Original Research

The sad weekend: A perilous North American tradition

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ABSTRACT

Obesity is a global concern and affects millions of Americans who consume poor-quality diets. Diets directly affect the gut microbiota, which can have subsequent effects on inflammation and contribute to other chronic states. Previously we have shown that a Standard American Diet (SAD) increased immune cell activation and prolonged recovery and that a beneficial diet could reduce these negative effects. Here, male and female mice were given access to regular chow (REG), SAD, our Anti-Inflammatory Diet (AID) or a combination of SAD and AID. This latter group was modeled on the commonplace dietary pattern of healthy eating during the week (AID: Monday-Friday) and relaxed eating patterns on the weekend (SAD: Saturday-Sunday). After 14 weeks of diet consumption and an inflammatory injury, we found that the SAD prolonged and the AID promoted recovery. However, recovery was significantly delayed in those mice consuming the AID-SAD, regardless of weekly healthy diet access. In addition, fecal samples taken during the study revealed dramatic differences in microbial community composition, relative abundance of abundant bacterial phyla and alpha diversity. These data confirm the impact of diet on gut microbiota and suggest a relation between abundance of specific bacterial taxa and susceptibility to prolonged recovery from injury.

1. Introduction

Obesity is a global concern, particularly in the Western world. In America, the number of overweight and obese individuals has surpassed the number of healthy-weight individuals (Flegal et al., 2013; Yang and Colditz, 2015). Obesity and chronic pain often co-occur (Hitt et al., 2007; Okifuji and Hare, 2015). Obesity is considered a risk factor for chronic pain and studies show that obese individuals report greater pain (Stone and Broderick, 2012).

Obesity is also accompanied by activation of the innate immune system which results in a chronic inflammatory state (Okifuji and Hare, 2015). Additionally, it has been demonstrated that there are increased levels of pro-inflammatory cytokines present in obese individuals (Ouchi et al., 2011; Bluher et al., 2005), and there is evidence that the presence of cytokines is related to severity of pain (Zhang and An, 2007).

One major environmental factor that contributes to obesity is a poor-quality diet. Evidence shows that a high-fat diet contributes to inflammation and pain behaviors in rats, even in the absence of obesity (Song et al., 2017). One study done in healthy adults found that diet quality mediated the relationship between body mass index and self-reported pain (Emery et al., 2017) even after controlling for medication, biomechanics, psychological distress, age and education. These data suggest that diet quality alone contributes greatly to pain. Therefore, both poor-quality diet and obesity contribute to inflammation and can increase the possibility of experiencing chronic pain.

Alternatively, diet can reduce inflammation and pain. A ketogenic (high-fat, low-carbohydrate) diet led to reduced nociception and peripheral inflammation in rats (Ruskin et al., 2009). A Mediterranean diet has been shown to reduce pain in patients with osteoarthritis (Veronese et al., 2016) and rheumatoid arthritis (McKellar et al., 2007). In addition, increasing omega-3 fatty acids and decreasing omega-6 fatty acids led to a reduction in headache pain in chronic headache patients (Ramsden et al., 2015). A number of studies in human chronic pain patients have reported a reduction in pain and/or inflammation as a result of diet (Kjeldsen-Kragh et al., 1991; Messier et al., 2004; Kaartinen et al., 2000; Skoldstam et al., 2005; Kremer et al., 1990; Arjmandi et al., 2004; Drozdov et al., 2012; Belcaro et al., 2010). This indicates that diet has the potential to be used as a non-pharmacological intervention for

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treating pain.

There is an established role of the gut microbiota in obesity (Brahe et al., 2016). This is evidenced in studies utilizing germ-free mice that are resistant to the obesogenic effects of a high-fat diet (Backhed et al., 2004, 2007; Rabot et al., 2010). One such study found that transferring the microbiota of obese mice to germ-free mice led to an obese phenotype in the recipient mice (Turnbaugh et al., 2006). Additionally, one group isolated an endotoxin-producing bacteria from obese patients and transferred it to germ-free mice. They found that recipient germ-free mice developed an obese phenotype comparable to wild-type mice on a high-fat diet (Fei and Zhao, 2013). High-fat diets can significantly alter microbiota and decrease overall microbiota diversity (Araujo et al., 2017). Specifically, it has been demonstrated that a high-fat diet can lead to a decrease in Bacteroidetes phyla and an increase in Proteobacteria and Firmicutes phyla (Hildebrandt et al., 2009; Zhang and Yang, 2016). Together, these data suggest a clear relationship between microbiota composition and obesity.

Less is known about the links between diet, the gut microbiota, and the inflammatory pain response. A pioneering study found that germ-free mice have a reduced pain response following the induction of inflammation compared with control mice (Amaral et al., 2008). This study established a direct role for the gut microbiota in the inflammatory pain response. Broad spectrum antibiotic treatment has been shown to attenuate mechanical hypersensitivity in adolescent rats (Yan and Kentner, 2017) and gout-induced inflammation and pain in mice (Vieira et al., 2015). However, the therapeutic use of diet interventions to manipulate the composition of the gut microbiota and reduce inflammatory pain has been largely untested. Here, we sought to investigate whether periodic access to the SAD impaired recovery from inflammatory injury when the primary diet was the AID and to determine whether changes in the gut may reflect alterations in recovery.

Previously, we developed a Standard American Diet (SAD) and an Anti-inflammatory Diet (AID) to investigate the effects of diet on pain behavior. The SAD was developed to model a typical American diet with elevated levels of processed carbohydrates and fats. The AID was developed as a low-glycemic diet with additions of food components that are known to reduce inflammation, including sulforaphane, resveratrol, ginseng, epigallocatechin gallate and omega-3 polyunsaturated fats. We demonstrated that the SAD led to prolonged recovery in rats and significant immune system activation in the spinal cord (Totsch et al., 2017). Using the AID as an intervention, we showed that AID consumption reversed the negative consequences of the SAD and promoted recovery in mice (Totsch et al., 2018a). These data supported the negative impact of a "Western" diet and the possibility to reduce the negative effects with a healthier diet, but we wished to model a pattern of eating that included "cheat days" on the weekends with healthy eating during the week to determine the effect of this intermittent exposure. All procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

2. Methods

To determine how alternating between the diets would affect pain and recovery from inflammatory injury, we switched subsets of animals to a poor-quality diet on the weekends. As in our previous studies, the SAD was used to mimic the poor-quality diet of Americans, while the AID served as the healthy alternative.

2.1. Animals

Male and female (n = 22, 11/sex, 6 weeks of age at onset) CD1 mice (ICR:Crl) were housed in groups of 2–3, under a 12 h light cycle (lights on at 07:00 h) and provided with standard chow (REG; NIH-31, Harlan Teklad) and sterile water. All mice were fed standard chow for two weeks before introduction to the experimental diets. Following baseline measures for mechanical sensitivity (see von Frey Testing below), male and female mice were randomized to diet groups: regular chow (REG, NIH-31, n = 4, 2 males), Standard American Diet (SAD, Envigo TD.140536, n = 6, 3 males), Anti-Inflammatory Diet (AID, Envigo TD.150364, n = 6, 3 males), and AID-SAD (n = 5, 3 males). The diet exposure lasted for 16 weeks (Fig. 1). All animals were obtained, housed, cared for and used in accordance with the University of Alabama at Birmingham Institutional Animal Care and Use Committee guidelines.

2.2. Diet compositions

The SAD and AID diets were developed by our group and used previously (Totsch et al., 2017, 2018a, 2018b). While both diets are considered higher-fat diets, the main sources of fat in the SAD are from lard, beef tallow, milkfat and hydrogenated vegetable shortening, whereas the main sources in the AID are from flaxseed and soybean oil. The SAD is also considered to be high in processed carbohydrates to reflect an American diet (Supplemental Table S1).

2.3. Body weight

All mice were weighed twice weekly on Monday and Friday for the duration of the experiment.

2.4. Von Frey testing

Mechanical sensitivity was measured at baseline (day 1) and once per week during diet consumption via von Frey Testing. Male and female mice were placed atop a perforated metal floor in individual custom-made transparent Plexiglas cubicles. Mice were given 1–2 h to habituate prior to behavioral testing. Nylon monofilaments (Stoelting Touch Test Sensory Evaluator Kit #2 to #9; ~0.02–1.4 g) were applied to the plantar surface of each hind paw for 1 sec. Data presented represent an average for the two hind paws. The up-down method of Dixon (1991) was used to estimate the 50% withdrawal thresholds.

2.5. Inflammatory chronic pain

After 14 weeks of diet consumption, mechanical sensitivity (as described above) was tested, and mice were injected with complete Freund's adjuvant (100% CFA, in a 20 μL injection volume) into the left hind paw. Mechanical sensitivity of mice was retested 24 h later to confirm the presence of mechanical allodynia and then on days 4, 6, 8, 11, 13, 15 and 18 following CFA injection. Groups were considered to have returned to their baseline level of sensitivity once the group mean was at least 90% of the pre-CFA withdrawal threshold taken on the day prior to CFA administration.
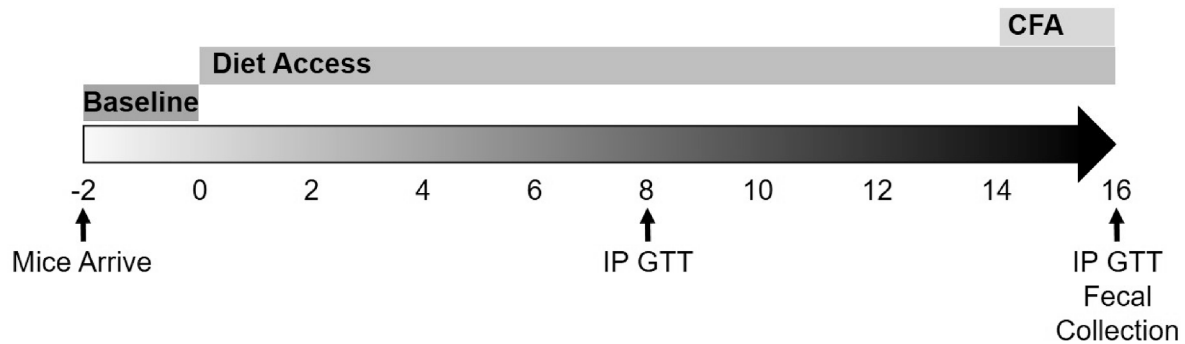
2.6. Intraperitoneal glucose tolerance test (IP GTT)

After 8 and 16 weeks of diet consumption, a glucose tolerance testing was performed. Male and female mice were fasted for 6 h, blood was collected by tail vein puncture and the droplet analyzed by standard glucometer (TRUEresult, Nipro Diagnostics). Following baseline readings, all mice were given injections of glucose (1.5 g/kg, IP). Blood was tested at 15, 30, 60 and 120 min post-injection. Following each test, pressure was applied to the puncture to stop bleeding and mice were returned to their home cage.

3. Microbiome analysis

3.1. Fecal collection

Microbiome analysis was performed on fecal pellets collected at week 16. Mice were individually removed from their home cage and isolated in a clean cage for a period of 5 min or until a minimum of 3

Fig. 1. Experimental timeline for all mice. Diet access began on week 0 and continued until week 16. CFA = complete Freund's adjuvant, IP GTT = intraperitoneal glucose tolerance test.

fecal pellets were produced. Pellets were immediately collected with sterile forceps and stored in centrifuge tubes at −20 °C until analysis.

3.2. 16S rRNA amplicon sequencing

DNA was isolated from the fecal matter ZR Fecal DNA MiniPrep™ kit (Zymo Research Corp., Irvine, CA, USA) according to the manufacture's instructions. The V4 region of the 16S rRNA gene was amplified using the LongAmp™ Taq PCR Kit (New England Biolabs, Ipswich, MA, USA) and using primers designed by Caporaso and colleagues (Caporaso et al., 2011). The amplification mixture contained 10 μL of 5 × LongAmp Taq Reaction Buffer, 1.5 μL of 10 mM dNTPs, 2 μL of 10 μM forward and reverse primers, 1.5 μL of LongAmp Taq DNA Polymerase, 30 μL of 2–5 ng per μL- template DNA, and 3 μL of H2O for a total reaction volume of 50 μL. These reactions were then cycled under the following conditions: 94 °C for 1 min, followed by 32 cycles at 94 °C for 30 s, 50 °C for 1 min, and 65 °C for 1 min, with a final extension step at 65 °C for 3 min. PCR products were electrophoresed on a 1.0% (w/v) agarose/Tris-borate-EDTA agarose gel, visualized by UV illumination, excised with a sterile scalpel and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. The 16S rRNA gene libraries were quantitated using Pico Green and adjusted to a concentration of 4 nM. Sequencing was performed at the University of Alabama Heflin Center for Genomic Sciences on an Illumina MiSeq instrument.

3.3. Bioinformatic analysis

Quality filtering, error estimation, merging of reads, dereplication, removal of chimeras, and selection of amplicon sequence variants (ASVs) was performed in the Qiime 2 platform (ver.2020.6.0) (Bolyen et al., 2019) with the DADA2 plugin (ver.2020.6.0) (Callahan et al., 2017). Taxonomy was assigned to ASVs using the SILVA small subunit ribosomal RNA database (Quast et al., 2013). Samples with less than 1000 sequence reads were removed from further analyses. Metadata associated with each sample including diet and cage assignment is provided in Supplemental Table S2.

The resulting biom table was imported into the R platform (ver.4.0.2) with the phyloseq package (ver.1.32.0) (McMurdie and Holmes, 2013). Samples with less than 1000 sequence reads were removed from further analyses. ASVs were filtered from the dataset if they were not present with a sequence count of five in at least two samples. Sequence counts were transformed to relative abundance and agglomerated at the order level in phyloseq to produce the taxonomic barchart with ggplot2 (ver.3.3.2) (Wickham, 2016).

The four most abundance phyla were analyzed with generalized linear models (GLMs) accounting for cage effects using the model: percent relative abundance ~ Diet * Cage Assignment with the R base stats package (ver.4.0.2). Frequency distribution plots (histograms) and normal probability plots were produced to check for normal distribution of the model residuals, and data were log transformed to improve normality when needed. Then, ANOVA F-tests were performed using the car package (ver.3.0.8) (Fox and Weisberg, 2019) on the GLMs to test the association between the percent relative abundance of microbial phyla and the main effects of *diet* and *cage* and the interaction between diet and cage. Post-hoc Tukey's honestly significant difference (HSD) tests, which report adjusted p-values to correct for multiple comparisons, were conducted to determine which diet groups differed from each other using the agricolae package (ver.1.3.3) (Mendiburu, 2015).

Alpha diversity values for the Shannon Index of evenness (using the phyloseq package) and Faith's phylogenetic diversity (PD) (using the btools package (Battaglia, 2020)) were calculated after rarefaction to 30,000 sequences per sample. Then, GLMs and ANOVAs were conducted as described for the bacterial phyla to test for the effects of diet on alpha diversity while accounting for cage effects. Tukey's HSD tests were conducted for alpha diversity metrics with significant F-tests as described above.

Compositional differences (beta diversity) were calculated based on the Aitchison distance of centered log-ratio transformed (CLR) counts. Zero counts were imputed using the Bayesian-multiplicative replacement function cmultRepl in the zCompositions package (ver.1.3.4) (Palarea-Albaladejo and Martin-Fernandez, 2015) prior to performing the CLR transformation with the CoDaSeq package (0.99.6) (Gloor et al., 2017). Principal component analysis of the Aitchison distance was performed with prcomp in the R base stats package and plotted with ggplot2. The effect of diet on microbial community composition assessed by the Aitchison distance method was tested with permutational multivariate analysis of variance (PERMANOVA) using the 'adonis' function in the vegan package (ver.2.5.6) with 999 permutations (Oksanen et al., 2017). Cage effects and the interaction between diet and cage affects were accounted for in PERMANOVA analysis with the linear model: Aitchison distance ~ Diet * Cage Assignment. Pairwise PERMANOVAs were conducted using the package RVAideMemoire (ver.09.68) (Hervé, 2017) to directly assess the significance of microbial compositional differences between diet treatments. Next, permutation tests for homogeneity in multivariate dispersion (PERMDISP) using the vegan 'betadisper', which quantifies the distance from each sample to its group's centroid (the group mean). Together PERMANOVA and PERMDISP provide a comprehensive analysis of changes in microbial composition by evaluating between-group and within-group differences.

We utilized the R statistical package indicspecies (ver.1.7.9) (De Cáceres et al., 2010) to analyze the strength and statistical significance of the relationship between individual genera and diet. Analysis was done at the genus level because taxonomic assignment to the species level is unreliable with short-read 16S rRNA amplicon sequencing. The 'tax_glom' function in phyloseq was used to summarize the ASV count data at the genus level. IndicSpecies was run using the 'multipatt' function with 999 permutations. Indicator taxa analysis incorporates two components, 'A' (specificity), where a value of 1 equals a taxa that is found exclusively in one group, and 'B' (fidelity), where a value of 1

equals a taxa that is found in all plots of one group and no plots in any other group. We set a sensitivity threshold (Bt = 0.75) to require that an genus must be present in 75% of the samples assigned of a specific diet group to be considered significant indicator of that diet. A false discovery rate (FDR) correction for multiple comparisons was applied to p-values from the indicspecies and genera were considered significant when the adjusted p-value was less than or equal to 0.05. All R scripts and data needed to recreate the analyses and figures regarding the microbiota data in this manuscript are available under the GitHub repository (https://github.com/KMKemp/ELSmicrobiome, DOI https://doi.org//10.5281/zenodo.4021079).

We further explored the relationship between individual genera and diet by testing for differential abundance at the genus level with the Analysis of Composition of Microbiomes (ANCOM-II) method, which handles zero-inflated data (Kaul et al., 2017; Mandal et al., 2015). The ANCOM-II script was downloaded from https://github.com/FrederickHuangLin/ANCOM and parameter settings included removing genera with zero counts in 90% or more of samples, applying a false discovery rate (FDR) correction for multiple comparisons was applied to p-values, setting a significance level of 0.05, and adjusting for Cage Assignment as a co-variant.

We visualized the relative abundance of significant genera detected by indicspecies and ANCOM-II using the pheatmap package (ver.1.0.12) (Kolde, 2019) to create a heatmap with hierarchical clustering of both rows (genera) and columns (samples). The relative abundance of genera was log10 transformed for visualization purposes.

3.4. Statistics

All data were analyzed with Sex as a variable as we have seen significant sex differences in diet effects previously (Totsch et al., 2017). Data were analyzed by univariate or repeated measures analysis of variance (ANOVA) where appropriate with Diet and Time as variables. Degrees of freedom were corrected for unequal variances using a Greenhouse-Geisser correction. Following CFA administration, a significant repeated measures ANOVA analyzing recovery led to further analyses. Percent baseline was calculated for each daily measure following injection. Groups were considered to have recovered when they reached 90% of their pre-CFA sensitivity. To reduce variability, the pre-CFA baseline was the average of the two weeks prior to CFA as there were no main effects of Time or Diet, and no Time × Diet interaction. Dunnett's tests were used to compare glucose tolerance test data (area under the curve, trapezoidal method) to the REG group. In all cases an

alpha level of 0.05 was considered significant.

4. Results

4.1. Body weight

For simplicity of presentation, all body weight data were analyzed and presented in two-week increments. There was a main effect of Time $(F_{(8, 112)} = 84.432, p < 0.001)$, Sex $(F_{(1, 14)} = 80.344, p < 0.001)$ and Diet $(F_{(3, 14)} = 3.613, p < 0.05)$ (Fig. 2). There was a Time by Sex by Diet interaction $(F_{(24, 112)} = 3.850, p < 0.01)$.

4.2. Von Frey testing

There was no effect of Time, Diet or Sex (p's > 0.05).

4.3. Inflammatory chronic pain

Diet groups were considered recovered once they reached 90% of their pre-CFA baseline measure. There was a main effect of Time $(F_{(6, 84)} = 25.484, p < 0.001)$ and a Time by Diet interaction $(F_{(18, 84)} = 1.943, p < 0.05)$. There was no main effect of Sex or Diet (p's > 0.05). Mice that consumed the SAD displayed allodynia on days 1–11 and the group returned to 90% pre-CFA sensitivity by day 13 (Fig. 3). Mice that consumed the REG displayed allodynia on days 1–6 and the group returned to 90% pre-CFA sensitivity by day 8. Mice that consumed the AID displayed allodynia on days 1–4 and the group returned to 90% pre-CFA sensitivity by day 6. Of note, mice in the AID-SAD group displayed allodynia on days 1–11 and they returned to 90% pre-CFA sensitivity by day 13. Full testing days are also available as percent of baseline (Supplemental Fig. S1) or as raw data (Supplemental Fig. S2).

4.4. Intraperitoneal glucose tolerance test (IP GTT)

After 8 weeks of diet consumption, glucose tolerance was assessed. There was a main effect of Time $(F_{(4, 52)} = 83.372, p < 0.001)$ and Sex $(F_{(1, 13)} = 10.022, p < 0.01)$. There was no effect of Diet.

Similarly, following 16 weeks of diet consumption, there was a main effect of Time $(F_{(4, 56)} = 22.487, p < 0.001)$ and Sex $(F_{(1, 14)} = 5.893, p < 0.05)$. There was no effect of Diet.

Following analysis of area under the curve, at 8 weeks there was a significant Sex effect $(F_{(1, 14)} = 5.080, p < 0.05)$, but no effect of Diet (Fig. 4A). At 16 weeks there was no effect of Sex or Diet (p's > 0.05)
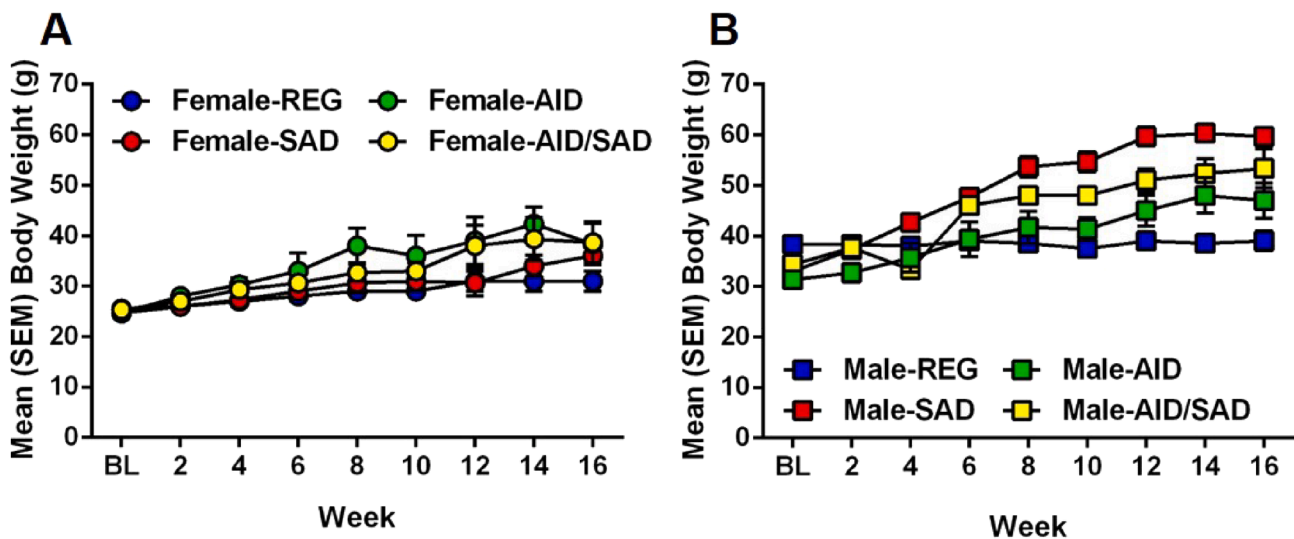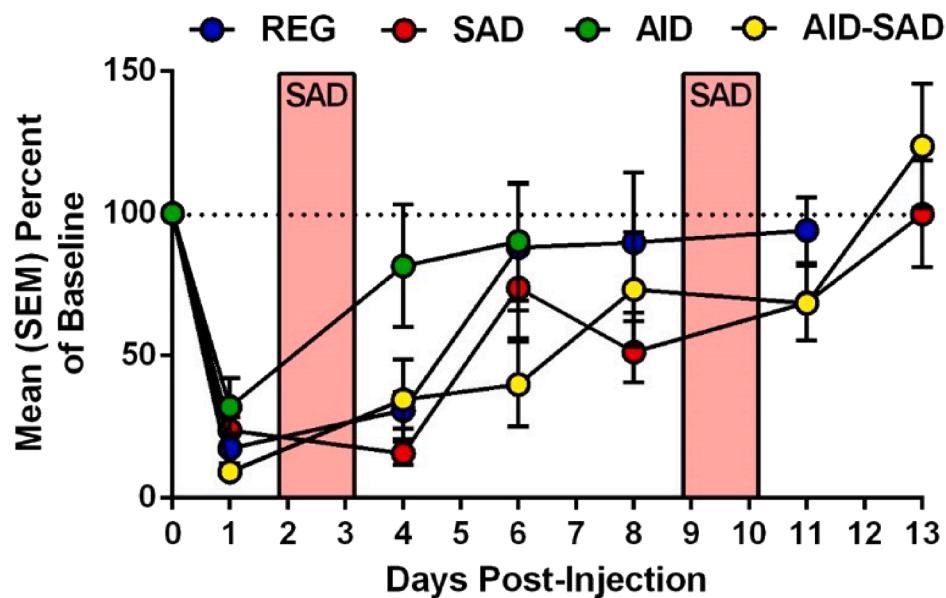

Fig. 2. Weight changes during diet exposure. Body weight (grams) for (A) female and (B) male mice during 16 weeks of diet consumption. All data are expressed as mean ± SEM.

Fig. 3. Effects of the SAD and AID on recovery. Hypersensitivity following CFA administration as measured by 50% withdrawal threshold. Dotted line represents baseline sensitivity. Experimental groups were considered as returned to baseline when the group threshold achieved 90% of pre-CFA sensitivity. All lines discontinue at the point of return to baseline thresholds. All data are expressed as mean ± SEM.
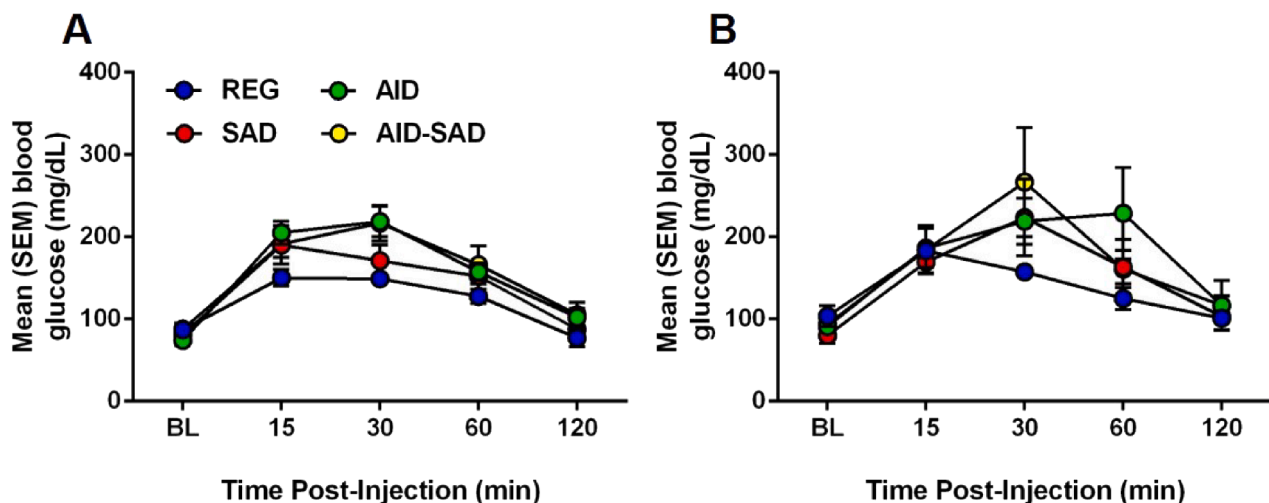

Fig. 4. Effects of the SAD and AID on glucose tolerance. Blood glucose levels (mg/dL) following glucose injection at (A) 8 and (B) 16 weeks of diet exposure. All data are expressed as mean ± SEM.

(Fig. 4B). At both 8 and 16 weeks, male mice tended to show greater responsivity to glucose administration, when compared to female mice.

4.5. Microbiome

Microbial amplicon sequence variants (ASVs) were classified for a total of 22 fecal samples collected after 16 weeks of diet consumption. Three samples had less than 1000 sequence counts and were excluded from further analyses. The final group sizes for microbiome analysis were: REG (n = 4, 2 males), SAD (n = 4, 1 male), AID (n = 5, 1 male), and AID-SAD (n = 5, 3 males). After quality-filtering with the Qiime2 DADA2 plugin and exclusion of samples with low counts, sequence libraries contained an average of 52,862 (31,863–83,610) sequences per sample. Overall, microbial community structure differed by diet treatment (Fig. 5). The most abundant phyla in all diet treatments were Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Fig. 6). Shapiro-Wilk normality tests, histograms, normal probability plots of

model residuals indicated that log transformation improved the normality of Actinobacteria and Proteobacteria data and the reciprocal log transformation improved the normality of Firmicutes data. Generalized linear models (GLMs) indicated there were no mains effect of Diet or Cage Assignment on the relative abundance of Bacteroidetes and Firmicutes (Supplemental Table S3). Diet treatment significantly affected the relative abundance of Actinobacteria (F (3,10) = 4.788, p = 0.03) and Proteobacteria (F (3,10) = 11.348, p = 0.001), while the main effect of Cage Assignment and the interaction between Diet and Cage Assignment were not significant. Tukey's honestly significant difference (HSD) tests (Supplemental Table S4) indicated that mice administered the SAD diet had the highest relative abundance of Proteobacteria (mean = 23.5 ± 7.2%), which was significantly greater than the relative abundance of Proteobacteria in the AID (mean = 1.5 ± 0.7%, padj = 0.002) and AID-SAD (mean = 1.6 ± 0.4%, padj = 0.004) groups. Actinobacteria had a greater relative abundance in AID (mean = 18.9 ± 14.3%, padj = 0.03) and AID-SAD (mean = 13.4 ± 6.2%, padj = 0.02)
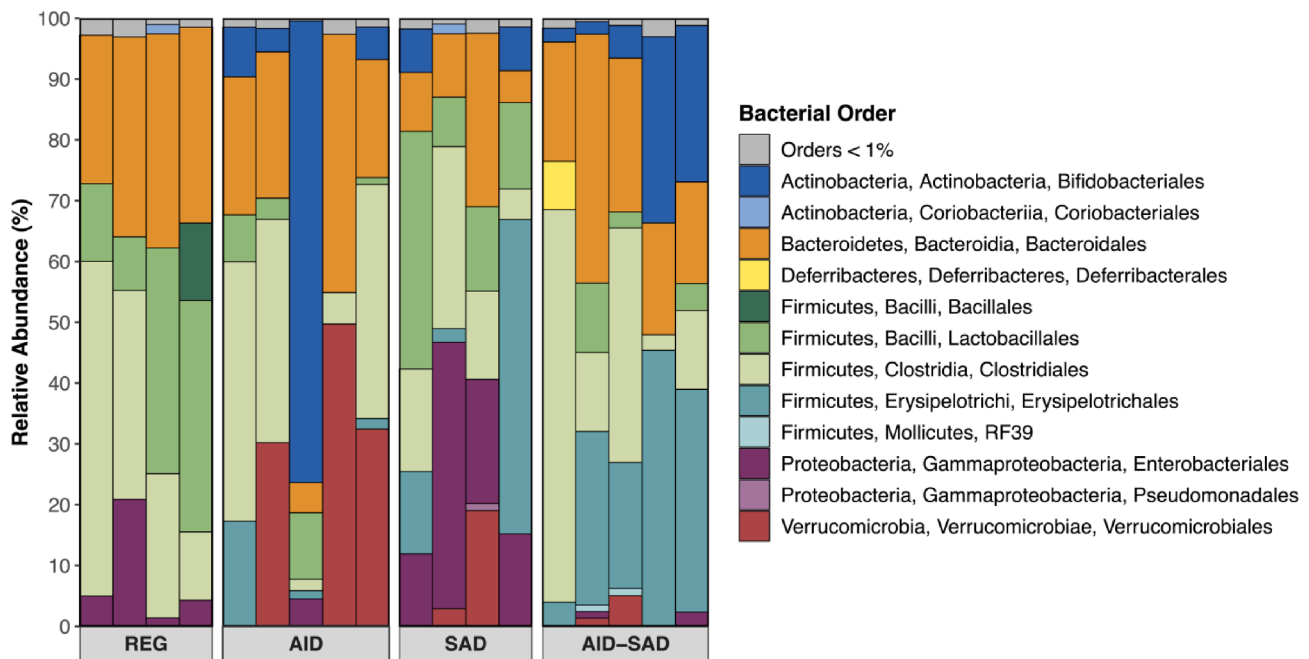
Fig. 5. Influence of diet on microbial community composition. 16S rRNA gene sequence-based microbial community composition expressed as the percent relative abundances of bacterial orders representing >1% of the total sequence reads.

groups compared to the REG group (mean = 0.7 ± 0.3%).

Shapiro-Wilk normality tests as well as histograms and normal probability plots of GLM model residuals indicated that normality of Faith's phylogenetic diversity was improved with log transformation while the Shannon Index of evenness did not need transformation. Diet significantly altered microbial alpha diversity (Fig. 7A, B) based on the Shannon Index of evenness (Diet: F (3,10) = 4.355, p = 0.03), and Faith's phylogenetic diversity (Diet: F (3,10) = 9.690, p = 0.003). Mice that consumed the AID had the lowest alpha diversity metrics, while mice that consumed the REG had the highest alpha diversity metrics, and consumption of AID-SAD and SAD yielded values in between (Supplemental Table S6). There was no main effect of Cage Assignment on either of the alpha diversity metrics, while there was a significant interaction between Diet and Cage Assignment for both metrics (Supplemental Table S5). Diet explained greater than 55% of the variation in alpha diversity (Shannon Index: Diet PES = 0.566; Faith's PD: Diet PES = 0.744), while Cage Assignment explained a lower proportion of the variation (Shannon Index: Cage Assignment PES = 0.124; Faith's PD: Cage Assignment PES = 0.348).

Permutational analysis of variance (PERMANOVA) indicated that Diet significantly affected microbial community composition based on the Aitchison distance metric (Diet: F (3,10) = 2.621, p = 0.001; Fig. 7C), while the main effect of Cage Assignment and the interaction between Diet and Cage Assignment were not significant (Supplemental Table S7). Moreover, R-squared values indicated 34% of the variation in microbial community composition was explained by Diet while Cage Assignment explained only 5% of the variation. Pairwise PERMANOVA indicated that the community composition of mice fed AID was significantly different from that of mice fed REG (FDR padj = 0.02) and SAD (FDR padj = 0.02; Supplemental Table S8). However, there was no difference between the microbial composition of AID-fed mice and AID-SAD-fed mice (FDR padj = 0.3). Permutation tests for homogeneity in multivariate dispersion (PERMDISP) revealed that level of dispersion among diet groups was not highly significant (F (3,14) = 3.351, p = 0.05). This indicates that compositional differences detected by the PERMANOVA analyses were largely due to samples occupying a different location in ordination space (i.e., a shift in community composition), rather than due to large differences in group dispersion.

4.6. Bacterial taxa as indicators of diet

We identified candidate 'indicator' taxa at the genus level that were significantly associated with each diet using both indicator taxa analysis (indicspecies) and differential abundance testing (ANCOM-II) (Supplemental Tables S9 and S10). We visualized the log10-transformed relative abundance of significant genera in a heat map (Fig. 8). Samples clustered into three groups based on the abundance patterns of the six 'indicator' genera, with AID and AID-SAD samples forming one cluster and SAD and REG samples forming two separate clusters. SAD samples contained *Pseudomonas* while these taxa were largely absent in AID and AID-SAD diets. Similarly, REG samples contained genera that were also nearly absent in AID and AID-SAD diets. Of the 350 amplicon sequence variants (ASVs) identified in this study, three belong to the *Pseudomonas* genus. All three *Pseudomonas* ASVs had greater relative abundance in SAD samples compared to AID and AID-SAD samples (Supplemental Fig. S3).

5. Discussion

Obesity is associated with many chronic disorders, including chronic pain. There is also a significant role for microbiota in obesity (Turnbaugh et al., 2006, 2008; Ley et al., 2005). Using two previously-developed diets, the Standard American Diet (SAD) and the Anti-Inflammatory Diet (AID) (Totsch et al., 2017, 2018a, 2018b), we were interested in investigating the impact of alternating between the diets on recovery from inflammatory injury and glucose tolerance. By feeding animals the AID on Monday to Friday, and the SAD on Saturday and Sunday, we were able to mimic dietary "cheat days" that characterize common eating patterns in Americans.

Previously, we have demonstrated that consuming a Total Western Diet resulted in enhanced hypersensitivity in male mice following CFA administration (Totsch et al., 2016). We then used our newly-developed, translatable SAD to mimic a poor-quality American diet in rats. We found the SAD significantly prolonged recovery time and increased the activation of immune cells in the spinal cord (Totsch et al., 2017). In mice, prolonged access to the SAD also increased T cell infiltration in the spinal cord and brain (Totsch et al., 2018b). Recently, we investigated
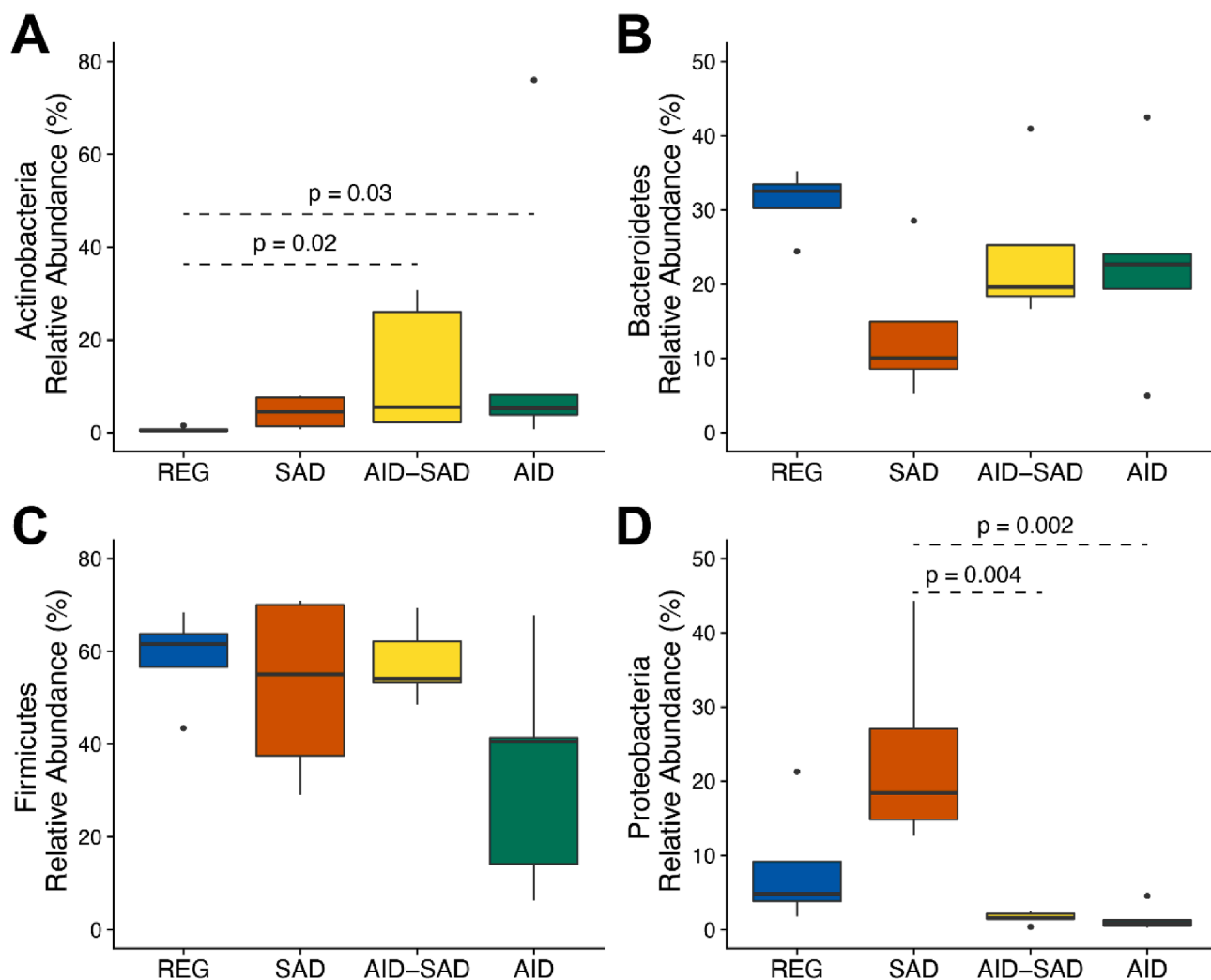
Fig. 6. Percent relative abundance of bacterial 16S ribosomal RNA gene sequences summarized at the phylum level for the top four most abundant phyla, (A) Actinobacteria, (B) Bacteroidetes, (C) Firmicutes, and (D) Proteobacteria. P-values (alpha < 0.05) are shown for Tukey's honestly significant difference (HSD) pairwise comparisons following significant one-way analysis of variance (ANOVA) tests on general linear models (GLMs). GLMs accounted for the main factors Diet and Cage Assignment, as well as their interaction. Whiskers represent 1.5 times the inter-quartile range and data beyond the end of the whiskers are plotted as outlier points.

the ability of our novel AID to be used as an intervention in mice. Similar to our work with rats, there was an increase in recovery time for SAD-consuming mice. Interestingly, we found that prophylactic consumption of the AID and AID-intervention at the time of injury led to improved recovery times (Totsch et al., 2018a). The present study, once again, replicated our previous findings with the SAD. Mice on the SAD were allodynic for 11 days while REG mice displayed allodynia for 6 days (Fig. 3). Of note, AID-consuming mice were only allodynic for 4 days. Interestingly, mice that consumed the AID during the week and SAD on the weekends displayed allodynia for 11 days, the same as that of SAD-fed mice. This suggests that switching to a poor-quality diet led to detrimental effects on recovery, despite consuming a healthier diet for the majority of the time. Chronic consumption of the AID promoted recovery, while continual or intermittent consumption of the SAD prolonged recovery.

When assessing glucose tolerance at weeks 8 and 16, there were no significant effects of consuming the SAD (Fig. 4). This is in contrast to our previous work using the SAD in which consumption of the SAD elevated blood glucose levels and reduced glucose tolerance (Totsch et al., 2017, 2018a). The SAD is considered a high-carbohydrate, high-fat diet. Therefore, it is plausible that the increased carbohydrate ratio resulted in tolerance to an intraperitoneal glucose injection. There is

evidence that a high-carbohydrate diet does not affect glucose tolerance following intravenous glucose and decreases blood glucose following oral glucose test (Silva et al., 1987). In contrast, consumption of the AID in the present study led to an increase in blood glucose in female mice. A previous study found that a low-carbohydrate, high-fat diet in rats resulted in impaired glucose tolerance following intraperitoneal glucose tolerance tests (Bielohuby et al., 2013). Similarly, another study found that a low-carbohydrate, high-fat diet increased glucose intolerance in mice (Lamont et al., 2016). Since the AID is a considered a high-fat diet, our data support the findings from these studies.

In the present study, we were also interested in the ways in which alternating diets would affect the gut microbiota of mice. Thus, we sequenced microbial communities in fecal samples following 16 weeks of diet consumption. Loss of microbial diversity (LOMD) has been considered a common feature of many digestive diseases (Caporaso et al., 2010; Matsuoka and Kanai, 2015) and is frequently reported to be associated with obesity (Turnbaugh et al., 2008). However, meta-analyses re-examining published microbiome data have found inconsistent relationships between alpha diversity and disease or obesity (Duvallet et al., 2017; Walters et al., 2014; Sze and Schloss, 2016). In the present study, mice fed AID and AID-SAD had lower alpha diversity in terms of species evenness and phylogenetic diversity compared to REG-
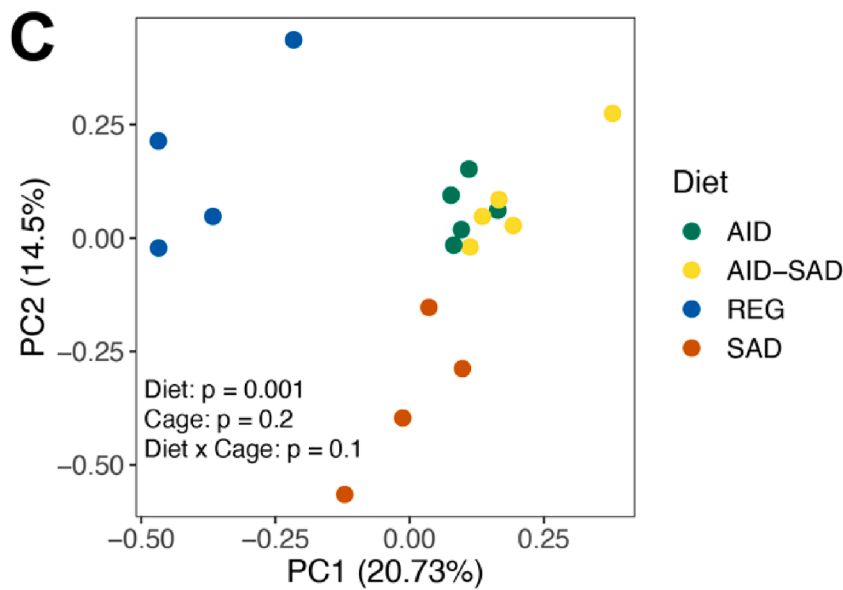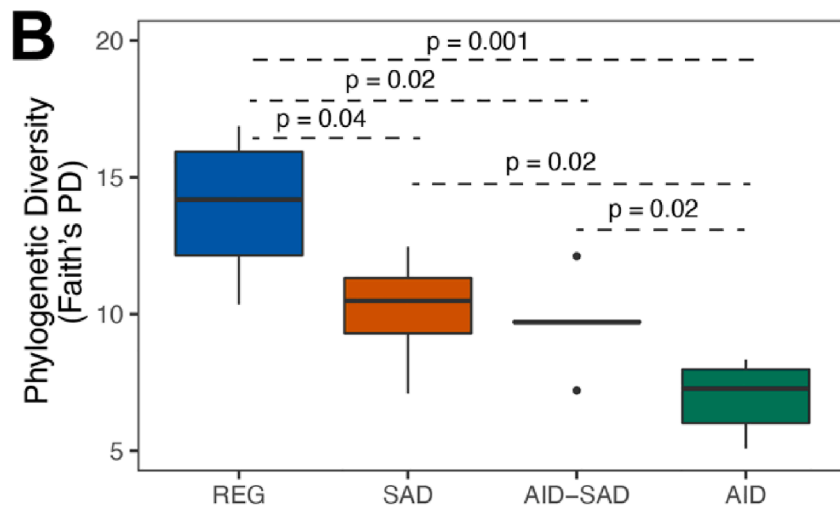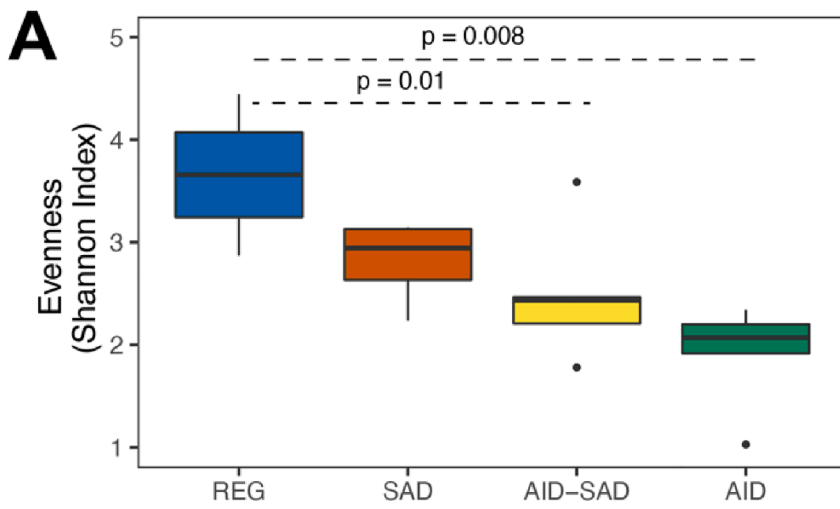
Fig. 7. Influence of diet on alpha diversity and overall community composition. Alpha diversity is shown in panels (A) Shannon Index of evenness and (B) Faith's Phylogenetic Diversity. In panels A and B, P-values (alpha < 0.05) are shown for Tukey's honestly significant difference (HSD) pair-wise comparisons following significant one-way analysis of variance (ANOVA) tests on general linear models (GLMs). GLMs accounted for the main factors Diet and Cage Assignment, as well as their interaction. Whiskers represent 1.5 times the inter-quartile range and data beyond the end of the whiskers are plotted as outlier points. Beta diversity is shown in panel (C) the centered log-ratio transformation Aitchison distance. In panel C, each solid point corresponds to an individual sample and points closer together represent samples with similar compositions. P-values from permutational analysis of variance (PERMANOVA) are shown for the main factors Diet and Cage Assignment, as well as their interaction.
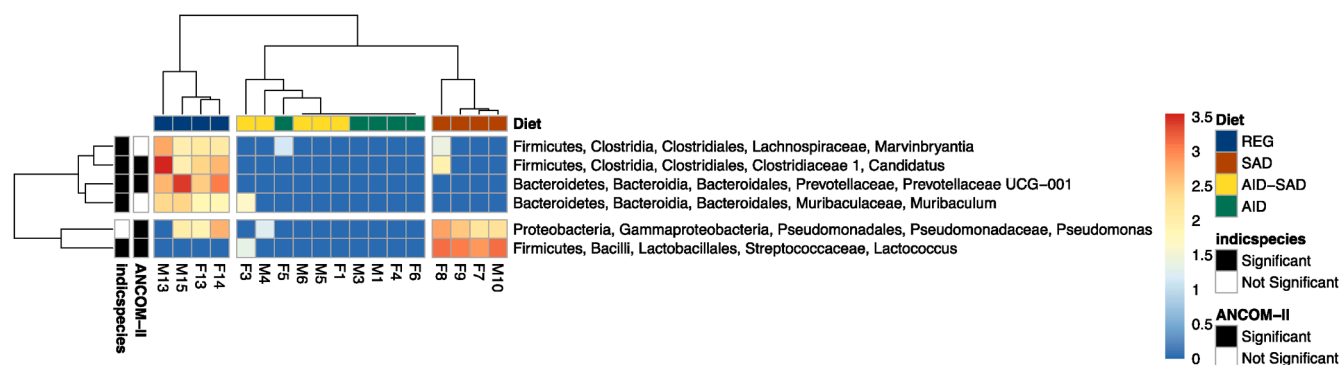
Fig. 8. Bacterial indicators of diet. 'Indicator' taxa were identified at the genus level using both indicator taxa analysis (indicspecies) and differential abundance testing (ANCOM-II). Relative abundances of the indicator taxa were log10 transformed for visualization and each column represents a sample from an individual mouse while each row represents an 'indicator' taxa. Hierarchical clustering of columns and rows is based on the abundance patterns of these 'indicator' taxa.

fed mice (Fig. 7). However, there was no apparent relationship between alpha diversity and body weight or recovery time. Thus, in agreement with previous meta-analyses, we found that microbial diversity was not associated with body condition or recovery time.

As in humans, microbial communities in the mouse gastrointestinal tract are generally dominated by the phyla Firmicutes and Bacteroidetes, followed by the lesser abundant phyla Actinobacteria and Proteobacteria. In our study, Proteobacteria were significantly lower in mice that consumed the AID and AID-SAD compared to mice that consumed the SAD. An increase in Proteobacteria is a sign of gut imbalance (Shin et al., 2015) and is seen in disease states such as metabolic disorders (Fei and Zhao, 2013) and in diabetic patients (Larsen et al., 2010). Numerous studies have shown that mice fed a high-fat diet have a significantly higher relative abundance of Proteobacteria compared to mice fed standard chow (Everard et al., 2014; Wang et al., 2015; Zhang et al., 2012). Both SAD and AID are considered high-fat diets (35.6 and 39.9% kcal from fat, respectively); however, the main sources of fat in SAD are lard, beef tallow, milkfat and hydrogenated vegetable shortening while the main sources in AID are flaxseed and soybean oil. In our study, the relative abundance of Proteobacteria in SAD-fed mice was ~15 times higher than in AID-fed and AID-SAD mice (Fig. 6**D**), indicating that the source, not just the content, of dietary fat may be an important factor in regulating Proteobacterial populations. Together with the results that SAD prolonged allodynia (Fig. 3), this suggests that diet-mediated changes in microbiome composition may play a role in regulating inflammatory chronic pain and are worthy of further future investigation.

We explored deeper taxonomic trends to identify statistically robust associations between diet and 'indicator' taxa at the genus level (Fig. 8). Consumption of the SAD diet increased the relative abundance *Pseudomonas* while these taxa were largely absent in the microbiota of mice fed AID and AID-SAD. Members of this genera have been implicated as plant, insect and human pathogens (Silby et al., 2011). However, full metabolic and physiologic repertoire of *Pseudomonas* has yet to be described and the specific metabolic roles and immunomodulatory properties of various *Pseudomonas* spp. in the mammalian gut is unknown.

The present data suggest that intermittent exposure to the SAD had similar detrimental effects on recovery to long-term exposure. Furthermore, SAD consumption promoted the growth of Proteobacteria potentially pathogenic taxa in the genus *Pseudomonas*. Alternatively, consumption of AID may reduce the growth of these taxa and limit their potential immunomodulation effects.

6. Data availability statement:

The raw fastq files generated for this study are available under the NCBI BioProject number PRJNA615647.

Author Contributions

RES and SKT designed the experiments, SKT tested the animals, SKT, TLQ, RYM and SAL performed the blood testing and specimen collection. KMK performed microbiota analyses. RES, SKT and BAG designed the SAD and AID with the assistance of Dr. Tina Herfel (Envigo). RES, SKT and KMK wrote the manuscript and all authors discussed the results and provided comments on the manuscript.

Conflict of Interest

The authors declare no conflict of interest regarding this publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynpai.2020.100053.

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