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Effects of a vinegar-based multi-micronutrient supplement in rats: a multi-pronged assessment of dietary impact

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Abstract

We determined the effects of continuous access to drinking of water with a vinegar-based multi-micronutrient (VMm) supplement containing rice and fruit vinegars, vitamins, organic acids and sugars during gestation, lactation, and early adulthood in rats. Pregnant rats were provided with reverse-osmosis water or VMm water from the start of pregnancy through the time of weaning. Weaned pups consumed the same drinking water for 3–12 additional weeks. We examined fecal metabolite and microbial profiles, and other physiological parameters. Body weights were less in rats that drank VMm water. Thirty fecal metabolites involved in amino acid and dipeptide metabolism were significantly altered in VMm-supplemented rats. Analysis of microbial 16S rRNA showed enrichment of bacteria in the family S24–7 in VMm-supplemented rats, and one in Ruminococcaceae in controls. Our data show that a VMm-containing beverage can alter growth,

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AUTHOR CONTRIBUTIONS

JDB, RMM and AV designed and performed experiments, analyzed data and wrote the manuscript. Y-HH performed statistical analyses of the metabolome data. AV and RM performed fecal microbiome and data analysis. JK performed the pharmacokinetic analysis. MC-S performed MRI analysis. MI and KA performed Pirogen analyses. All authors read, revised and approved the manuscript.

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and gut metabolism and microbial community. Future work to correlate these parameters is warranted.

Keywords

Dietary supplement; fruit vinegar; fecal metabolomics; fecal microbiome; iron metabolism; bone

1. Introduction

A dietary supplement is a product that a) is meant to augment the diet, and b) contains vitamins, minerals, amino acids, botanical extracts or other similar ingredients (FDA, 1994). Common among supplements are micronutrients and vitamins, since certain dietary patterns may not provide all the optimal nutrients from food and water intake alone. Vinegars have been ingested for millennia and are an important element in Asian, European, Western and other traditional cuisines. Vinegar has been used for preservation of various foods and is often used for flavoring and pickling.

Recent research has indicated that vinegar affects glucose metabolism and alters lipid profiles in rats and humans (Hlebowicz, Darwiche, Bjorgell, & Almer, 2007; Johnston, Steplewska, Long, Harris, & Ryals, 2010; Naziroglu et al., 2014; Petsiou, Mitrou, Raptis, & Dimitriadis, 2014). Vinegar has also been shown to attenuate experimentally induced colitis in mice, via suppression of inflammation (Nishidai et al., 2000; Shen et al., 2016; Shimoji et al., 2002). Fruit vinegars have been reported to improve immune function (Bounihi et al., 2014; Cha, Moon, Soh, Oh, & Choi, 2006; Lee, Kim, Do, Kim, & Kwon, 2014). Many of these potential benefits from consumption of vinegars in animals and humans might be explained by their ability to alter the gut microbiome and the metabolite profile. Increasing evidence suggests that alterations in gastrointestinal flora and metabolites can affect health via systemic processes (Arpaia et al., 2013; Chen et al., 2014; Huang et al., 2013).

Pairogen® (Akatsuka Co., Tsu, Japan) is a beverage that contains ferrousferic chloride (FFC®) water, rice and fruit vinegars (e.g., apple, Japanese apricot and persimmon), sugars, citric and malic acid and vitamins B and C (Hirobe, 2009). The rice and fruit vinegars are obtained by fermentation of rice and fruit sugars, respectively. Adding Pairogen to drinking water has been reported to improve mouse survival when challenged with intravenous administration of *Rhodococcus* (Yimin et al., 2012). Pairogen-supplemented mice cleared bacteria from liver and spleen significantly faster. These effects were passed on to their F1 progeny. Enhanced IL-10 and heme oxygenase-1, decreased TNF- α and IL-6 expression in *Rhodococcus aurantiacus*-stimulated peritoneal macrophages were also shown in F1 mice consuming Pairogen (Yimin et al., 2012).

Although the effects of Pairogen have been studied in a disease model, as described above (Yimin et al., 2012), no data on the effects of chronic consumption of this drink in healthy animals are available. The objective of this study was to determine the effects of continuous access to a vinegar-based multi-micronutrient supplement (Pairogen) in drinking water during gestation, nursing (lactation) and early adulthood in rats. We examined fecal metabolite and microbial community profiles, as well as selected physiological parameters

such as body weight, body fat and bone composition, blood chemistry, and gut absorption of iron, in order to evaluate the effects of ingestion of a fruit vinegar-containing beverage.

2. Materials and methods

2.1. Vinegar-based Multi-micronutrient Supplement

The multi-micronutrient supplement used in this study was obtained as a concentrated formulation (Pairogen®) from Akatsuka Garden Company (Tsu City, Japan). The Pairogen concentrate contains water, rice and fruit vinegars (e.g., apple, Japanese apricot and persimmon), sugars, citric and malic acid and vitamins B and C. The sample was diluted 1:100 with reverse-osmosis water and filtered through a 0.45 µm pore size membrane and analyzed at Akatsuka Co. (Japan). Organic acids, vitamins, amino acids and sugars were measured using liquid chromatography on an Agilent 1100 Series liquid chromatograph (Agilent Technologies, Santa Clara, CA) with auto sampler. Elemental analysis was performed by inductively coupled optical emission spectroscopy (ICP-OES) (Optima 5300 DV, Perkin Elmer, Billerica, MA). Anions were measured by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA). The column composition, temperature, mobile phase, flow rate and detector varied depending on the type of compounds being analyzed (See Online Supplement, Methods). Additional analyses for small molecules in Pairogen concentrate were performed at Metabolon, Inc. (Durham, NC) using liquid chromatography/mass spectroscopy (LC/MS) or gas chromatography/MS (GC/MS). Details of methods for VMm analyses are available in Online Supplement.

2.2. Experimental Design

The animal protocols in this study were approved by the Harvard Medical Area Animal Care and Use Committee. Figure 1 outlines the overall experimental design. A total of twelve female Sprague-Dawley rats 2 days after conception were obtained from Taconic Farms (Germantown, NY) and housed individually in standard pasteurized polycarbonate microisolator cages under controlled conditions of temperature, humidity, and light at the Harvard Center for Comparative Medicine. They had access to commercial chow (PicoLab Rodent Diet 5053, Framingham, MA) and designated drinking water *ad libitum* throughout pregnancy and lactation.

These pregnant rats were provided with either reverse-osmosis water or with the same reverse-osmosis water with 1% VMm supplement. In a previous pilot experiment, higher VMm concentrations resulted in a decrease in water consumption. The designated drinking water was provided throughout the period of pregnancy (21–22 days). Right after birth, the litter size was randomly culled down to 10 pups/litter each with male to female ratio of 5 to 5. The designated drinking water was continuously provided throughout lactation. As the pups aged, they might have consumed not only their mother's milk but also the drinking water and chow provided to their mothers. At age 21 days, the pups were weaned and were then provided with the same drinking water assigned previously until euthanasia at times up to age 105 days. This protocol provided a long-term consumption of VMm, encompassing gestational, lactational and early developmental stages including the onset of puberty. In an initial experiment, 10 pups from 1 control and 10 pups from 1 VMm dam were used for

fecal metabolomics analysis at age 21 and 42 days. Then, in a larger experiment, 10 pups from 5 dams/treatment group (1 male and 1 female per dam) were used for fecal metabolomics and microbiome analyses at age 21 and 42 days. This experimental design explored the importance of greater genetic variability among rats in each treatment group. These same rats were analyzed for hematological parameters (42 and 63 days) and for body composition (bone density and body fat, 63 days). Additional rats were also analyzed for body composition at age 105 days and for gut absorption of iron at age 49 days.

2.3. Assessment of iron absorption in the gastrointestinal tract

We used radioisotope of iron (^{59}Fe) as tracer in a pharmacokinetic study to determine if consumption of VMm affects iron bioavailability from the gastrointestinal tract. We determined if iron absorption in the gut, as well as iron clearance from the blood, and tissue distribution were affected in VMm-exposed pups. $^{59}\text{FeCl}_3$ was purchased from Perkin Elmer (Boston, MA) and diluted with 1:50 molar excess of ascorbic acid immediately prior to the experiment to reduce ^{3+}Fe to ^{2+}Fe . A total of 6 rats from each treatment group were dosed by gavage with ^{59}Fe in this buffer at 1 ml/kg volume dose and equivalent radiation dose of 150 μCi $^{59}\text{Fe}/\text{kg}$. Each rat was anesthetized with up to 4% vaporized isoflurane (Halocarbons Lab, North Augusta, SC) prior to gavage dosing. Blood samples were sequentially obtained from the tail vein over a 72-hour period (15, 30, 60, 90, 120, 240, 480 m, 24, 48, and 72 h). Plasma and red blood cells were separated for radioisotope analysis.

Since the blood levels of ^{59}Fe represent the amount absorbed from the gut minus the amount cleared from the circulation, another set of 4 rats/group was intravenously injected with the same dose of ^{59}Fe via the penile vein. Blood samples were similarly obtained from the tail vein. At 72 hours post-dosing with $^{59}\text{FeCl}_3$, all rats were humanely killed with overdose of isoflurane anesthesia, exsanguinated via the abdominal aorta, and tissue samples collected. The gastrointestinal tract (GIT) was divided into segments after removal of the luminal contents. Radioactivity of samples of tissue, plasma, and red blood cells was measured using a WIZARD 1410 gamma counter (Perkin Elmers, Waltham, MA). Data were analyzed and expressed as tissue concentration ($\mu\text{Ci}/\text{g}$) and % of the administered dose.

Pharmacokinetic (PK) analyses were performed on plasma and RBC levels during the first 24 hours to compare PK parameters for ^{59}Fe between control and VMm groups using the WinNonlin Software version 5.2 (Pharsight Corp, Mountain View, CA). PK parameters such as half-life (time during which $1/2$ of ^{59}Fe dose is cleared from the plasma), total area under the plasma concentration-time curve (AUC) (an estimate of ^{59}Fe in the plasma), total body clearance (CL) (rate of loss of ^{59}Fe from the body), and bioavailability (F, the extent of absorption of ^{59}Fe from the gut) were calculated. Half-life was calculated as 0.693 divided by the slope of the regression line between 4 and 24 hours from a plot of the natural log of ^{59}Fe plasma concentration vs. time. A non-compartmental analysis was performed to quantify the degrees of exposure and loss of iron in the pups. AUC was calculated by the trapezoidal rule-extrapolation method. CL was computed by dividing the administered dose by AUC. F was then calculated as the ratio of AUC post-gavage to AUC post-IV injection. We used MANOVA (SAS statistical analysis software: SAS Institute, Cary, NC) analyses on

tissue concentration and distribution data to determine the effects of VMm on tissue uptake of iron.

2.4. Assessment of bone mineral density and mineral content, body fat and blood parameters

At age 42 and 56 days, blood samples from the same rats were analyzed for various hematological parameters using Hemavet 950 FS hematology analyzer (Drew Scientific Group, Miami Lakes, FL). Complete blood cell counts including white cell numbers and differentials, hematocrit, and hemoglobin were measured in anti-coagulated whole blood.

At age 63 days, the same rats used for microbiome and metabolome studies, and additional rats at 105 days (6 control and 6 VMm) were analyzed for leg bone mineral density (BMD, g/cm²) and bone mineral content (BMC, g) of the right hindleg using peripheral dual-energy x-ray absorptiometry (PIXImusII, GE Lunar, Madison, WI). The percentages of body fat in these rats were also analyzed using MRI. Measurements were performed at a preclinical 7-T MRI (Bruker BioSpin, Ettlingen, Germany). Rats were scanned in a Bruker quadrature volume coil (RF RES 300 1H 112/86 QSN. TD AD) with a 3D gradient echo pulse sequence (commonly called 3D FLASH). All rats were studied in a prone, head-first position. The field of view (FOV) was either $9.6 \times 9.6 \times 9.6$ cm³ or $7.2 \times 7.2 \times 9.6$ cm³, depending on the size of the animal. The third dimension was oriented along the head/feet direction. The imaging resolution was $128 \times 128 \times 256$ for the bigger FOV and $96 \times 96 \times 256$ for the smaller FOV, yielding a spatial resolution of $0.75 \times 0.75 \times 0.375$ mm³ for all rats. Each rat was scanned three times with three distinct echo times (TE), 2.416, 2.892, and 3.368 ms to generate separate fat and water images using the Dixon technique. (Dixon, 1984). Other imaging parameters were: TR 15 ms, flip angle 15¹⁷, bandwidth 200 kHz. Data were expressed as % fat of the imaged body sections from the thorax to the pelvis.

2.5. Fecal microbiome and metabolome analyses

In the first experiment using all pups from each dam, we collected 24-hour fecal samples from 10 pups from one control and 10 pups from one VMm dam at age 21 and 42 days using separate metabolism cages. Freshly-voided fecal samples were collected, frozen, and sent to Metabolon, Inc. (Durham, North Carolina) for metabolomic analyses. In the second experiment with greater genetic diversity, we also collected fresh fecal samples (from 1 male and 1 female pup from each control and VMm dam) at age 21 and 42 days using separate metabolism cages. The total number of samples was 10 per group. The fecal samples were divided into 2 aliquots; one was used for metabolomic analyses and the other for microbiome analyses. Details of microbiome and metabolome analyses are available in Online Supplement.

2.5.1. DNA extraction and 16S rDNA Sequencing—DNA from fecal samples was extracted according to the manufacturer's instructions using the MoBio Ultra Kit (Mo Bio, Carlsbad, CA, USA). The V1 to V3 variable region of the 16S rRNA gene was sequenced (with the primer pair 28F-519R) using 454 FLX Titanium technology. Sequencing procedures were performed at Research and Testing Laboratories (Lubbock, TX) based on RTL protocols (www.researchandtesting.com). Bacterial tag-encoded FLX amplicon

pyrosequencing (bTEFAP) was performed as described previously (Dowd, Sun, Wolcott, Domingo, & Carroll, 2008).

2.5.2. DNA Sequence Analyses—A pipeline in the software program mothur v.1.31.6 was used to extract, clean up, and trim sequences so that only high quality sequences were obtained (Edgar RC, 2011; Quast, 2013; Schloss, 2009). The sequences in the resulting filtered and cleaned dataset were binned into OTUs as described in the supplementary methods (Online Supplement). The resulting OTU-based analyses included alpha- and beta-diversity testing, Principal Coordinates Analysis (PCoA) and analysis of molecular variance (AMOVA) to identify putative effects of VMm intake on the microbiome. LEfSe analysis was employed to identify OTUs that were differentially abundant in VMm versus control groups. LEfSe was employed to identify OTUs that were differentially abundant in VMm versus control groups (Segata et al., 2011).

2.6. Untargeted Metabolomic Analyses of Feces

Metabolomic profiling analyses were performed by Metabolon, Inc. (Durham, NC) as described in the online supplementary information (Online Supplement). Statistical analysis of metabolite profiles was achieved by linear mixed-effect regression analysis, principal component analysis (PCA) and hierarchical clustering (heat maps) and used to identify putative differences between VMm and control groups. Pathway enrichment analysis (Goh KI, 2012; Janji V, 2012; Shlomi T, 2009; Subramanian et al., 2005) based on Kyoto Encyclopedia of Genes and Genomes (KEGG) (M. Kanehisa, & Goto, S., 2000; M. Kanehisa, Goto, S., Sato, Y., Kawashima, M., Furumichi, M., & Tanabe, M., 2014) pathways was performed to identify pathways altered by VMm consumption.

3. Results

3.1. Vinegar-based Multi-micronutrient Supplement analyses

The composition of VMm supplement is summarized in Table 1. LC/MS and GC/MS analyses of VMm concentrate revealed the presence of the following small molecules: asparagine, phenylalanine, glutamate, maltotetraose, isomaltose, erythulose, ribose, ribulose, maltose, maltopentose, 1,6-anhydroglucose, mannitol, fumarate, citrate, glycerate, gluconate, 1,3-dihydroxyacetone, vanillin, benzyl alcohol, and ascorbate. As administered to the rats, the VMm concentrations were 2 orders of magnitude lower.

3.2. Effects on body weight and composition, and blood cell counts

The weekly weights of male and female pups are shown in Figure 2. Significant differences were observed in the mean weights of male versus female. In addition, VMm rats had significantly lower body weights compared to those of controls in both male and female rats evident from age 63 days and later. No clinical differences such as activity were noted.

We examined the effects of VMm supplementation on femur bone density using DEXA. Bone mineral density (BMD, g/cm²) and bone mineral content (BMC, g) in male rats were not altered with VMm supplementation. The BMD of female rats did not change at 56 and 63 days of age. However, at 105 days, BMD was ~15% higher in female control versus

VMm ($p<0.05$) (Figure S1a, Online Supplement). The percentage of body fat determined by MRI image analysis showed no significant difference between control and VMm at age 21 and 42 days (Figure S1b, Online Supplement).

We measured the effects of VMm supplementation on various hematological parameters. There were significant increases in blood neutrophils, monocytes and lymphocytes, and mean platelet volume (MPV) in VMm rats at day 42 (Figure S2, Online Supplement). Other blood parameters were not different. No alterations in measured blood parameters were seen at day 56 (data not shown).

3.3. Effects on iron absorption in the gut

We examined whether VMm supplementation may alter the absorption of essential micronutrients in the GIT such as iron. The plasma and RBC profile of ^{59}Fe post-IV injection or post-gavage are shown in Figure 3. PK analyses showed that the bioavailabilities of ^{59}Fe post-gavage in control (0.558) and in VMm group (0.536) were not significantly different. Clearance of ^{59}Fe from the blood after IV administration was also unchanged. Multivariate analyses of variance on all tissue concentration and % dose data at 72 hours post-gavage or post-IV injection showed no significant effect of VMm on tissue concentration and distribution of absorbed iron (data not shown).

3.4. Effects on fecal metabolome

3.4.1. Effects on fecal metabolite profiles—In the multiple-dam cohort with greater genetic variability in the pups, we detected a total of 399 metabolites, compared to a library of 2,400 known metabolites. Principal components analysis of the fecal metabolite profiles (control at 21 days, VMm at 21 days, control at 42 days and VMm at 42 days) is shown in Figure 4. The four experimental groups form distinct clusters. The clusters of control and VMm groups at both 21 and 42 days are closer to each other in terms of the Euclidean distance. However, the clusters for 21 do not overlap those for 42 days. Temporal changes are easily observed. As seen in the clustering analysis, the heat maps show an increase in concentrations of metabolites in the VMm group at both 21 and 42 days (Figure S3, Online Supplement). Metabolites in the dipeptide KEGG pathways were increased in VMm group versus controls at 21 days. A fewer number of dipeptides were represented in the top 50 metabolites in the VMm group at 42 days. Principal Component Analysis and clustering analysis of metabolite profiles from the single-dam cohort are presented in online supplement (Figure S4).

By comparing data from the multiple-dam with those from the single-dam cohort, we examined the impact of genetic diversity on the gut metabolome. The majority of the data above were from an experimental design with five different mothers of this outbred rat strain. Interestingly, the number of significantly different metabolites was greater in pups from the single-dam experiment (Table 2).

3.4.2. Significantly altered metabolites—The numbers of metabolites whose concentrations were altered in pups from both multiple-dam and single-dam experiments are summarized in Table 2. The total numbers of significantly altered metabolites were lower in

the multiple-dam than single-dam cohort. ANOVA contrasts of the metabolomic data from rat fecal samples showed that VMm supplementation resulted in significant changes in the concentrations of 35 (day 21) and 10 (day 42) metabolites compared to control. A greater number of metabolites were changed in pups from a single mother (178 at day 21 and 142 at day 42). However, using a linear mixed regression model that controls for time effects revealed that 30 (multiple-dam) and 55 (single-dam) metabolites were significantly altered with VMm supplementation ($p < 0.05$). The 30 metabolites and their metabolic pathways altered by VMm in the multiple-dam cohort are shown in Table S1 (Online Supplement). The concentrations of 29 metabolites increased with VMm supplementation. The only metabolite that decreased with VMm was delta-tocopherol. A larger number of metabolites (266) were significantly altered between age 21 and 42 days. This larger degree of changes between 21 and 42 days were also observed in pups from the single-dam experiment (207 metabolites).

3.4.3. Significantly altered metabolic pathways—To identify which metabolic pathways were impacted by VMm supplementation in the multiple-dam cohort, pathway enrichment analyses were performed. As seen in results of the linear mixed regression model, the consistent fecal metabolite changes were associated with amino acid and dipeptide metabolism. At 42 days, only tryptophan and purine metabolic pathways were affected. In contrast to the 21 days results, although the enrichment of these pathways at 42 days was statistically significant, the application of a false discovery rate (FDR) for a more stringent analysis rendered the differences insignificant (data not shown).

3.5. Effects on fecal microbiome

The microbiome was analyzed from fecal samples only in the multiple-dam cohort. Eleven bacterial phyla were represented in the microbial community profiles obtained by 16S rRNA gene sequencing. The phyla Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria, accounted for 98% of sequences in all samples. These phyla are well established as the dominant members of the microbial community in mammalian hosts (Mahowald et al., 2009; Nelson, Poroyko, Morowitz, Liu, & Alverdy, 2013; Pflughoeft & Versalovic, 2012; Ridaura et al., 2013).

3.5.1. Relative abundance of bacteria in VMm and control samples—At the phylum and class levels, the relative abundance was similar between VMm and control samples (data not shown). Nine families accounted for > 90% of the sequences in every sample. At the family level (Figure S5, Online Supplement), there were minor differences between VMm and control groups; however, these were not statistically significant, according to the Student's t-test. The families Bacteroidaceae, Lactobacillaceae, Ruminococcaceae and Turicibacteraceae were significantly different between 21d and 42 d (Student's t-test, $p < 0.05$). The abundances of Bacteroidaceae, Lactobacillaceae and Turicibacteraceae decreased with age, whereas that of Ruminococcaceae increased at 42 days. Thus, three weeks of development produced significant changes in both the fecal metabolite and microbial contents.

3.5.2. Linear discriminant analysis effect size (LEfSe) analysis—To further investigate whether VMm supplementation altered microbial communities, we analyzed the microbial community profiles using LDA Effect Size (LEfSe). One OTU belonging the family Ruminococcaceae (phylum Firmicutes) was enriched in control rats (Figure 5a) and one in the family S24-7 (phylum Bacteroidetes) was enriched in VMm rats (Figure 5b). A greater number of OTUs were enriched when fecal samples from day 21 were compared with day 42. Two OTUs belonging to the family Ruminococcaceae (phylum Firmicutes) and one belonging to S24-7 family were enriched at day 42 versus day 21 (Figure 5c–e). On the other hand, OTUs belonging to families Bacteroidetes, Streptococcaceae and Peptotryptococcaceae were greater at day 21.

3.5.3. Alpha diversity—The within-group microbial diversity (alpha-diversity) in VMm and control groups at 21 and 42 days were assessed using indices such as Shannon and Simpson' index, and then compared using the non-parametric Kruskal-Wallis and Wilcoxon tests. No significant differences were observed in alpha diversity between VMm and Control groups.

3.5.4. Beta diversity - Principal coordinates analysis (PCoA) and analysis of molecular variance (AMOVA)—To investigate the beta diversity in VMm and control groups, we tested community structure indices, including thetaYC, BrayCurtis and Jaccard, using Unifrac and PCoA. Unifrac analysis using these metrics did not reveal significant differences between VMm and control. Principal coordinate analysis provided a way to visualize the separation of microbial communities in treatment and control groups at both sampling times. The communities in VMm-supplemented rats did not differ significantly from control rats (Figure 6).

The results from AMOVA reinforced these results for three separate indices. There was no significant difference in microbial community structure or membership between VMm and control groups. However, there was a significant difference in these indices over time (Table 3).

4. Discussion

4.1. Effects on growth and body composition

Our study sought to determine the effects of consumption of VMm-supplemented drinking water during gestation, nursing (lactation) and early adulthood in rat offspring. We explored the effects of continuous drinking of VMm-supplemented drinking water on fecal metabolite and microbial community profiles, as well as selected physiological parameters such as body weight, body fat and bone composition, blood chemistry, and gut absorption of iron.

Significant differences were observed in the mean weights of male versus female as expected. Interestingly, VMm supplementation also resulted in significantly lower body weights in both male and female rats from age 63 days and later. The presence of VMm in drinking water did not reduce their daily water consumption. However, the VMm-supplemented rats did ingest additional glucose and fructose from the supplement. Aside from weight reduction, no apparent clinical effects were noted such as changes in

spontaneous activity. We examined the effects of VMm supplementation on bone mineral density (BMD, g/cm²) and bone mineral content (BMC, g). The only significant finding was ~15% reduction in BMD in female rats consuming VMm-supplemented water. The correlation of reduced body with the lower bone mineral density in VMm-treated female rats needs further investigation. The percentage of body fat determined by MRI image analysis showed no significant difference between control and VMm rats.

We examined whether VMm supplementation altered the absorption of essential micronutrients in the GIT such as iron. Pharmacokinetic analyses showed that absorption of iron from the gut or the clearance of iron from the blood were not affected by drinking VMm nor were the tissue concentration and distribution of absorbed iron. Thus, VMm had no significant effects on iron metabolism. Since these data were obtained from 7-week-old rats, it is still possible that iron absorption at an earlier/younger age, the critical period for hematopoiesis might be altered. Testing this hypothesis in younger (weanling, 21 days) rats might be useful.

4.2. Effects on fecal metabolome

Fecal metabolites were significantly altered in rats drinking VMm-supplemented water. In the multiple-dam cohort with greater pup genetic diversity, we detected a total of 399 metabolites, compared to a library of 2,400 known metabolites. Principal components analysis of the fecal metabolite profiles (control at 21 days, VMm at 21 days, control at 42 days and VMm at 42 days) revealed that the age (21 vs. 42 days) effect was stronger than the VMm effect. By comparing data from the multiple-dam with those from the single-dam cohorts, we examined the impact of genetic diversity of the mothers (5 vs. 1 mother) on the gut metabolome in the offspring. Importantly, the number of significantly different metabolites was greater in pups from single dam. The total numbers of significantly-altered metabolites diminished in the multiple-dam versus single-dam cohort probably due to greater genetic heterogeneity in pups from different mothers. This suggests that the rat gut metabolome was influenced by genetics as well as by environment.

VMm supplementation resulted in significant changes in the concentrations of 35 (day 21) and 10 (day 42) metabolites compared to control. A higher number of metabolites were changed in pups from a single mother (178 at day 21 and 142 at day 42). However, using a linear mixed regression model that controls for age effects revealed that only 30 (multiple-dam) and 55 (single-dam) metabolites were significantly altered with VMm supplementation. Out of the 30 significantly altered metabolites, the concentrations of 29 metabolites increased with VMm supplementation and only one decreased. The only metabolite that decreased with VMm was delta-tocopherol. It is an easily absorbed form of vitamin E and is also used as a food additive (E309, FDA Food Additives Status list). The decrease may be due to increased absorption of this vitamin in VMm-supplemented rats. A larger number of metabolites (266) were significantly altered between age 21 and 42 days. This larger degree of changes between 21 and 42 days were also observed in pups from the single-dam cohort (207 metabolites).

Using pathway enrichment analyses, we identified which metabolic pathways were affected by VMm supplementation in the multiple-dam cohort. The fecal metabolite changes most

consistent were associated with amino acid and dipeptide metabolism. The increases in amino acid, lipid and carbohydrate metabolites may be related to VMm effect on protein digestion in the gut. Protein, lipid and carbohydrate breakdown products are absorbed from the intestinal lumen as free amino acids, glucose or fatty acids. These increased metabolites in the feces may be indicative of decreased gut absorption, more efficient breakdown, increased uptake of glucose and fructose from the VMm supplement, or influence of VMm on the microbial community in the gut.

4.3. Effects on fecal microbiome

The microbiome was analyzed from fecal samples only in the multiple-dam cohort. Eleven bacterial phyla were represented in the microbial community profiles obtained by 16S rRNA gene sequencing. The phyla Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria, accounted for 98% of sequences in all samples. These phyla are well established as the dominant members of the microbial community in mammalian hosts (Mahowald et al., 2009; Nelson et al., 2013; Pflughoeft & Versalovic, 2012; Ridaura et al., 2013).

At the phylum and class level, the microbial community profile as measured by relative abundance was similar between VMm and control samples. However, at the family level, Bacteroidaceae, Lactobacillaceae, Ruminococcaceae and Turicibacteraceae were significantly different between 21d and 42 d. The abundances of Bacteroidaceae, Lactobacillaceae and Turicibacteraceae decreased with age, whereas that of Ruminococcaceae increased at 42 days. An increase in Bacteroidaceae has been observed in patients with depression (Naseribafrouei et al., 2014) and irritable Bowel Syndrome (Ng et al., 2013). In contrast, daily intake of a probiotic strain of *Lactobacillus casei* was correlated with lower abundances of Bacteroidaceae as well as an improvement in stress-induced abdominal symptoms (Kato-Kataoka et al., 2016). Members of the Lactobacillaceae have traditionally been associated with beneficial effects on digestive health (Ventura et al., 2009). A decrease in the abundance of Lactobacillaceae with age might be indicative of the changing microflora in response to dietary changes. Increase in the abundances of the Ruminococcaceae, bacteria that perform fermentation of plant fibers, is possibly due to a change in diet. Up to day 21, the rat pup diet was predominantly milk, whereas from time of weaning until day 42, their diet consisted of standard rat chow.

Using LDA Effect Size (LEfSe) analysis, we found that one OTU belonging to the family S24-7 (Phylum Bacteroidetes) was enriched in VMm rats and another OTU in the family Ruminococcaceae (Phylum Firmicutes) was enriched in control rats. These OTUs have the potential as biomarkers for effects of VMm supplementation. An increase in the abundance of S24-7, a bacterial group that has not been cultivated, has been associated with dietary changes (Evans et al., 2014; Serino et al., 2012). Mice on a high-fat diet had lower percentage of S24-7 than those on a low-fat diet. In the same study, exercise increased relative abundance of S24-7 in mice on both diets (Evans et al., 2014). This family is known to include bacterial taxa that can ferment dietary fiber to produce butyrate, a short-chain fatty acid (SCFA), which helps maintain immune function in the colon (Arpaia et al., 2013; Furusawa et al., 2013). An enrichment of S24-7 in VMm-supplemented rats suggests a potential health benefit of this vinegar-containing beverage. Recent genomic characterization

of this family (Ormerod et al., 2016) will enable a better understanding of its function in host-microbe interactions, particularly relating to the physiology of the host. The family Ruminococcaceae comprises fermentative bacteria in the phylum Firmicutes and has been associated with consumption of dietary fiber. More information on the specific genus that was enriched in the control group would be required to accurately define the role for this taxon. Using LDA Effect Size (LEfSe) analysis, we found that 3 OTUs (2 in the family Ruminococcaceae (Firmicutes) and one in S24-7 (Bacteroidetes) were enriched in 42 day samples. Similarly, 3 OTUs (one in the family Peptostreptococcaceae and one in the genus Streptococcus, both Firmicutes) and one OTU in phylum Bacteroidetes were enriched in the 21-day samples.

5. Summary

We observed significant differences in the fecal metabolome and minor differences in fecal microbiome between control and VMm-supplemented animals. This VMm supplement was provided for three separate three-week periods: gestation, lactation, and three weeks post-weaning. Multiple factors can determine the concentration of each fecal metabolite. While many of the metabolomic changes were associated with enhanced/decreased absorption of metabolites from the diet, these changes may also reflect VMm-induced differences in the microbiome that may in turn influence absorption from the lumen. Some molecules are present in the diet, others are produced by the gut microbiome, others represent metabolism of molecules by the gut microbiome and finally, the absorption of metabolites from the GI tract also affects concentrations. Our data clearly show an effect of diet (a vinegar-containing beverage), but also of development. From weaning (21 days) to early adolescence (42 days), there were more changes in fecal metabolites and microbiome. The total numbers of significantly altered metabolites were also lower in the multiple-dam than in the single-dam cohort probably due to greater genetic heterogeneity in pups with different mothers.

Although LEfSe analyses indicated treatment-specific significant enrichment of certain OTUs, our data indicate that overall, the effects of VMm supplement on fecal microbial communities were not as dramatic as on fecal metabolites. It is possible that the major components of this beverage, vinegars and fruit sugars, are metabolized and/or absorbed early on in digestion and therefore, do not affect colonic microbiota.

Our data indicate that VMm supplementation had no overt toxic effects on rats despite the reduction in body weight and bone mineral density at later ages. Whether a lower body weight in older animals correlates with changes in metabolic pathways and shifts in microbial communities require further investigation. We also do not know whether differences in body weight persist throughout the life span. This paper documents the importance of drinking water composition *in utero* and during early development. It is also a paradigm for investigations of other ingested nutrients, probiotics, or toxins, such as metals or nanomaterials in food and drink. We believe that an integrated approach of studying the microbiome, the metabolome, and a variety of physiologic parameters is essential in understanding how ingested materials affect the GI tract and thus the entire body.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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APPENDIX A.: Supplementary data

The supplementary materials associated with this article can be found in the online version.

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Highlights

Vinegar-based multi-micronutrient supplemented water:

- Alters fecal metabolites especially amino acids and dipeptides
- Alters the gut microbiome
- Reduces body weight gain later in life
- Can be best evaluated by linking changes in the gut microbiome with fecal metabolites and physiological parameters

Figure 1.

Experimental design. Pregnant rats were obtained at gestational age E2. Pregnant rats were provided with drinking water supplemented with 1% v/v Pairogen™ (n=6 rats) or with control tap water (n=6 rats). The designated drinking water was provided throughout the period of pregnancy and lactation. At age 21 days, the pups were weaned and were then provided the same drinking water assigned previously until euthanasia at times up to age 105 days. Fecal samples were collected at age 21 days and 42 days. Additional rats were tested for gut absorption of iron at age 49 days. Body composition (bone density and body fat) was analyzed in selected rats at age 63 and 105 days.

Figure 2.

Body weights of male and female rats over the course of the experiment. Each rat was weighed once weekly from weaning (21 days) until the end of the experiment (105 days). The numbers of rats in each group decreased over time. There were significant decreases in body weights from day 70 until day 105 in both male and female rats in the VMm group versus controls (* $P < 0.05$, MANOVA).

Figure 3. Pharmacokinetics of ^{59}Fe in control and VMm-supplemented rats. **a.** Plasma concentrations of ^{59}Fe over 24 hours post-injection (**b.**) Plasma concentrations of ^{59}Fe over 24 hours post-gavage. Note: n= 4 males and 6 females/group.

Figure 4.

Principal components analysis of the metabolite profile in VMm and control rats at day 21 and day 42. The percentage of variation explained by the plotted principal components is indicated on the axes. Each symbol represents the metabolite profile of an individual rat. The clusters of control and VMm groups at both 21 and 42 days are closer to each other in terms of the Euclidean distance. The clusters for 21 do not overlap those for 42 days in both control and VMm groups.

Figure 5.

Linear discriminant analysis effect size analysis of fecal microbiome data. **a, b.** Relative abundance plots of OTUs enriched in control and VMm groups, as identified by LefSe analysis of microbial community profiles. Bacteria belonging to Ruminococcaceae were enriched in control (**a**) while those belonging to S24-7 were enriched in VMm-supplemented rats (**b**). **c-h.** Age-related changes in fecal microbial community. Three OTUs belonging to families S24-7 and Ruminococcaceae were enriched on day 42 (**c,d,e**), and 4 OTUs belonging to 3 families (Bacteroidetes, Streptococcaceae and Peptostreptococcaceae)

were enriched on day 42 (**f,g,h**). Note: solid horizontal lines and dashed horizontal lines represent the mean and median relative abundances.

Figure 6. Principal Coordinates Analysis (PCoA) of the *theta*YC index of microbial community structure in VMM and control rats at day 21 and day 42. The clusters of VMM rats overlap with those of control rats. The percentage of variation explained by the plotted principal coordinates is indicated on the axes. Each symbol represents the microbial profile of an individual rat.

Table 1.

Analysis of Vinegar-based Multi-micronutrient Supplement (Pairagen® concentrate)

Organic acid (mg/mL)	Malic acid	10.8
	Acetic acid	8.4
	Citric acid	31.5
Vitamins (mg/mL)	Vitamin C	7.7
	Vitamin B2	0.08
	Vitamin B6	0.08
Sugar (mg/mL)	Fructose	200
	Glucose	220
	Sucrose	3.1
	Maltose	30
	Lactose	5.1
	Raffinose	5.1
Amino acid (µg/ml)	ASP	73.8
	GLU	709.2
	SER	10.2
	HIS	4.8
	GLY	1160
	THR	13.8
	ARG	8.4
	ALA	807.6
	TYR	3
	VAL	4.8
	MET	0.6
	PHE	3.6
	ILE	4.2
	LEU	6
	PRO	1.8
Elements (µg/ml)	Cl	5873
	Na	3496
	K	736
	Si	42
	Mg	22
	Br	8

Organic acids, amino acids and sugars were detected and measured using liquid chromatography. Elements were measured by ICP-OES.

Table 2:

Summary of statistical analyses of fecal metabolites in VMm and control groups. Data shown are from two separate experiments, one in which rat pups were chosen from multiple rat dams and a second in which all rat pups came from a single dam. Results from ANOVA contrasts and a linear mixed-effect regression model are presented. Only the statistically significant metabolites are represented in this table.

	Multiple-Dam Cohort		Single-Dam Cohort	
ANOVA contrasts	Total # metabolites (p 0.05)	Metabolites (↑ ↓)	Total # metabolites (p 0.05)	Metabolites (↑ ↓)
Control: 21 vs. 42 d	193	33 160	227	117 110
VMm: 21vs. 42 d	231	50 181	242	159 83
21 d: VMm vs. Control	35	33 2	178	22 156
42 d: VMm vs. Control	10	9 1	142	39 103
Pedigree Linear Mixed Regression Model				
VMm effect	30		55	
Age effect	266		207	

Data shown were obtained from the multiple-dam (5 dams, 2 pups per dam) and single-dam (10 pups from each dam) cohorts.

Table 3:

Analysis of molecular variance (AMOVA) based on three indices of microbial community composition and structure.

	thetaYC	jclass	Braycurtis
Comparison	p value	p value	p value
Control vs. VMm	0.74	0.04	0.59
Among 4 groups	0.007	<0.001	0.003
Post-hoc pairwise comparison			
21 d: Control vs. VMm	0.12	0.11	0.16
42 d: Control vs. VMm	0.93	0.07	0.70
Control: 21 vs. 42 d	0.007	0.03	0.007
VMm: 21 vs. 42 d	0.03	0.004	0.006*

Theta YC and *BrayCurtis* are indices of similarity and *jclass* measures the community membership.

Experiment-wise error rate: 0.05; Pair-wise error rate (Bonferroni): 0.008