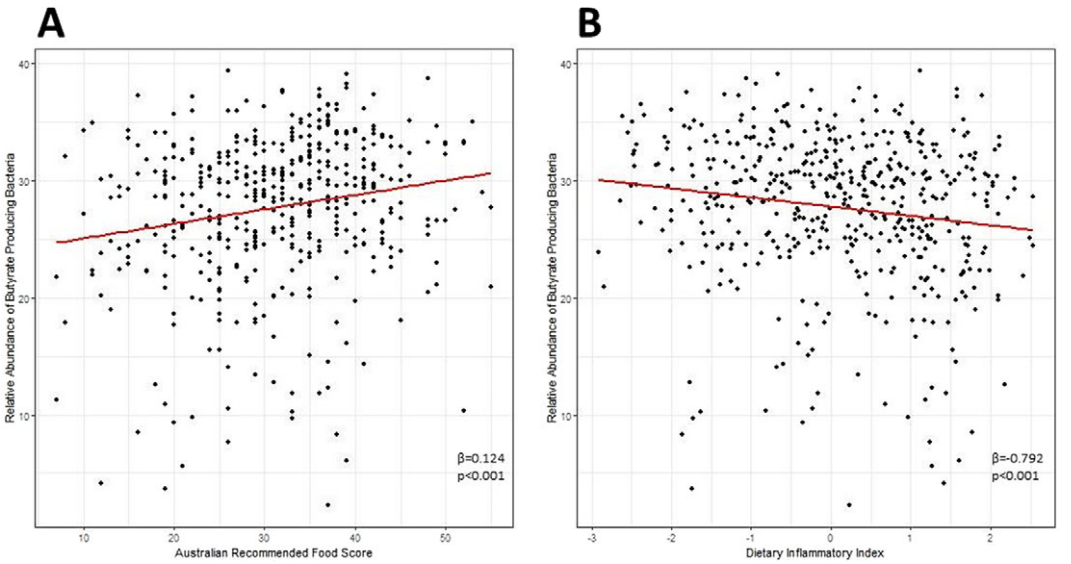

Figure 1. Associations between diet quality indices and the relative abundance of butyrate producing bacteria. (A) The Australian Recommended Food Score is positively associated with the relative abundance of butyrate producing bacteria [0.12 (95% CI 0.07, 0.18)], and (B) the Dietary Inflammatory Index is negatively associated with the relative abundance of butyrate producing bacteria [−0.80 (95% CI −1.27, −0.34)].

Table 4. Regression results for associations between butyrate-producing bacteria and muscle mass, strength and function outcomes.

	β	95%CI	R^2	Covariates found to attenuate the relationship
Skeletal muscle index				
Model 1	0.02*	0.01, 0.04	0.03	
Model 2	0.01	−0.01, 0.02	0.30	ARFS (diet quality) and PA
Handgrip strength (log10 transformed)				
Model 1	0.01*	0.004, 0.009	0.05	
Model 2	0.002	−0.0001, 0.004	0.37	Age, PA and muscle medical conditions
Timed Up-and-Go (log10 transformed)				
Model 1	−0.01*	−0.01, −0.004	0.03	
Model 2	−0.003	−0.01, 0.00	0.37	Age

Note: Model 1 unadjusted, Model 2 adjusted for age, fat mass, Australian Recommended Food Score, gut medical condition, muscle medical condition, PA, smoking and batch effects.
*$p < 0.001$.

Discussion

The current study investigated the relationships between diet, butyrate-producing bacteria and muscle mass, strength and function in a group of Australian men. We showed that a healthy ARFS score and an anti-inflammatory diet were positively associated with the relative abundance of butyrate-producing bacteria. There was also weak evidence for differences in beta-diversity of those with high and low muscle strength. While a higher relative abundance of butyrate-producing bacteria was associated with better measures of all muscle indices, the effect sizes were small and the relationships were marginally attenuated after adjustment.

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Our findings that measures of diet quality – a healthy Australian and an anti-inflammatory diet – were related to an increased abundance of butyrate-producing bacteria supports previous work in this area. A study investigating the ARFS and the gut microbiome (Harbison et al., 2021) reported that a healthier ARFS was associated with the relative abundance of members of the *Bacteroides* and *Lachnospiraceae* genera, and that these abundances differed between healthy controls and participants with type 1 diabetes mellitus. Diet quality has also been associated with higher concentrations of SCFA and/or greater abundance of bacterial species related to desirable health outcomes, such as *Akkermansia muciniphila* and *Lactobacillus* (Ruiz-Saavedra et al., 2020). Ruiz-Saavedra et al. also reported that, in addition to other diet quality indices, the DII was associated with the abundance of *Faecalibacterium praunsnitzii*, a well-established butyrate-producer. This bacterial species, along with other butyrate producers, was also increased by a one-year Mediterranean-style diet in older adults of the NU-AGE study (Ghosh et al., 2020). Our study supports these findings, and those from Zheng et al., which found that a pro-inflammatory diet was associated with greater abundance of bacterial species considered either pathogenic or associated with pro-inflammatory biomarkers (Zheng et al., 2020).

The range of fibre-rich plant foods present in a healthy diet may explain the positive associations we observed between diet quality and butyrate-producing bacteria. Dietary fibre is metabolised by several members of the bacterial microbiome. These primary degraders breakdown fibre from complex polysaccharides to mono- or oligosaccharides, which are fuel for butyrate producing bacteria (Baxter et al., 2019). While the current study focused on the relative abundance of butyrate-producing bacteria, future research may benefit from the inclusion of primary fibre degraders to account for this cross-feeding between bacteria (Soto-Martin et al., 2020). In addition to fibre, a higher ARFS score may also reflect greater dietary diversity, indicating consumption from several food groups. Dietary diversity is associated with bacterial diversity (McDonald et al., 2018), which is in turn related to better health outcomes (Claesson et al., 2012). Dietary diversity increases the likelihood of exposure to a wider array of nutrients that may contribute to the complex, symbiotic community of bacteria associated with butyrate production (Tavakoli et al., 2016). Thus, elucidating the potential role of dietary diversity that is inherent in all diet quality indices, independent of fibre consumption, in the diet quality-butyrate-producing bacteria relationship may also be beneficial. In addition to the known anti-inflammatory effects of fibre (Shivappa et al., 2013), the inflammatory potential of overall diet, which is measured by the DII, also may play a role in the relative abundance of butyrate producers. Other food components of the DII have been observed to contribute to the gut microbiota composition, including alcohol, fat type and quantity, and protein (Bishehsari et al., 2017; Wu et al., 2011). However, it is likely that no single component decides the inflammatory effect of diet in the gut, and more likely is the interplay between these components, helping or hindering the environment in which butyrate-producing bacteria can thrive.

Consistent with reports from previous studies that investigated alpha-diversity and frailty and lean mass in humans (Barger et al., 2020; Zhang et al., 2020), we did not observe associations between alpha-diversity and muscle health. Furthermore, the evidence for an association between greater abundance of butyrate-producing bacteria and healthier muscle mass, strength, and function was weakened following inclusion of covariates. The associations observed in unadjusted models were variably attenuated by age, physical activity, diet quality and muscle-related medical conditions, indicating complex relationships between a number of inter-related variables. While there is a dearth of evidence in humans investigating the relationship between butyrate and muscle health, Barger et al. (2020) reported associations between greater muscle mass and both butyrate-producing bacteria and butyrate production genes. However, while we observed attenuations in significance for all three muscle outcomes, associations reported by Barger et al. withstood adjustment. This may be because Barger et al. did not adjust for gut or muscle medical conditions, which the current study did. This discrepancy in findings may be further explained by the age range of 33–96 years in the current study compared to the >65 years inclusion criteria for Barger et al. Indeed, age was responsible for attenuating the relationships between butyrate-producing bacteria and muscle strength and function in the current study, however no age interaction was observed.

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In the current study age attenuated the butyrate-producing bacteria and muscle relationship for strength and function, but not mass. This may be due to age-related declines of the neurological component of muscle function which precede the physical manifestations (ie. mass) (McGregor et al., 2014). Furthermore, age-related changes in body composition cause intermuscular adipose infiltration, resulting in muscle quality degradation prior to reductions in muscle mass (Addison et al., 2014). Age is also associated with changes in the gut microbiome, including reduced abundance of certain beneficial bacteria and alpha-diversity (Jackson et al., 2016). However, it remains unclear whether these changes in the microbiome are due to chronological age itself or the dietary and lifestyle changes concomitant with age. Alterations in food intake and food preferences can occur as individuals age. These changes can impact peristalsis, which in turn can cause motility changes, altering the gut microbiome composition (Roager et al., 2016). Therefore, age also was considered in the relationship between diet quality and butyrate producers. Prebiotic, probiotic, or butyrate supplementation trials in older people may elucidate whether the impact of age on butyrate-producing bacteria could be circumvented, delivering potential benefits to muscle health.

Butyrate-producing bacteria directly influence the levels of intestinal butyrate (Pryde et al., 2002). While the current study did not observe an association between butyrate-producing bacteria and muscle health following adjustment, investigation in other study samples, such as women and very old participants (≥85 years), may provide insight into this potential association across a wider range of people. A positive effect of butyrate-producing bacteria on muscle health may relate to butyrate's role in retaining muscle health with increasing age. In mice, butyrate supplementation appears to attenuate age-related muscle atrophy (Walsh et al., 2015), and findings from a study of energy-restriction diets in pigs suggest that SCFA supplementation resulted in increased nitrogen retention, a marker of protein synthesis (Pacy et al., 1994). This suggests that increased butyrate concentration may help to attenuate muscle loss observed in energy-deficient diets, which are common in older age (Morley, 1997). The physiological mechanisms through which gut-derived butyrate may assist in muscle retention may be via healthy glucose and insulin measures. Supplemental butyrate has improved insulin measures in mice liver (Mollica et al., 2017), and in humans, fibre supplementation improved insulin measures in adipose tissue and muscle, independent of the liver (Robertson et al., 2012). These findings suggest that butyrate may improve the glucose disposal process, which is often hindered in older age (Ferrara et al., 2006), via either skeletal muscle and/or the liver. These potential glucose and insulin pathways and the limited systemic concentration of gut-derived butyrate (den Besten et al., 2013) warrant further investigation in humans.

Strengths and limitations

Data from this study were derived from the GOS, which is a well characterised, long-term population study. The wide range of data collection within the GOS allowed for several important covariates to be considered, including medications and medical conditions. In addition, the Baecke Physical Activity Questionnaire is a robust measure of work, leisure, and sport-related PA in older adults, which is an important consideration for muscle health research. However, this questionnaire has been validated only in participants aged <60 years and may therefore not accurately reflect the PA levels in older people. The use of DXA-derived SMI, HGS, and TUG provided objective measures for three important aspects of muscle health, and dietary assessment was conducted with a thorough and validated instrument. This study also reported a 78 per cent return rate of stool samples from the GOS cohort, suggesting good representation of the general community.

However, the cross-sectional nature of the study means that causality cannot be assumed. With few cases in all low muscle health categories, the current sample is considered a healthy one. Therefore, associations reported here may be more subtle than those detectable in a case–control study. Participants were men only and, considering differences in SCFA profiles have been observed between sexes

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(Sun et al., 2021), these results may not be applicable to women. Known limitations in the resolution of taxonomic identification in 16S sequencing may have affected the identification of butyrate-producing bacteria. Self-reported dietary data are known to be associated with reporting biases and other sources of error (Shook et al., 2018). While all ARFS components were extractable from the FFQ, only 22 of the total 45 DII components were available. Therefore, the DII scores used herein may not have captured the full inflammatory potential of participants' diets (Phillips et al., 2019).

Conclusions

Healthier diet quality and an anti-inflammatory diet were positively associated with butyrate-producing bacteria in the gut microbiome. However, we did not observe compelling associations between either alpha-diversity, beta-diversity, or the relative abundance of butyrate-producing bacteria and muscle health. These findings support the role of diet quality in achieving a healthier gut microbiome, however, further evidence is required to support the presence of a gut-muscle axis in humans. Future research including human dietary interventions may assist to further elucidate the role of diet quality in muscle health and the potential mediating role of the gut microbiome.

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Data availability statement. Data are available from the Geelong Osteoporosis Study Data Steering Committee upon reasonable request. Please direct enquiries to the corresponding author.

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