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Original research

Distinct microbiome composition and metabolome exists across subgroups of elite Irish athletes

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ABSTRACT

Objectives: The gut microbiome has begun to be characterised in athlete groups, albeit, to date, only across a subset of sports. This study aimed to determine if the gut microbiome and metabolome differed across sports classification groups (SCGs) among elite Irish athletes, many of whom were participating in the 2016 Summer Olympics.

Methods: Faecal and urine samples were collected from 37 international level athletes. Faecal samples were prepared for shotgun metagenomic sequencing and faecal and urine samples underwent metabolomic profiling.

Results: Differences were observed in the composition and functional capacity of the gut microbiome of athletes across SCGs. The microbiomes of athletes participating in sports with a high dynamic component were the most distinct compositionally (greater differences in proportions of species), while those of athletes participating in sports with high dynamic and static components were the most functionally distinct (greater differences in functional potential). Additionally, both microbial (faecal) and human (urine) derived metabolites were found to vary between SCGs. In particular *cis*-aconitate, succinic acid and lactate, in urine samples, and creatinine, in faeces, were found to be significantly different between groups. These differences were evident despite the absence of significant differences in diet, as determined using food frequency questionnaires, which were translated into nutrient intake values using FETA.

Conclusions: Differences in the gut microbiome and metabolome between groups, in the absence of dietary changes, indicates a role for training load or type as a contributory factor. Further exploration of this hypothesis has the potential to benefit athletes, aspiring athletes and the general public.

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Practical implications

- The gut microbiome and metabolome of athletes differs across sports classification groupings.
- Diet is not the driver of differences in the gut microbiome and metabolome across these sports classification groups.
- The difference in microbiomes and metabolome across groupings, in the absence of significant dietary differences, suggests a role for exercise type as a contributing factor.
- The identification of a causative factor of these separations was not feasible within this study and potential future studies would need to assess parameters such as detailed exercise tracking in a larger cohort to strive towards the identification of causative factors.

1. Introduction

It has been established that a variety of lifestyle and environmental factors impact on the gut microbiome. Modulators include diet and pharmacological agents, including antibiotics.[1] Physical fitness has also been recognised as an influencer of the gut microbiome, although the impact of exercise has been variable across different studies.[2,3] As individuals who participate in exercise at an extreme level and over an extended period, the gut microbiome of elite-level athletes has been the focus of several studies. The gut microbiome of these athletes has been found to be distinct from

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both a composition and functional perspective from that of controls, while training load across cyclists was found to be associated with differences in gut microbiome composition.[4,5] While different types and intensities of training, for example forced versus voluntary wheel running, have been found to impact on the gut microbiome in murine models,[6] to date the impact of different training types on the human microbiome has not been determined. Nonetheless, different sports have been found to have distinct impacts on other parameters, including cardiovascular measurements, bone turnover and muscle mass.[7,8]

This study set out to investigate if the gut microbiome and/or metabolome of elite-level athletes reflect the type of sport in which they participated. To achieve this goal, 37 elite Irish athletes, who competed across 16 different sports, were studied.

2. Methods

Ethical approval for the study was granted by the clinical research ethics committee of the Cork teaching hospitals under project code APC073. Faecal and urine samples were collected from female (N = 14) and male (N = 23) Irish athletes across 16 different sports (Fig. 1A). FFQs were collected and converted to nutrient intake values using FETA (Food Frequency Questionnaire European Prospective Investigation into Cancer and Nutrition Tool for Analysis).[16] DNA was extracted from faecal samples using the repeated bead beating plus column (RBBC) method and prepared for shotgun sequencing on an Illumina NextSeq. The datasets generated and analysed during the current study are available in the ENA under the study accession number PRJEB32794. Resulting FastQ reads were quality checked using a combination of BMTagger, Picard and SAM tools.[9,10] Taxonomic classifications were determined using Kraken 2 and Bracken.[11,12] Functional profiling was completed using HUMAnN2.[13] Urine samples underwent GC—MS, UPLC-MS and 1H-NMR analysis, while faecal samples underwent 1H-NMR and UPLC-MS analysis in Imperial College London, as previously described. Analysis of resulting data sets was completed in R. A hclust tree was created in the R package ape and visualised using iToL.[14] LEfSe was used to determine species and pathways which characterise specific groups.[15] Full detailed methods available as Supplementary material 1.

3. Results

This study set out to determine if differences existed in the gut microbiome and metabolome of elite-level athletes participating in different categories of sports. To gain an understanding of the impact of dynamic versus static components of exercise, each sport was classified into a broader sports classification groups (SCGs) (Fig. 1A).[7] Dietary intake was established using food frequency questionnaires which were converted into energy, food group and nutrient intakes using FETA[16] (Supplementary material 7). While individual variation in nutrient intakes were observed, no significant differences were found between any nutrients or food groups based on SCGs, gender or location of sample collection following correction for multiple comparisons (Supplementary material 8). Although microbial diversity within samples was not found to differ based on SCGs (Supplementary material 9), microbial diversity between samples, as illustrated with MDS, revealed that samples from athletes within the same SCG generally cluster together (Fig. 1B and C), from both a compositional ($R2 = 0.20396$, $p = 0.003$, Stress = 0.1663576) and functional potential ($R2 = 0.23542$, $p = 0.001$, Stress = 0.143677) perspective.

Analysis of compositional variation at species level revealed samples were dominated by one or a combination of five species, namely, *Eubacterium rectale*, *Polynucleobacter necessarius*, *Faecalibacterium prausnitzii*, *Bacteroides vulgatus* and *Gordonibacter massiliensis* and, while many of these species (including *F. prausnitzii*) were present across all samples, the clustering of samples reflected the relative abundance of these species. These five species were found to account for 19–72% relative abundance within these samples. Samples in this study were identified as being dominated by species from the genus *Bacteroides*, with samples comprised of $19.8 \pm 11.6\%$ *Bacteroides* species.

Discriminatory species were identified with linear discriminant analysis (LDA) effect size (LEfSe), which allows for the identification of species or pathways which explain differences between groups.[15] LEfSe analysis revealed a number of species that discriminated between SCGs (Fig. 2A). *Streptococcus suis*, *Clostridium bolteae*, *Lactobacillus phage LfeInf* and *Anaerostipes hadrus* were found to be associated with those groups with a moderate dynamic component (B1 and B2), which includes sports such as fencing. *Bifidobacterium animalis*, *Lactobacillus acidophilus*, *Prevotella intermedia* and *F. prausnitzii* were found to be associated with the SCG C1 (high dynamic and low static components, including sports such as field hockey), while *Bacteroides caccae* was found to be associated with the SCG C3 (high dynamic and static components, including sports such as rowing). *Bacteroides caccae*, *F. prausnitzii* and *Anaerostipes hadrus* exhibited greatest discriminatory potential, with LDA effect sizes of >4, with respect to the SCGs C3, C1 and B, respectively. Samples from the group C3 were found to have 4.5 times greater relative abundance of *Bacteroides caccae* than other groups, while *Anaerostipes hadrus* was present at 3 times greater relative abundance in the group B relative to other groups. *F. prausnitzii* was present at 1.5 times greater relative abundance in samples from group C1 in comparison to other groups. Notably, samples from the group C1 were found to have 25 times greater relative abundance of *Lactobacillus acidophilus*. Interestingly, no species were identified as being associated with the SCGs A3 (low dynamic and high static components, including sports such as judo) or C2 (high dynamic and moderate static components, including sports such as swimming) when the LDA effect size cut off was set to 3.

Substantial variability exists at the functional level across all groups. A heatmap was used to visualise 25 pathways that were the most variable between samples and samples were separated into five clusters based on the reads per million of these pathways. Samples from individuals OM007 (middle distance running; SCG C2) and OM032 (swimming; SCG C2) were seen to have a higher abundance of many pathways than was apparent in their counterparts, and these samples cluster together, away from all other samples in cluster 1. Samples from cluster 2 were mostly collected in Ireland and can be characterised by samples which have greater proportions of 5 pathways (SO4ASSIM-PWY, PWY-821, ARGININE-SYN4-PWY, P162-PWY and ARGORNPROST-PWY), which are involved with acetate biosynthesis and sulphate degradation. Those samples from cluster 4 contain a lower proportion of reads for these 25 pathways in comparison to other samples. Samples from cluster 5 have greater proportion of 3 pathways (PWY-7007, PWY-7315 and P562-PWY), which are involved with methyl ketone and outer membrane biosynthesis.

In order to identify pathways which were significantly different between groups, LefSe was again utilised (Fig. 2C). A greater number of pathways (10) were found to be associated with the SCG C3 than any other group and this included pathways involved with folate and amino acid biosynthesis which were found to be 1.5 times greater in this group in comparison to other groups. 5 pathways were found to be associated with the SCG B and this included pathways involved with flavin biosynthesis and fermentation. Notably, the pathway P461-PWY was found to be present in the group B in proportions 2 times greater than in other groups. This pathway is responsible for fermentation of sugar alcohols and has been identified in a number of *Streptococcus* species. *S. suis*, a species

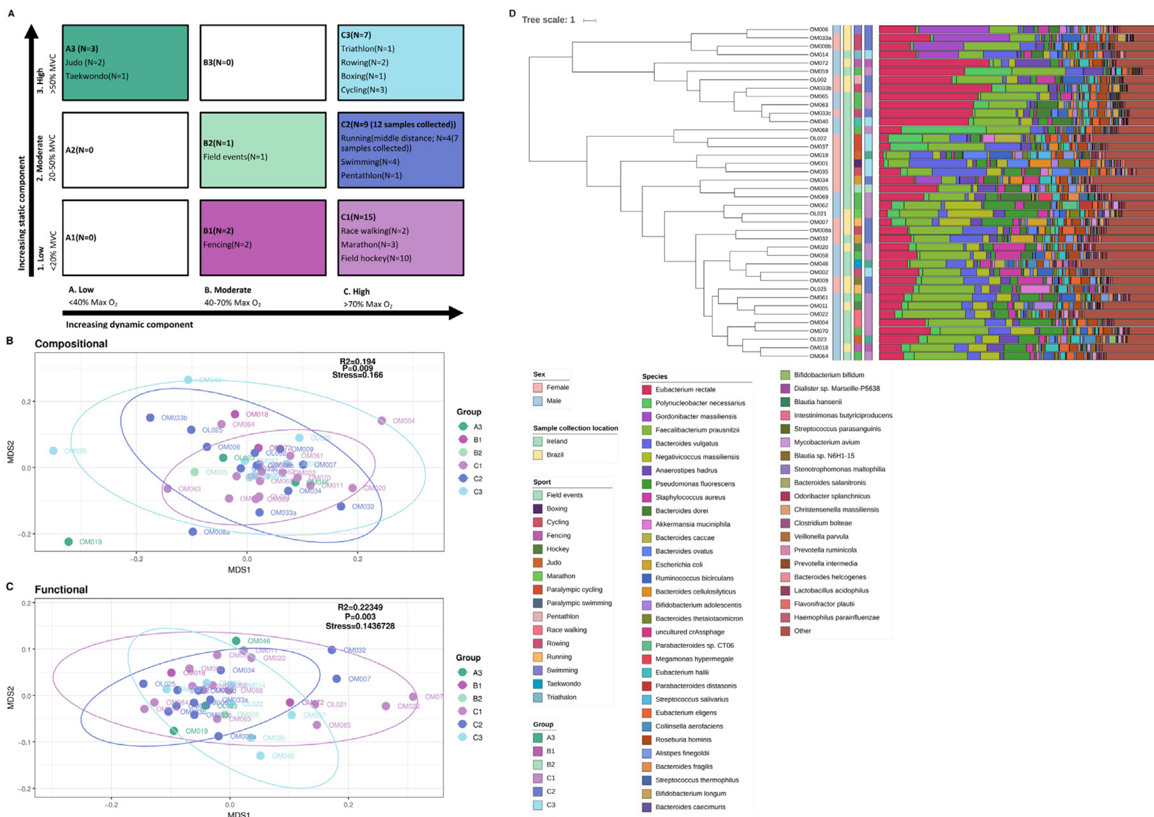

Fig. 1. Diversity and composition of the gut microbiome.

A) Classification of sports based on peak static and dynamic components. Adapted from Mitchell et al., to only include those sports from which samples were collected from during this study and coloured by classification group.[7] With an increasing dynamic component an increase in estimated percent of maximal oxygen uptake (Max O2) is achieved, resulting in an increased cardiac output. An increasing static component is related to estimated percent of maximal voluntary contraction reached and results in an increased blood pressure load. The lowest cardiovascular demands (cardiac output and blood pressure) are in those sports grouped to B1 (in this analysis), while the highest are grouped to C3. No classification was available for pentathlon and so this was classified to highest score of those sports involved in pentathlon (i.e. swimming and running which are classified as C2).

B + C) Multidimensional scaling (MDS) result from (B) species level compositional profiles from Bracken following taxonomic classification with Kraken 2[11,12] and (C) HUMAnN2 profiles of functional potential,[13] as determined using vegan in R, show the differences between faecal samples based on group (A3 (N = 3), B1 (N = 2), B2 (N = 1), C1 (N = 15), C2 (N = 12), C3 (N = 7)). Clustering of samples was observed based on classification group for both compositional and functional data.

D) Hierarchical cluster analysis, with species level taxonomic profiles. Each bar represents an individual sample. Each colour represents an individual species, as classified with Bracken following taxonomic classification with Kraken 2. Only those species which were present at >1% relative abundance in at least 1 sample shown, with all others grouped as other. Colours of stacked bars alongside tree represent sex of participant, location at which sample was collected, sport and sports classification group. No clustering is observed based on metadata available.

identified as associated with this group may therefore be responsible for this observation. 5 pathways were found to be associated with the SCG A3 while, interestingly no species were identified as associated with this group. No pathways were identified as being associated with the SCG C2, although 4 species were identified as being significantly increased in this group in comparison to other groups, indicating that while the composition of this group was unique from other groups, the functional potential did not vary. No species or pathways were identified as being significantly different in the SCG C1 in comparison to other groups. None of the collected metadata (gut health or dietary intake) was found to be correlated with the species or pathways identified in this study.

Faecal and urine samples underwent metabolomic analysis (NMR and UPLC-MS analysis for faecal samples and NMR, GC–MS and UPLC–MS analysis for urine samples) to explore variations in microbial and human derived metabolites, respectively. MDS analysis (Fig. 3) revealed significant separation between SCGs based on urine GC–MS (A) ($R2 = 0.471$, $p = 0.003$, Stress = 0.021), urine NMR (B) ($R2 = 0.212$, $p = 0.033$, Stress = 0.158) and faecal NMR (C) ($R2 = 0.246$, $p = 0.036$, Stress = 0.178) analysis. No separation was seen between SCGs based on UPLC-MS analysis (faecal and urine; see Supplemental material 10). OPLS-DA analysis revealed separation between the SCGs A3 and B1; B1 and C2; and C1 and C2 based on urine GC–MS, between the SCGs A3 and B1; and A3 and C2 based on urine NMR and between the SCGs A3 and B1 based on faecal NMR (Supplementary materials 11–13).

21 metabolites were found to be significantly different between SCGs (Supplementary material 14), while pairwise analysis between SCGs found that only 4 of these were significantly different between at least 2 SCGs, 3 urine metabolites and 1 faecal metabolite (Fig. 3D). *cis*-Aconitate and succinic acid were found to be in significantly greater concentrations in GC—MS analysed urine samples from the SCG C2 in comparison to C1. While these metabolites were outside the limit of quantification for some samples, where the metabolites were quantified they were in concentrations 2 and 2.5 times greater than group C1. Lactate was found to be significantly different between the groups C1 and C2, and C1 and C3 from NMR analysis of urine samples. Although concentrations of this metabolite were small, differences observed in the mean concentrations found that the SCG C1 had concentrations of lactate which were 8 and 2 times lower than those found in the SCG's C2 and C3, respectively. Creatinine was the only faecal metabolite identified as being significantly different between SCGs ($p = 0.017$), with this metabolite identified as being in a greater concentration in the group C1 in comparison to the groups A3, C2 and C3 following NMR analysis. Interestingly, all 4 metabolites identified as

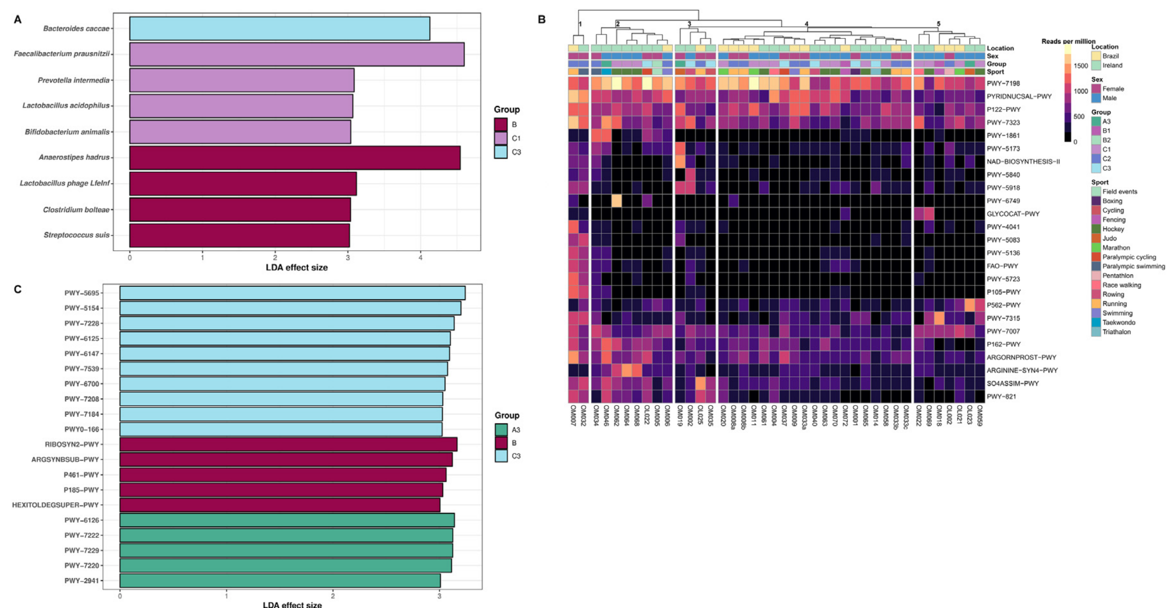

Fig. 2. Composition and functional potential of the gut microbiome vary based on the sports classification groupings.

A) LEfSe plots identifying discriminatory species, as classified with Bracken[12] following taxonomic classification with Kraken 2,[11] between sports classification groups (A3 (N = 3), B (grouped samples with a moderate dynamic component for statistical analysis; N = 3), C1 (N = 15), C2 (N = 11), C3 (N = 7)) (see Fig. 1A for more information regarding groupings). An LDA effect size cut off of 3 was used.

B) HUMAnN2[13] heat map reporting the 25 most variable pathways that were greater than 1% relative abundance in at least one sample. Clustering completed using Wards method. Annotations below tree represent sex of participant, location at which sample was collected, sport and sports classification group. Variability in pathway abundance was observed between samples which was not accounted for by metadata available.

Pathways; PWY-4041:Gamma glutamyl cycle, PWY-5136:Fatty acid beta oxidation II (peroxisome), PWY-5840:Superpathway of menaquinol-7 biosynthesis, PWY-7007:Methyl ketone biosynthesis, PWY-5083:NAD/NADH phosphorylation and dephosphorylation, FAO-PWY:Fatty acid beta oxidation I, P122-PWY:Heterolactic fermentation, PWY-5723:Rubisco shunt, PWY-5173:Superpathway of acetyl-CoA biosynthesis, GLYCOCAT-PWY:Glycogen degradation I (bacterial), P562-PWY:Myo-inositol degradation I, P162-PWY: L-glutamate degradation V (via hydroxyglutarate), NAD-BIOSYNTHESIS-II:NAD salvage pathway II, ARGORNPROST-PWY :Arginine, ornithine and proline interconversion, PWY-5918:Superpathay of heme biosynthesis from glutamate, ARGININE-SYN4-PWY:L-ornithine de novo biosynthesis, SO4ASSIM-PWY:Sulfate reduction I (assimilatory), P105-PWY:TCA cycle IV (2-oxoglutarate decarboxylase), PYRIDNUCSAL-PWY:NAD salvage pathway I, PWY-7315:dTDP-N-acetylthomosamine biosynthesis, PWY-6749:CMP-legionaminate biosynthesis I, PWY-7198:Pyrimidine deoxyribonucleotides de novo biosynthesis IV, PWY-7323:Superpathway of GDP-mannose-derived O-antigen building blocks biosynthesis, PWY-1861:Formaldehyde assimilation II (RuMP Cycle), PWY-821:Superpathway of sulfur amino acid biosynthesis (Saccharomyces cerevisiae).

C) LEfSe plots identifying discriminatory pathways, as classified with HUMAnN2, between sports classification group (A3 (N = 3), B (grouped samples with a moderate dynamic component for statistical analysis; N = 3), C1 (N = 15), C2 (N = 11), C3 (N = 7)). Unstratified pathway abundance (reads per million) assignments as determined using HUMAnN2 were analysed. An LDA effect size cut off of 3 was used.

Pathways; PWY-5695:Urate biosynthesis/inosine 5′-phosphate degradation, PWY-5154: l-arginine biosynthesis III (via N-acetyl-l-citrulline), PWY-7228: Superpathway of guanosine nucleotides de novo biosynthesis I, PWY-6125: Superpathway of guanosine nucleotides de novo biosynthesis II, PWY-6147: 6-hydroxymethyl-dihydropterin diphosphate biosynthesis I, PWY-7539: 6-hydroxymethyl-dihydropterin diphosphate biosynthesis III, PWY-7208:Superpathway of pyrimidine nucleobases salvage, PWY-7184: Pyrimidine deoxyribonucleotides de novo biosynthesis I, PWY0-166: Superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis (E. coli), RIBOSYN2-PWY: Flavin biosynthesis I (bacteria and plants), ARGSYNBSUB-PWY: L-arginine biosynthesis II (acetyl cycle), P461-PWY: Hexitol fermentation to lactate, formate, ethanol and acetate, P185-PWY: Formaldehyde assimilation III (dihydroxyacetone cycle), HEXITOLDEGSUPER-PWY:Superpathway of hexitol degradation (bacteria), PWY-6126: Superpathway of adenosine nucleotides de novo biosynthesis II, PWY-7222: Guanosine deoxyribonucleotides de novo biosynthesis II, PWY-7229:Superpathway of adenosine nucleotides de novo biosynthesis I, PWY-7220: Adenosine deoxyribonucleotides de novo biosynthesis II, PWY-2941: l-lysine biosynthesis II, PWY-6700: queuosine biosynthesis.

significantly different between groups were found to be significantly different between the SCGs C1 and C2, which only differ based on their static component. As these samples have a comparably larger sample size (N = 15 and 12) in comparison to other groups it is possible that this is the reason for this observation. Notably, the SCG C2 was found to have no significant differences in relation to microbial composition and functional potential when compared to other groups; however variation was observed in this group in relation to metabolites highlighting the importance of a multi-faceted approach utilising both sequencing and metabolic approaches to identify differences between groups. No significant correlations were observed between metabolites and any individual species or pathways. Furthermore, no correlations were identified between any of the metabolites and any available metadata for this study.

4. Discussion

Exercise has been found to have a beneficial impact on a number of body systems including the prevention and treatment of obesity, and alleviation of symptoms of depression, anxiety, and disorders affecting the cardiovascular system.[17,18] In addition, exercise is thought to influence, or correlate with differences in, the gut microbiome across animal and human studies.[2,5,6] However, little is known about the variation in the gut microbiome and metabolome between sports. This study set out to gain an initial insight into the possibility that the gut microbiome or metabolome differ across athletes from different sports or SCGs.

SCGs were used to distinguish between sports based on the static and dynamic components of the sport allowing for a comparison of sports based on their impact on cardiorespiratory measures.[7] Blood pressure (static exercise) and VO_2 max (dynamic exercise) have been linked with gut microbiome composition previously, however most of these associations were found in obese cohorts or those with cardiovascular disease.[19,20] Although cardiorespiratory measures were not taken in the current study, the classification into SCGs, and correlations with gut microbiome composition and functionality as well as metabolome, provides further evidence of a link.

Samples from this study were found to be significantly different from a microbial composition and functional potential perspective

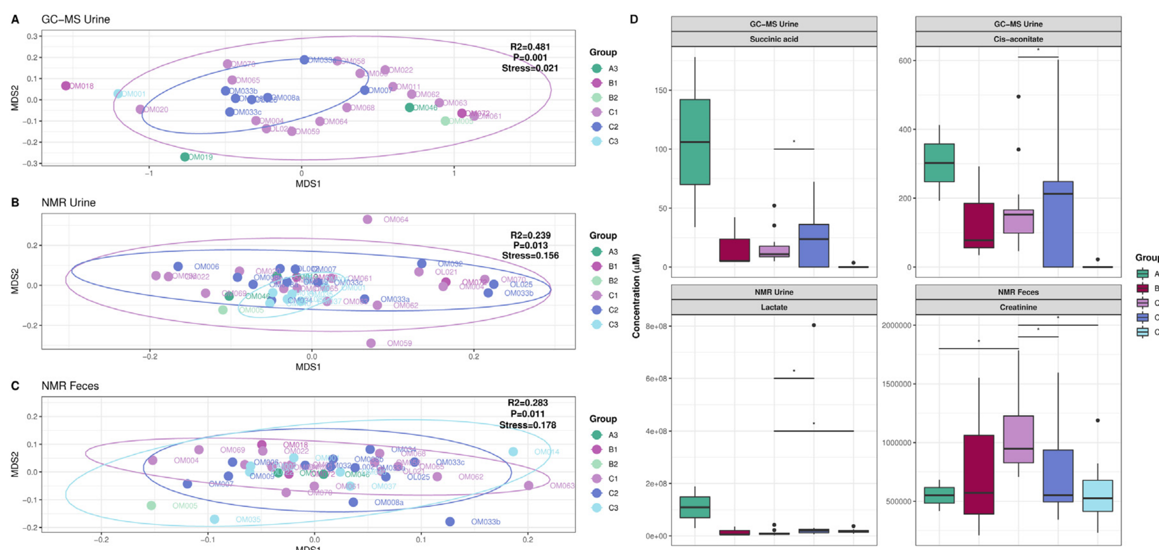

Fig. 3. Metabolomic analysis reveals separation in microbial and human derived metabolites based on sports classification groups.
A + B + C) Multidimensional scaling (MDS) result from (A) GC—MS analysis of urine samples, (B) NMR analysis of urine samples and (C) NMR analysis of faecal samples, as determined using vegan in R, show the differences between metabolites contained in samples based on sports classification group (A3 (N = 3), B1 (N = 2), B2 (N = 1), C1 (N = 15), C2 (N = 12), C3 (N = 7)) (see Fig. 1A for more information regarding groupings). Clustering of samples was observed based on sports classification group for both GC—MS urine and NMR analysis of urine and faecal samples.
D) Significantly different metabolites in urine and faecal samples, as determined with NMR and GC—MS analysis, between sports classification groupings (A3 (N = 3), B (grouped samples with a moderate dynamic component for statistical analysis; N = 3), C1 (N = 15), C2 (N = 11), C3 (N = 7)) (see Fig. 1A for more information regarding groupings). Statistical analysis was completed using the Kruskal-Wallis test, with a Mann Whitney U test performed for significant results to determine the groups between which this difference applied. All reported p values were adjusted using Benjamini-Hochberg. The significance threshold was set at 0.05. * P < 0.05.

between SCGs, while the metabolome was also identified as variable between groups. Individual variability exists across athletes with clustering of the majority of samples driven by the relative abundances of five species, namely *E. rectale*, *P. necessaries*, *F. prausnitzii*, *B. vulgatus* and *G. massiliensis*, which have previously been identified as common gut inhabitants. Although *E. rectale* has been found to be enriched in participants with a low VO_2 max,[20] this finding was not identified in this study, potentially as a consequence of a high maximal voluntary contraction being associated with the group with the lowest VO_2 max (A3). *F. prausnitzii*, a butyrate producer, has previously been found in greater relative abundance in active women, with an increase seen with exercise training.[2] This species, which was also identified in this study as being associated with the SCG C1, is a dominant taxon in healthy cohorts, with depletion evident in disease-associated cohorts, including in IBD,[21] indicating their presence may be beneficial. Among the species found to discriminate for SCGs, *L. acidophilus* was found to discriminate for the SCG C1. This is not surprising given that eight out of the fifteen participants from hockey reported taking a product which contains this species, demonstrating compliance with reported supplementation rather than a reflection of the species of the general SCG. Another species used in probiotic supplements, *B. animalis*, was also identified in this group, although this species was not listed as a component of the reported supplement.[22] Species associated with the SCG B include those with the potential to produce butyrate, a short chain fatty acid found to promote gut homeostasis, specifically *A. hadrus* and *C. bolteae*.[23,24] Variation in species composition may be as a result of the variance in demands across different types of sports, including duration of activity and training modes. This in turn may result in an alteration in gut microbiome composition as a consequence of the alteration of gut transit time, or some other physiological change.[25,26]

Both the faecal and urine metabolome was found to be distinct between SCGs indicating differences in microbial and human derived metabolites, respectively. Succinic acid was found to be greater in the SCG C2 relative to the SCG C1. Conversely, succinate was previously found to be lower in rugby players relative to inactive controls,[5] while in the current study succinic acid was found to increase with an increased cardiac output. Lactate, produced in the muscles during normal metabolism and exercise,[27] was found to be increased in SCGs with an increased static component (i.e., in the SCGs C2 and C3 relative to C1). An increased static component involves the development of intramuscular strength,[7] potentially resulting in an increase in the production of lactate. Creatinine is the breakdown product of creatine phosphate in muscle and is related to muscle turnover.[28] This metabolite was found to be greatest in the SCG C1 relative to A3, C2 and C3 indicating muscle turnover may have been greatest in this cohort with a high dynamic and low static component. Dynamic exercises are involved with changes in muscle length and joint movement,[7] potentially resulting in the increase in the production of creatinine. Previously, those sports involving resistance training have been indicated as resulting in the greatest muscle turnover,[29] however the extended endurance component involved with the sports in this cohort may give rise to a similar effect.

These differences in microbiome and metabolome were observed despite no significant variation in dietary intakes across athletes from different SCGs; suggesting that variations in training loads and competition requirements contributed to these microbiome and metabolome-related patterns. Studies of athlete groups across various laboratories have also highlighted microbiome differences, and while this may be as a result of variation in other methodologies used (e.g., sequencing analysis methodologies), differences in sport may also play a role.[4,5]

5. Conclusion

In conclusion, the gut microbiome of the athlete cohorts tested in this study is distinct based on SCGs with differences also established in the urine and faecal metabolome. While studies have identified variation in the gut microbiome within cyclists based on time per week spent exercising,[4] to our knowledge this is the

first study identifying a separation in the gut microbiome between different sports. The identification of a causative factor of these separations was not feasible within this study and potential future studies would need to assess parameters such as detailed exercise tracking in a larger cohort to strive towards the identification of causative factors. The identification of species, pathways and metabolites driving separations between SCGs could help identify an optimal gut microbiome in the context of the sport an individual is participating in. Additionally, an understanding of the variation in an athlete microbiome could help guide future studies surrounding the impact of exercise on the gut microbiome within general populations. Furthermore, species identified here as increased for individual SCGs could form the basis of future investigations to identify their influence on performance, as was recently determined for *Veillonella*.[25]

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsams.2019.08.290.

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