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Original Article

Polypharmacy With High Drug Burden Index (DBI) Alters the Gut Microbiome Overriding Aging Effects and Is Reversible With Deprescribing

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Abstract

Aging, medication use, and global function are associated with changes in the microbiome. However, their interrelationships and changes over time require further characterization. In a longitudinal aging mouse study, we investigated the effects of aging, chronic polypharmacy with a high Drug Burden Index (DBI, measure of total anticholinergic and sedative medication exposure) and gradual cessation (deprescribing) on the microbiome, further exploring any association with global outcomes. Chronic administration of high DBI polypharmacy attenuated the aging-related reduction in alpha diversity, which was not sustained after deprescribing. Beta diversity and LEfSe (Linear discriminant analysis Effect Size) features varied with age, polypharmacy, and deprescribing. Aging with and without polypharmacy shared decreases in *Bifidobacteriaceae*, *Paraprevotellaceae*, *Bacteroidaceae*, and *Clostridiaceae*, while only aging with polypharmacy showed increased LEfSe features. Microbiome diversity correlated with frailty, nesting, and open field performance. Polypharmacy deprescribing reversed changes that occurred with treatment. However, the microbiome did not recover to its pretreatment composition at 12 months, nor develop the same aging-related changes from 12 to 24 months as the control group. Overall, aging, chronic polypharmacy, and deprescribing differentially affected the diversity and composition of the gut microbiome, which is associated with frailty and function.

Keywords: Anticholinergics, Frailty, Gut microbiota, Sedatives

Globally adults aged 65 years and older are a growing population, and with a high prevalence of multimorbidity, have high medication use (1). Polypharmacy, the use of 5 or more concurrent medications, is prevalent in one to two third of older people in studies internationally (1–3). The World Health Organization recognizes polypharmacy as a major risk to patient health when inappropriately prescribed, promoting "medication safety in polypharmacy" as a target to achieve the current "*Medication Without Harm*" Global Patient Safety Challenge (4). For older adults living in the United States, Europe, and Australia, antihypertensive and lipid-lowering medication classes have some of the highest prescription rates (5–7). Use of anticholinergic and sedative medications is also increased in older adults, consistent with an increase in the prevalence of indications including pain, urinary incontinence, mental health disorders, and sleep changes (8).

The Drug Burden Index (DBI) is a clinical risk assessment tool that measures total anticholinergic and sedative medication exposure (9). DBI for each prescribed sedative/anticholinergic medication is calculated from an estimate of the dose–response equation (daily dose taken/[minimum recommended daily dose

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+ daily dose taken]), and DBI for the individual is the sum of the DBIs of each sedative/anticholinergic medication that they take (9). Polypharmacy and increasing DBI are associated with adverse geriatric outcomes, including impaired physical and cognitive function (10). Inappropriate polypharmacy can be reduced by withdrawing medicines (deprescribing) (11). Furthermore, physiological changes during aging, including body composition, renal function, and hepatic function, alter the individual's response to medications (12,13).

The gut microbiome changes significantly in a wide range of health conditions and geriatric syndromes, including cardiovascular diseases (14), dementia (15), and frailty (16,17). It also undergoes physiological changes at different life stages, including extensive compositional and functional alterations during aging (15,18).

While the gut microbiome influences disease processes that are indications for medication prescription, it also influences the pharmacokinetics and pharmacodynamics of medications, and medications influence the microbiome (15). Enterohepatic circulation provides direct communication with the gut microbiome, enabling secondary metabolite production and pharmacotherapy, including prodrug activation (15,19). The first comprehensive in vitro study of nonantibiotic medications showed 24% of approximately 1 200 medications, inhibit at least 1 of 40 common human gut microbes (19). Recent case–control and cross-sectional human studies have further characterized microbiome changes with the use of nonantibiotic medications, including antihypertensives, lipid-lowering agents, anticholinergics, and sedatives (20–25). Furthermore, polypharmacy, independent of the type of medications used in combination, is associated with changes in the microbiome (20,21).

Further studies are needed to elucidate the effects of multiple medications on the microbiome, in vivo effects, and whether microbiome changes are related to functional performance. While interpretation of human studies is limited by variations in diet, disease, and medications (number and type), animal studies can control these variables to elucidate the biological effects of aging, or of a treatment regimen on the microbiome.

We previously developed a chronic polypharmacy aging mouse model and found that mice chronically treated with high DBI polypharmacy became frailer and had impaired physical function and activities of daily living (26), while deprescribing reversed some of these findings. Here we use our aging mouse model of polypharmacy to evaluate the effects of aging and polypharmacy on the microbiome in vivo. This study aims to investigate the effect of aging, polypharmacy, and aging with polypharmacy, on the microbiome as measured by alpha and beta diversity indices, and LEfSe (Linear discriminant analysis Effect Size) features. It further explores whether polypharmacy-related microbiome changes are reversible with deprescribing. Finally, we investigate any correlations between microbiome changes and in vivo frailty/function, affected by this polypharmacy regimen.

Materials and Methods

Animals

In this study, the C57BL/6J (B6) male mice were obtained from and housed at the Kearns facility (Kolling Institute of Medical Research, Sydney, Australia). All procedures and experiments were approved by the Animal Ethics Committee of the Northern Sydney Local Health District, Sydney, Australia (RESP/15/21). Animal monitoring (health status checks) and food and water replenishments occurred weekly. "Breeder" mice at the Kearns facility are replaced every ten generations with breeders from the Animal Resource Centre in Perth, WA, Australia, to maintain genetic similarity with the Jackson laboratory. At 6–8 weeks, mice were housed in cages of up to 5, maintained on a 12-hour light–dark cycle (lights on 07:00, lights off 19:00) with ad libitum access to water and regular chow provided by the facility (Rat and Mouse Premium Breeder Diet containing 23% protein, Gordon Specialty Feed, NSW, Australia).

The study design is summarized in Supplementary Figure 1. At 10 months, all animals were switched to the base-diet (Standard Meat Free Mouse and Rat Feed, 20% protein, Specialty Feeds, Western Australia, Perth, Australia), which they were maintained for the remainder of the study. Animals were randomly allocated to treatment groups after the first round of functional testing at 12 months. This consisted of receiving medication in their water and/or food (polypharmacy) or received no added medication at all (control). As detailed in (26), the larger study comprised 13 cohorts of mice, each group staggered 3–4 weeks from the preceding group when obtained, and in all scheduled experimental procedures that followed. Each cohort comprised a maximum of thirty mice, with n = 2–5 mice representing each treatment group. Mice involved in the microbiome study were from cohorts 6–14.

Drug Treatment Groups

Within each cohort, animals were randomly assigned to either control or high DBI polypharmacy. Animals assigned to high DBI polypharmacy received: Simvastatin (20 mg/kg/d), metoprolol (350 mg/kg/d), oxybutynin (27.2 mg/kg/d), citalopram (15 mg/kg/d) in food, and oxycodone (5 mg/kg/d) in water. Criteria to select these medications ensured drug classes are commonly used by older Australians, are rarely dose-adjusted in old age, show similar pharmacokinetic and pharmacodynamic profiles in mice and humans, and did not show toxic effects in previous mouse studies when administered alone (26).

After completing all functional tests at 21 months, animals receiving medication treatment were stratified to be deprescribed or to remain on the medication, ensuring an equal distribution of animals into each group. This stratification was based on their frailty index score (27) (frailty assessment detailed later), equally distributing animals according to frailty into each group, to ensure prescribed and deprescribed animals had the same frailty distribution at 21 months.

Animal Handling and Frailty, Nesting, Open Field, and Blood Pressure Measurement

Animals were routinely handled by the researchers during weekly monitoring checks and additional scheduled experiments. To reduce anxiety, the same researchers were responsible for conducting the experiments, and apart from the Kearns facility staff, the animals were not exposed to any other individuals. Additionally, animals were tested during their light cycle and habituated to the room for 30 minutes before undertaking any experimental procedure.

The frailty, nesting and open field assessments, and blood pressure measurement on the same mice are described briefly later with further details in (26). Utilizing the Mouse Clinical Frailty Index as an assessment tool, frailty was assessed as a cumulation of deficits over 31 parameters (scored as 0, 0.5, or 1) that included systemic features (weight/temperature) and changes in various systems, including the nervous system, musculoskeletal, integumentary, respiratory, and gastrointestinal (27). Frailty assessment was carried out by one researcher (J.M.) throughout the study, who was blinded to animal ID and treatment group.

Nesting assessment indicated the in-cage daily activities of mice in a naturalistic setting, using methods developed by Hess et al. (28). Scoring was completed at each age point by 2 independent treatment-blinded researchers. Nests were built by all cage members, so mice in the same cage received the same nesting score. Each nest was scored in 4 separate quadrants that were summed together for the total score (max. 20). Each quadrant scored 0–5 according to the criteria: 0—undisturbed; 1—disturbed; 2—flat; 3—cup; 4—incomplete dome; 5—full dome.

Open field provided an enclosed spaced (open at the top) in which each mouse was placed alone for 5 minutes under red light, allowing recording of their movement from a camera mounted above. These methods were adapted from Justice et al. (29), and developed to translationally capture the functional assessment of locomotion in humans. The ANY-maze program (ANY-maze, Stoelting Co., Wood Dale, IL) was then used to measure the distance traveled by each mouse from the video recordings, which were labeled blinded to treatment condition.

Blood pressure measurement occurred on the same 2 days for mice in the same cohort, and at the same time of the 12-hour light cycle (1–5 PM) for different cohorts of mice staggered 3–4 weeks apart to ensure testing at the same age. Systolic and diastolic measurements were automated using the CODA noninvasive blood pressure system (Kent Scientific Corporation, Torrington, CT), and the tail-cuff method was used.

Fecal Boli Collection

Animal feces were collected during the routine blood pressure measurements (within 12-hour light cycle between 1 PM and 5 PM), during which animals remained immobile and isolated from other cage members for about 15 minutes at a time. On rare occasions where the mouse did not produce feces, samples were collected, under identical conditions, during the following blood pressure measurement 2 days later. Collected samples were snap frozen in liquid nitrogen and transported to be stored in a −80°C freezer. All equipment, including the blood pressure cuffs were sanitized with 70% ethanol and wiped dry between use in different animals.

DNA Extraction and 16S Sequencing

Microbial DNA was extracted from samples using FastPrep DNA extraction kits. Extracted samples were then aliquoted onto a PCR plate and sent to Ramaciotti, UNSW, for 16S sequencing of the V3-V4 (341f-805r) regions, with MiSeq v3 2x300bp sequencing.

Microbiome Sequencing Data Processing and Statistical Analysis

Amplicon data were denoised with dada2 (30) embedded in qiime2 (31). Host contamination was removed using Bowtie 2 (version 2.4.2) (32). Taxonomy annotation of the data was performed using qiime2 feature classifier plugin with the relevant greengene database and ITS database, respectively.

R (version 4.0.4) packages qiime2R (33) and phyloseq (34) were employed for diversities analysis. Mann–Whitney–Wilcoxon test was applied in the comparison of the means of the Alpha diversities between different groups. To access the significance of treatments and other metadata variables between 2 distance matrices in the Beta diversity analysis, adonis (Permutational Multivariate Analysis of Variance Using Distance Matrices) was used to permute the distance matrix 999 times to yield p values and ESS (explained sum of squares). In the identification of treatment-associated signatures, LEfSe (35) were evaluated with alpha value for the factorial Kruskal–Wallis test among classes equals 0.5 and threshold on the logarithmic LDA score for discriminative features equals 2.

To interpret findings from the microbiome data, aging effects independent of polypharmacy use were observed in microbiome changes within control mice from 12 to 24 months. The effect of aging with high DBI polypharmacy was assessed in high DBI polypharmacy mice from 12 to 24 months. Changes from 12 to 15 months in high DBI polypharmacy mice characterized the main medication effects. In the subset of high DBI polypharmacy mice that were deprescribed from the medications, changes from 15 to 24 months and 21 to 24 characterized were assessed for deprescribing effects.

Results

Aging and Deprescribing Polypharmacy Both Affect Alpha Diversity. These Relationships Are Reflected Through Correlations With Nesting and Frailty

Several measures of alpha diversity were obtained (Figure 1A and B; Supplementary Figure 2A–D), and comparisons made between groups and at different timepoints (ages). Among nonmedicated aged control mice, Shannon alpha diversity decreased from 12 to 21 months (p = .021) and from 12 to 24 months (p = .041; Figure 1A and B). During the first age-associated changes at 21 months, Shannon alpha-diversity was negatively correlated with frailty (Supplementary Figure 3C), with no significant relationship at any other timepoint, including 24 months (Supplementary Figure 2 A, B, and D). At 12 months, alpha diversity was positively correlated with higher nesting performance (Supplementary Figure 3A), and there was no relationship with nesting at other timepoints.

Comparing alpha diversity in high DBI polypharmacy to control at each timepoint, we saw no significant difference to suggest polypharmacy effects (Supplementary Table 2). However, unlike the control, the polypharmacy group showed no significant change between timepoints on either measure of alpha diversity (Figure 1A and B; Supplementary Table 2), suggesting treatment effects of this specific polypharmacy regimen might have overridden aging

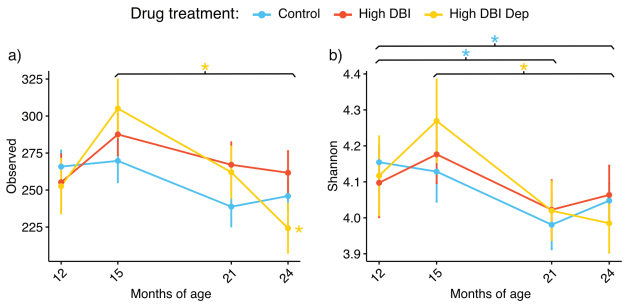

Figure 1. Alpha diversity measures: (**A**) Observed features and (**B**) Shannon index, of all mouse treatment groups (control = unmedicated mice; High DBI = mice receiving high DBI polypharmacy from 12 months; High DBI Dep = mice receiving high DBI polypharmacy from 12 months with deprescribing starting at 21 months). Animal numbers in each treatment group at each timepoint are summarized in Supplementary Table 1. Comparisons between age points and treatment groups were conducted using Mann–Whitney–Wilcoxon test. Supplementary Table 2 lists statistically significant comparisons; p values less than .05 are reported as significant and indicated by an * in the color of the treatment group between timepoint comparisons at which there is a significant difference. Comparing observed features, there was an overall significant change in polypharmacy deprescribed overtime (p = .026), and significant decrease from 15 to 24 months in polypharmacy deprescribed animals (p = .011). Analyses were performed using "R (version 4.0.4) packages. DBI = Drug Burden Index; Dep = deprescribed. Refer to online version for access to the colour figures.

effects. This is further supported by polypharmacy deprescribed mice showing an overall decrease (p = .026) in observed alpha diversity over time, which decreased significantly from 15 months (first timepoint following medication prescription) to 24 months (first timepoint following deprescription; p = .011).

This high DBI polypharmacy regimen prevented age-related changes seen in alpha-diversity of the microbiome of control animals, and deprescribing removed medication effects. Further examining the correlations, we found that alpha-diversity and frailty/function correlated with each other in polypharmacy-treated mice, which occurred at 15 months (main medication effects) and 24 months (deprescribing effects). Mice administered high DBI polypharmacy showed a significant positive correlation between frailty and alpha-diversity only at 15 months (Supplementary Figure 4A), and in deprescribed mice, alpha-diversity was negatively correlated with nesting at 24 months (Supplementary Figure 4D).

Beta Diversity Changes With Aging, Polypharmacy, and Deprescribing, and Correlates With Frailty, Nesting Performance, and Open Field Performance

Beta diversity changes were measured by comparing the Principal Coordinates Analysis (PCoA) plots evaluated by Bray-Curtis distances, between groups. Several measures of beta-diversity were obtained (Figure 2A and B; Supplementary Figure 5A–C), and associations with metadata including mouse food intake, water intake and number of mice per cage were explored (Supplementary Figure 6A-C). Analyses showed significant overall change with age and drug treatment (p < .05; Supplementary Table 3; Figure 2A and B). Comparing successive age points for control mice indicated a significant change only occurred between 21 and 24 months.

Comparing control and high DBI polypharmacy mice, at 12 months, we saw no difference between the groups (Figure 2A). After randomization to treatment groups, control and high DBI

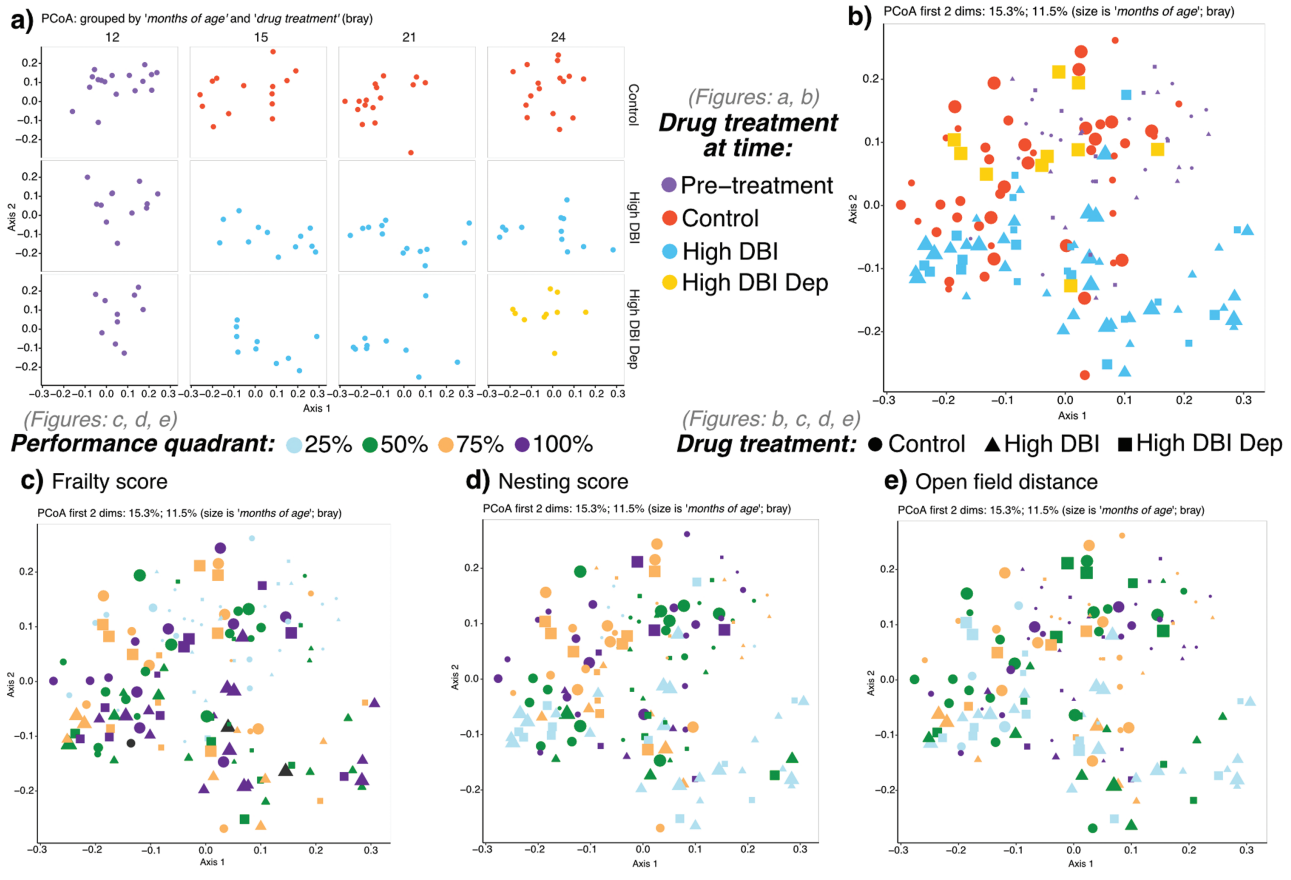

Figure 2. Principal Coordinates Analysis (PCoA) plots using Bray-Curtis dissimilarities of mouse microbiome samples at 12 months to 24 months. (**A**) Displays beta-diversity at each age point (12, 15, 21, and 24 months) sorted according to allocated treatment group (pretreatment = at 12 months before treatment group randomization; control = unmedicated mice; High DBI = mice receiving high DBI polypharmacy from 12 months; High DBI Dep = mice receiving high DBI polypharmacy from 12 months with deprescribing starting at 21 months). Figure 2(**B**) Superimposes all beta-diversity data points in (**A**). In Figure 2(**A** and **B**) color represents treatment group at timepoint (purple = pretreatment; red = control; blue = high DBI; yellow = high DBI deprescribed). In Figures 2(**B**–**E**) shape represents final treatment group at 24 months (circle = control; triangle = High DBI; square = High DBI deprescribed), and size of indicator displays different timepoints (12 months = smallest indicator size, increasing through 15 and 21 months, to 24 months = largest indicator size). Statistically, significant comparisons in Figure 2(**A** and **B**) are summarized in Supplementary Table 3; p values less than .05 are reported as significant and indicated by an *. Figures 2(**C**–**E**) display: (**C**) total frailty score where higher frailty score indicated higher frailty, (**D**) nesting score where higher nesting score indicates better nesting, and (**E**) open field distance traveled where higher distance traveled indicates more activity. Performance is sorted by quadrants for the relative performance of all mice (indicated by color; 25% = lowest to 100% = highest). Beta diversity had a significant relationship with frailty (R2 = 0.022, p = .001), nesting (R2 = 0.025, p = .001), and open field distance traveled (R2 = 0.013, p = .015). Animal numbers in each treatment group at each timepoint are summarized in Supplementary Table 1. Comparisons between age points and treatment groups were conducted using Mann–Whitney–Wilcoxon test. Analyses were performed using "R (version 4.0.4) packages. DBI = Drug Burden Index; Dep = deprescribed. Refer to online version for access to the colour figures.

polypharmacy administered mice were significantly different at 15 months (R^2 = 0.117, p = .002), 21 months (R^2 = 0.108, p = .002), and 24 months (R^2 = 0.098, p = .002; Figure 2A). After deprescribing a subset of high DBI polypharmacy animals at 21 months, high DBI polypharmacy deprescribed was significantly different to high DBI polypharmacy at 24 months (R^2 = 0.089, p = .022), but not to control (R^2 = 0.047, p = .276), indicating a reversal of polypharmacy effects after deprescribing (Figure 2A).

Finally, beta diversity was assessed in relation to metadata variables (Figure 2C–E). Our analyses showed that animals with a higher total frailty score, and performance in the lowest quadrant for nesting and open field, generally clustered at the bottom of the PCoA plots (Figure 2C–E). Consistently, mice with lower frailty scores clustered at the top of the PCoA plots. The low performers in nesting and open field, and mice with higher frailty were mostly treated with high DBI polypharmacy, as published previously (26). Using Bray-Curtis dissimilarities, we also discovered that beta diversity had a significant relationship with frailty (R^2 = 0.022, p = .001), nesting (R^2 = 0.025, p = .001), and open field distance traveled (R^2 = 0.013, p = .015; Figure 2C–E).

Mice Aging With and Without Polypharmacy Share Decreases in *Bifidobacteriaceae, Paraprevotellaceae, Bacteroidaceae,* and *Clostridiaceae,* While Mice Receiving Polypharmacy Show More Increased Features

LEfSe feature analyses were utilized to understand how aging, polypharmacy, and deprescribing affected specific microbes, by comparing LEfSe features within each group across all timepoints. Comparisons between timepoints within treatment groups are summarized at the phylum level (Supplementary Table 4), class level (Supplementary Table 5), order level (Supplementary Table 6), family level (Supplementary Table 7), genus level (Supplementary Table 8), and species level (Supplementary Table 9). Aging effects in the control mice involved decreases across various phyla, specifically *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Tenericutes* (Figure 3A). Only *S24_7* from the *Bacteroidetes* phylum showed an increase over these timepoint comparisons. Breakdowns into 12vs15, 15vs21, and 21vs24 month comparisons (Supplementary Figure 7A–C) suggest aging changes in control mice were acquired at various timepoints and increases of certain bacterial populations were outnumbered by decreases.

Aging with polypharmacy effects characterized by high DBI polypharmacy changes from 12 to 24 months (Figure 3B and C), shared features with control while showing additional decreases in *Actinobacteria* and *Bacteroidetes* phyla; increases in *Verrucomicrobia* and *Firmicutes*; and both increases and decreases in *Coriobacteriaceae* (p_Actinobacteria) and *Desulfovibrionaceae* (p_Proteobacteria) families. To assess the stability of changes in features induced by the polypharmacy regimen, the high DBI polypharmacy group that remained on the medications after 21 months were analyzed separately to the deprescribed, showing a similar overall pattern of change from 12 to 15 months (Figure 4A and B), which then reversed in the high DBI deprescribed group from 21 to 24 months (Figure 4D). From 12 to 15 months, both high DBI polypharmacy groups showed a significant decrease in *Bifidobacteriaceae*, *Clostridiacea*, *Lactobacillaceae*, and

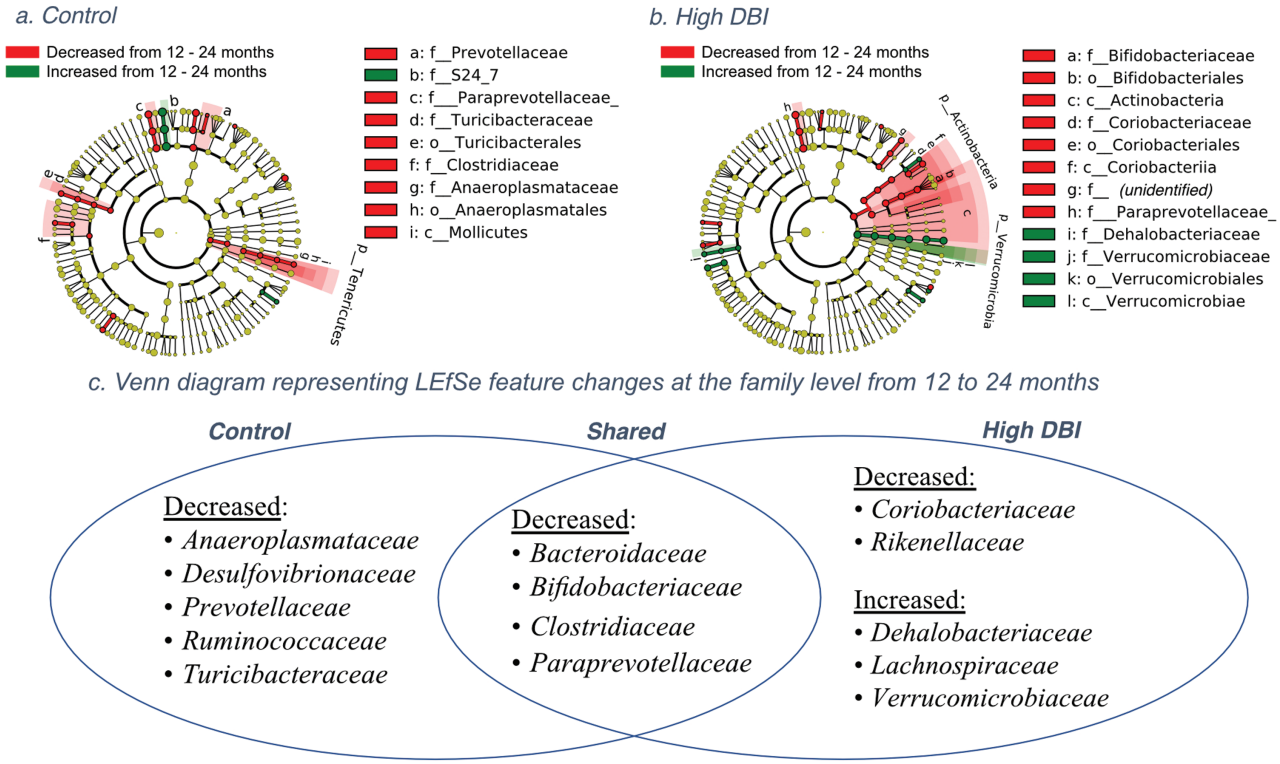

Figure 3. Aging in control and high DBI polypharmacy groups showing shared and disparate changes in microbial composition between 12 and 24 months. In the cladograms comparing control and high DBI polypharmacy changes from 12 to 24 months (**A** and **B**), red color indicates LEfSe features that decreased between the 2 timepoints, while green indicates increase. Venn diagram summarizes LEfSe feature changes at the family level from 12 to 24 months in control and polypharmacy groups that were shared, and those only found in that group (**C**). Animal numbers in each treatment group at each timepoint are summarized in Supplementary Table 1. DBI = Drug Burden Index; LEfSe = Linear discriminant analysis Effect Size. Refer to online version for access to the colour figures.

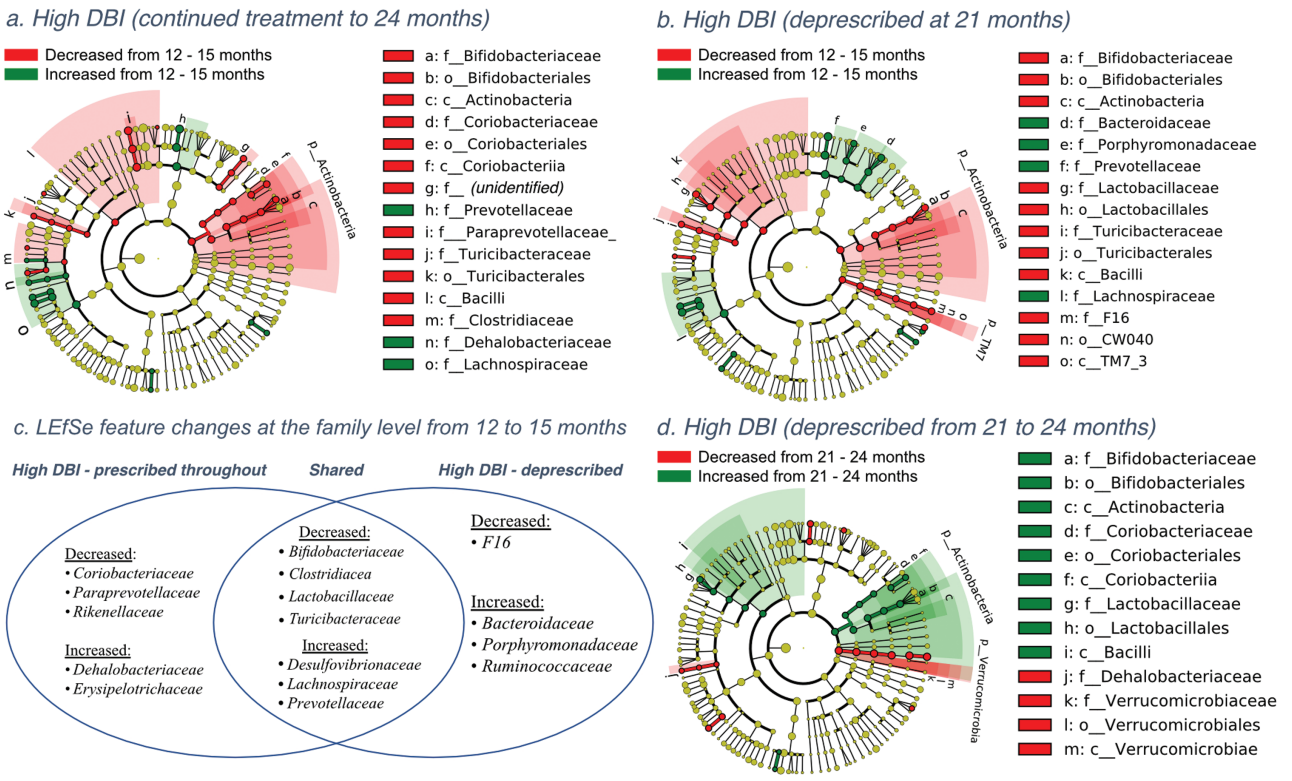

Figure 4. Polypharmacy administration starting at age 12 months affects specific microbes evident in both groups taking polypharmacy from 12 to 15 months (**A–C**). Deprescribing at 21 months leads to recovery of these microbes between 21 and 24 months (**D**). Red color indicates LEfSe features that decreased between the 2 timepoints, while green indicates increase. Animal numbers in each treatment group at each timepoint are summarized in Supplementary Table 1. DBI = Drug Burden Index; LEfSe = Linear discriminant analysis Effect Size. Refer to online version for access to the colour figures.

Turicibacteraceae families, and increase in *Desulfovibrionaceae*, *Lachnospiraceae*, and *Prevotellaceae* families (Figure 4C).

Unlike the control group, perturbations specific to the high DBI polypharmacy LEfSe features mainly occurred between 12 and 15 months (following the initiation of medication), which persisted throughout the study (Supplementary Figure 8A–C). There were no significant changes between 15 and 21 months (Supplementary Figure 8B), which may indicate medication use dominated aging effects. Changes from 21 to 24 months suggest this timeframe is more sensitive to polypharmacy and aging interaction effects.

LEfSe Features in High DBI Polypharmacy Deprescribed Mice Did Not Recover Completely to Pretreatment Levels, and These Changes Were Not Found With Aging in Control Mice

Pertubations in the gut microbiome of the high DBI polypharmacy group that were deprescribed are summarized in Supplementary Figure 9A–C. Deprescribing at 21 months reversed a lot of changes following polypharmacy at 15 months (Figure 4D), however, deprescribing medications did not recover LEfSe features completely (Figure 5). From 12 months (pretreatment) to 24 months (3 months postdeprescribing), high DBI polypharmacy deprescribed mice showed a significant increase in the *Erysipelotrichaceae* family, and reductions in *Porphyromonadaceae*, *Turicibacteraceae*, *Clostridiaceae*, *Lachnospiraceae*, and *Ruminococcaceae* families (Figure 5A and C). Between 12 and 24 months, control mice only shared a decrease in the *Clostridiaceae* family (Figure 5B and C).

LEfSe Correlations With Global Function

LEfSe features were correlated with different functional outcomes to identify any characterizing microbes. Control mice at 12 months showed a positive correlation between frailty score and *Turicibacteraceae* and *Anaeroplasmataceae* families. The correlation did not persist at 24 months (Supplementary Figure 10A and B). Nesting performance at both 12 and 24 months showed a significant negative correlation with the *Lactobacillaceae* family (Supplementary Figure 10A and B). Open field performance showed significant correlations with different family members of the same phylum at 12 months, and significant positive correlations with several families in the *Firmicutes* phylum at 24 months (Supplementary Figure 10A and B). In the 2 groups of mice given high DBI polypharmacy feed (prescribed throughout vs. deprescribed), there were no consistent correlations between metadata variables and LEfSe features (Supplementary Figure 10C–E).

Discussion

Nonantibiotic medications, especially administered together in polypharmacy regimens, affect the gut microbiome (19–21). This study utilized a longitudinal in vivo aging mouse model of polypharmacy, and 16s sequencing of the microbiome, showing microbiome changes with age, polypharmacy, and deprescribing. Aging effects on microbiome diversity were detected at 21 and 24 months, while polypharmacy-related changes were greater and overrode aging-related changes as measured using diversity indices and LEfSe features.

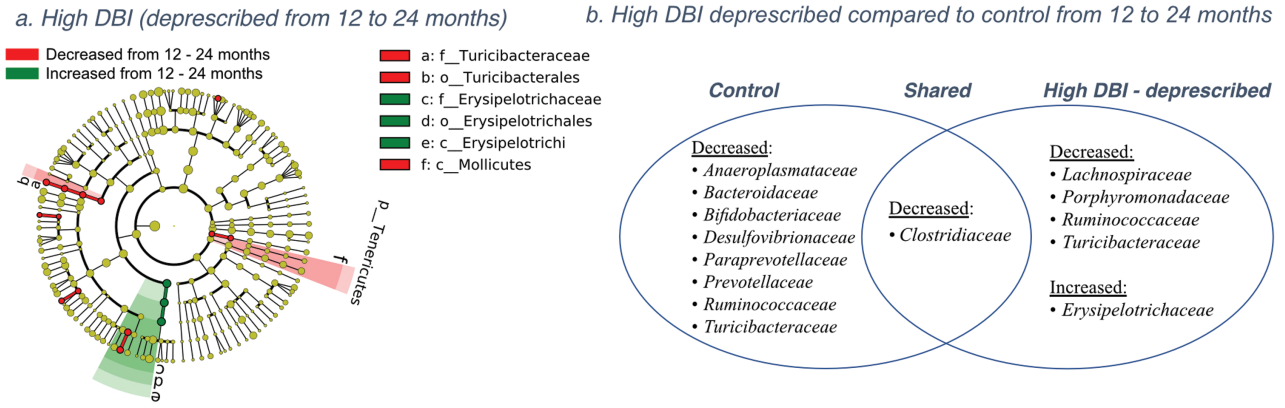

Figure 5. Recovery from polypharmacy medication effects on LEfSe features is seen by subtracting normal aging changes (seen in control mice from 12 to 24 months) from pre- to posttreatment changes in the high DBI polypharmacy deprescribed group. Cladogram compares the microbiome of polypharmacy mice pretreatment at 12 months, with the microbiome posttreatment in the same mice randomly allocated to the high DBI polypharmacy deprescribed group (**A**). Red color indicates LEfSe features that decreased between the 2 timepoints, while green indicates increase. Venn diagram compares changes in high DBI polypharmacy deprescribed from 12 to 24 months (showing which microbes did not recover to pretreatment levels) to control from 12 to 24 months (showing aging effects; **B**). Animal numbers in each treatment group at each timepoint are summarized in Supplementary Table 1. DBI = Drug Burden Index; LEfSe = Linear discriminant analysis Effect Size. Refer to online version for access to the colour figures.

Alpha-diversity decreased with increasing age in the control but not in the polypharmacy group and changed following deprescribing of medications. Beta-diversity changed with aging, polypharmacy treatment, but not aging plus polypharmacy. A cross-sectional study of older adults of different ages who may have taken a range of medications, found that alpha-diversity increased with age, but decreased in centenarians (18). Other studies report alpha-diversity remains constant in adulthood until the development of morbidities (or the increase of biological age—sometimes measured by frailty) (36,37). Some clinical observational studies report associations between frailty and alpha diversity (17,37), inconsistent to others showing no association (38). Discrepancies may be attributed to factors difficult to control in human studies, including diet and varying medication combinations. Increasing medication number accounts for more changes in alpha diversity than frailty or multimorbidity, according to multivariate analyses (21). The polypharmacy regimen increasing alpha-diversity in this study is contrary to a negative correlation between a number of medications and alpha-diversity observations in hospitalized older people (21) and in community-dwelling participants with a mean age of 66 years (39). This difference might be explained by differences in the type of medications comprising the polypharmacy regimens, or by residual confounding by indication for the drug in the human studies. In humans, increased alpha-diversity is associated with higher physical function (40), while sarcopenia, frailty, and activities of daily living are also associated with microbiome changes (41). However, in this study correlation between alpha diversity and metadata variables, including frailty are likely separately driven by medication effects, as significant relationships occur at timepoints following medication introduction (15 months) and medication deprescription (24 months), and nonmedicated control mice show reverse correlation relationships with alpha-diversity.

Bacterial changes consistent across control and high DBI polypharmacy mice, indicate aging effects uninterrupted by polypharmacy. Short-chain fatty acid (SCFA) producers are generally reduced with aging. The *Clostridiaceae* family, important in maintaining inflammation "homeostasis" and potential contributors to longevity (42), decreased in control and high DBI polypharmacy mice. The Clostridium cluster XIVa similarly decreased in another study of 18-to-24-month mice (42). Consistent with their involvement in longevity, in another study, mice aged 800 days had more *Clostridiaceae* than mice aged 600 days (43). The *Lactobacillaceae* family, also SCFA producers, decreased in control mice between 15 and 21 months and in high DBI polypharmacy from 12 to 15 months. Interestingly, both high DBI polypharmacy groups (remained on polypharmacy, and deprescribed), had increased *Lactobacillaceae* from 21 to 24 months, when control animals showed no change. Aging-associated changes that were only found in the control group included decreased *Firmicutes* phyla (also SCFA producers), and *Bacteroidetes* initially decreasing from 12 to 15 months, to then increased at 21 months. Similarly, in cross-sectional human gut microbiota studies, *Firmicutes* decreases with increasing age, while *Bacteroidetes* increases (44), trends most pronounced in adults living in nursing homes compared with those living in the community (16). Interestingly animals treated with high DBI polypharmacy did not show this pattern.

Polypharmacy effects change over the course of treatment, whereby different microbes were affected by the drugs at different timepoints. From 12 to 15 months, the *Bacteroidetes* phyla declined in control, and specifically the *Prevotellaceae* family, which increased in a subset of high DBI polypharmacy animals. Medication-associated increase of *Bacteroidetes* phyla has been described following selective serotonin reuptake inhibitor (SSRI) use (45), while statin use reduced *Bacteroidetes* levels (46). From 15 to 21 months, *Bacteroidetes* increased in control animals, while the *Bacteroidaceae* family declined in a subset of high DBI polypharmacy animals. Between 21 and 24 months, *Bacteroidetes* phyla members remained unchanged in control, while the *Rikenellaceae* family and *Parabacteroides distasonis* decreased in high DBI deprescribed animals. Comparable changes in members of the Bacteroidetes phylum have been associated with type 2 diabetic statin users with increased SCFAs (47), and mice that responded poorly to statin treatment (48). However, these studies did not control for the age of participants as a factor influencing medication-microbiome interactions. From 21 to 24 months control microbiome decreased in *Actinobacteria* phyla, as found in older adults (49), while increasing in both polypharmacy and polypharmacy deprescribed. Statins have increased *Actinobacteria* levels in acute coronary syndrome patients (47), and may be driving

the polypharmacy effects. Our findings suggest that the microbiome may be more sensitive to specific drug effects at certain ages, and this polypharmacy regimen could be a beneficial mediator of aging effects on the microbiome.

It is not clear which constituents of the high DBI polypharmacy regimen are driving these effects. Focusing on *Firmicutes*, for example, which only reduced in nontreated control mice suggesting the polypharmacy regimen attenuates aging effects by increasing levels; opioids increased *Firmicutes* in an animal study (22), statins have both increased *Firmicutes* in humans and animals (23,24) and decreased *Firmicutes* abundance in animals (46), while SSRIs reduced *Firmicutes* in humans and animals (45,50) beta-blockers have reduced *Firmicutes* in humans (25). A recent multiomic study of patients with the cardiometabolic disease found, taking statin and beta-blocker medication combinations led to microbiome changes that opposed the effects of disease severity markers on the microbiome (51). This is consistent with our polypharmacy regimen also shifting the microbiome toward a healthier state, which may be driven by the additive effects of simvastatin and metoprolol.

Despite the polypharmacy regimen showing different effects at different timepoints, reversal of polypharmacy effects observed from 12 to 15 months, were the main changes in polypharmacy deprescribed animals from 21 to 24 months. Therefore, a significant number of features affected by polypharmacy remained unchanged during the length of chronic medication intake and reversed when deprescribed. Similarly, following ciprofloxacin antibiotic cessation, communities showed an incomplete return to their initial state and composition of the gut microbiota stabilized, but was altered from its initial stable state (52). Alterations from a pretreatment (12 months) steady state at 24 months (3 months postdeprescribing) in our study, could be a consequence of aging, however, demonstrating a similar change over the course of weeks (52) (or in our study months) suggests medications can also have permanent effects. While the specific microbiome changes seen with polypharmacy differed between our interventional polypharmacy mouse model and the observational studies in humans, after ceasing medications, there are consistent trends across both study types toward recovery to pretreatment microbiome. Nagata et al. found an increasing number of medications used positively correlated with several *Streptococcus* and *Lactobacillus* species (39) (contrary to our current mouse study finding *Lactobacillaceae* decreased at 15 months for mice administered high DBI polypharmacy), and patients who reduced the number of drugs showed decreased *Streptococcus* and *Lactobacillus* (39) (current study—*Lactobacillaceae* increased following deprescribing, as well as in mice that continued on high DBI polypharmacy at 24 months). Reexposure to microbes may be a critical factor contributing to recovery. In a mouse study, recovery of S24-7 species from ciprofloxacin only occurred in specific cages via coprophagia, while single-housing disrupted recovery (53).

Deprescribing from polypharmacy also showed disparate changes in aging, as polypharmacy deprescribed mice had no common changes with control from 21 to 24 months. Opposite to control, *Bifidobacterium* increased in high DBI polypharmacy deprescribed, which usually decreases with age (49). Furthermore, high DBI polypharmacy deprescribed animals had increased *Lactobacillus* and decreased *Rikenellaceae*, shared with high DBI polypharmacy animals remaining on the medication. Studies have reported a decreased abundance of *Bifidobacteria* and *Lactobacilli* in older adults (43), suggesting the medications are countering aging-related effects. In mice, Bifidobacterium inhibits proinflammatory cytokines and colonic senescence while showing protective effects that facilitate colonic tight junctions and mucus production. In older adults and centenarians, *Bifidobacterium* abundance negatively correlates with increased inflammatory status (inflammaging) (54). Like our findings with control and contrary to high DBI polypharmacy and deprescribed, there was overrepresentation of the *Rikenellaceae* family in middle-aged and older aged mice (43). Finally, high DBI polypharmacy deprescribed were also the only group to show a decrease in the Verrucomicrobia phylum, specifically *Akkermansia Muciniphila*. This bacterium has beneficial health properties, known to reduce in old age, it is inversely correlated with metabolic syndromes and inflammatory disease severity, with supplementation showing beneficial effects on metabolic syndrome and disease reversal (55). Interestingly, the *Desulfovibrio* genus, which also decreased in this timepoint, has been described to have the opposite effects to *Akkermansia Muciniphila*, with reductions in Desulfovibrio associated with reduced plasma inflammatory markers, and increase associated with type 2 diabetes and obesity (56).

The strengths of the current study include its longitudinal design, randomized clinically relevant interventions, detailed robust metadata, and wide range of analytic techniques used, however, further improvements could be made. Utilizing whole genome sequencing to functionally assess the microbiome, analyzing fecal and serum fatty-acid levels, assessing the single drug constituents of the polypharmacy regimen individually in different doses, and assessing the effects of other common medication classes in polypharmacy combinations, will all provide higher explanatory power to the effect of medications. Female mice may yield different findings, as polypharmacy affects female mice differently on global function measures, including activities of daily living (57), and microbiome sex differences exist across the life span (36). Furthermore, this is a mouse study, which may limit its generalizability in humans. Polypharmacy regimens in humans vary in medication combinations and concentrations and are prescribed to manage morbidities—which develop with contribution from a varying assortment of modifiable (including diet, exercise, medication intake, epigenetics, and environment), and nonmodifiable (including genetics) risk factors. Our study isolates polypharmacy medication intake to study its effect, aiming to contribute toward understanding the complex physiological interactions involved in aging.

Further studies are needed to elucidate the polypharmacy-microbiome interactions in different medication combinations. Characterizing these changes in animal models where usual confounders like diet are controlled between groups will also help understand whether there is a core microbiome signature for all polypharmacy combinations, or whether the perturbations that occur are specific to the medication constituents of the regimen.

In conclusion, treatment effects with the polypharmacy regimen override aging effects, are robust when both high DBI polypharmacy groups (receiving polypharmacy from 12 to 24 months, vs. 12 to 21 months) are analyzed separately, and deprescribing shows evidence for reversal. While this high DBI polypharmacy combination increased frailty and reduced functional performance on a phenotypic level, it prevented normal age-related changes in the microbiome.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

None declared.

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Author Contributions

G.G. contributed to design of the study, acquisition, analysis, and interpretation of the data, drafting, and finalizing the manuscript. J.M. contributed to design of the study, acquisition, and interpretation of the data, and finalizing the manuscript. F.Z. completed the bioinformatic analysis of the data and contributed to interpretation of data, and finalizing the manuscript. M.B. and T.T. both contributed to acquisition of the data, and finalizing the manuscript. E.E.-O. contributed to analysis and interpretation of the data, and finalizing the manuscript. S.N.H. contributed to design of the study, interpretation of the data, supervised acquisition, and analysis of the data, and finalizing the manuscript.

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