RESEARCHREPORT
HUMAN NUTRITION

Title: Metabolism of Vitamin K Forms in Fresh Pork - #**17-003 IPPA**

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Industry Summary:

1. **Objectives:**
    a. Determine if vitamin K forms found in pork are absorbed and contribute to vitamin K function in the body.
    b. Determine if there is sex difference for vitamin K forms absorption.
    c. Determine if tissue accumulation of vitamin K forms are independent of gut microbiota (the microbe population living in our intestines) production of menaquinones. Menaquinone forms are also produced by the gut bacteria.

2. **How research was conducted:** Male and female mice were fed a diet containing (1) pork; (2)purified forms of menaquinones (vitamin K2) found in pork; or (3) a vitamin K deficient diet. Vitamin K forms in blood, tissues, and feces were measured. As vitamin K is essential for blood clotting, a blood clotting time test was conducted to test vitamin K function.

3. **Research findings:** Our results show that menaquinones found in pork are absorbed. Female mice has higher vitamin K concentrations than male mice in most analyzed tissues. Vitamin K forms in the liver and feces reflect the forms provided in the diet, both from pork and from the purified dietary forms. No vitamin K forms were found in blood. Although the blood clotting time test did not show differences between the mice on different diets, all mice fed pork or purified vitamin K forms were healthy, whereas several mice on a vitamin K deficient diet died prior to the end of the study. Despite an abundance of menaquinones produced by the microbiota (as indicated by measurement in feces) independent of diet, animals on the vitamin K deficient diet contained minimal vitamin K in tissues. These results together demonstrate multiple dietary vitamin K forms contribute to tissue accumulation of vitamin K independent of gut microbiota.

4. **What these findings mean to the industry**: Fresh pork cuts and processed pork products are a rich dietary source of menaquinones, which are forms of the essential nutrient vitamin K. We have demonstrated in a mouse model that menaquinone forms in the pork products can be absorbed and effectively metabolized. Vitamin K is considered a shortfall nutrient for older adults, which means that many older adults consume less vitamin K than consumed by other age groups. These data suggest that pork may have a role in increasing vitamin K intakes. However, these findings generated in a mouse model must be extended to human populations prior to promoting pork as a vitamin K-rich food.

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Scientific Abstract:

Objective: Vitamin K (VK) exists in multiple forms. Plant-based phylloquinone (PK) is considered the predominant dietary VK form. However, recent studies have shown bacterial and animal-based forms of VK, called menaquinones (MKn; n=number of prenyl units in side chain), are prevalent in foods such as dairy, fermented products, and meat, including pork. Bacterially-produced MKn are also synthesized by the gut microbiota. It is unknown if MKn are absorbed and metabolized. The objective of this study was to compare MKn concentrations in blood, tissue, and feces of mice given purified and food-based MKn to mice fed a VK-deficient (VKD, "control") diet.

Methods: Sixty male and 60 female 10-week old C57BL6 mice were acclimated on a VKD diet. After 4 weeks, mice were randomized to six groups and given a diet containing purified dietary VK forms ("PK", "MK4", "MK9", or an equimolar combination, "Combo"), pork (24% of diet, "Pork"), or maintained on "VKD" diet for 4 weeks. VK forms in diets, blood, tissues, and feces were measured using LC-MS, and compared by diet group and sex using 2-way ANOVA. Bacterial DNA was extracted from fecal samples and sequenced using Illumina HiSeq to analyze bacterial community composition. Differences in composition by sex and diet group were determined using non-parametric PERMANOVA, and bacterial richness was compared by sex and by diet group using Welch's t-test.

Results: The VK content of the study diets were as follows (in μg/kg diet): **control**: 20.6 ± 2.0 PK; **PK**: 3200 ± 932 PK; **MK4**: 10.2 ± 0.4 PK and 2150 ± 184 MK4; **MK9**: 9.2 ± 1.6 PK and 3820 ± 598 MK9; **Combo**: 925 ± 173 PK, 821 ± 130 MK4, and 1310 ± 214 MK9; and **Pork**: 11.1 ± 0.6 PK, 38.5 ± 7.8 MK4, 34.7 ± 9.8 MK9, and 33.2 ± 10.5 MK10. In all analyzed tissues, tissue accumulation of VK was greater in females as compared to males. VK content of the small intestine and liver reflected vitamin K present in the diet, such that PK, MK4, and MK9 were significantly higher in groups supplemented that form as compared to the control group (all $p<0.03$). Female mice had significantly higher bacterial richness compared to male mice (Welch's t-test, t = 2.2699, df = 92.739, p-value = 0.02553) while no significant differences in bacterial richness were observed between dietary regiments.

Conclusions: MKn forms in small intestine and liver reflect dietary MKn intake. There is sex difference for VK forms absorption. Fecal MKn partially reflected intake, but endogenous production of MKn was largely unaffected by dietary VK. Despite abundant endogenous MKn

production, tissue VK was low in the control group suggesting tissue accumulation of VK is independent of MKn produced in the gut.

Introduction:

The current U.S. dietary guidelines for intakes of Vitamin K (VK) are 90 and 120 µg/day for women and men, respectively. Dietary sources of VK are found in two natural forms: phylloquinone (PK; vitamin K1) and menaquinones (MK; vitamin K2). All forms of this fat-soluble vitamin share the common structure, 2-methyl-1,4-napthoquinone. MK differ in structure from PK in their 3-substituted lipophilic side chain, and are designated by the number of isoprenoid units, i.e. MK-n. MK-n with up to 13 isoprenoid units have been identified and are classified as either shorter chain or longer chain MK forms. Whereas PK is widely distributed in the food supply, MK forms are limited to animal products and fermented foods. Very little is known about the contribution of dietary MK to overall VK nutrition. Estimated intakes of PK and MK in Western Europe suggest that between 10% and 25% of total VK intake are provided by MK. No comparable data exist for the U.S. However, our data (NPB project #14-100) suggest that pork products contribute more MK to the total dietary VK intake than previously thought.

Based on completed NPB project #14-100, pork did not contain appreciable amounts of PK, which historically was considered a poor dietary source of VK. In contrast, all fresh pork cuts and processed pork products contained MK4, MK10 and MK11. The fresh pork cuts also contained MK9, which was not detected in any of the processed pork products. However, stating that fresh pork contributes to VK nutrition requires data on metabolism and function of these longer-chain MK forms. As a fat-soluble vitamin, all VK forms are thought to be absorbed by a passive diffusion process. However, our current understanding regarding VK absorption does not explain the current discrepancies in the scientific literature, which suggest that VK bioavailability varies according to side chain length. Previous studies of MK bioavailability and function have relied on purified supplements. There are no data on the interaction of different VK forms obtained from foods on VK metabolism and physiological function despite their co-existence in multiple foods, including pork. This knowledge is requisite to developing informed nutritional recommendations for optimizing VK nutriture.

Our research team was the first to directly examine interrelationships between the gut microbiota and MK biosynthesis *in vivo*, the result of which indicated that diet-mediated modulation of the gut microbiota alters fecal MK content. However, we found no evidence of these same MK forms in circulation so it is not clear that MK produced by bacteria in the human gut are absorbed and contribute to vitamin K nutriture. An animal model of VK metabolism is therefore essential to testing our hypothesis that pork is an important dietary source of longer-chain MK forms, and that these MK are absorbed and stored in liver and other tissues independent of MK production by gut bacteria. We also posit that these MK forms confer physiological function. Successful completion of the proposed research will lay the foundation for future studies to evaluate the importance of pork products to VK nutriture and human health.

Recent reports from Europe attribute unique heart health benefits to MK forms obtained from the diet. Growing evidence suggests that VK protects against the progression of abnormal calcification in coronary arteries, thereby decreasing risk of cardiovascular disease. As our previous work demonstrates that fresh pork cuts and pork products are a rich dietary source of MK, demonstrating bioavailability and bioactivity of pork-derived MK will establish an important beneficial role for pork in VK nutrition and related health and disease states.

Objectives:

a. Determine if menaquinone (MK) forms present in pork have equivalent absorption and function compared to the plant-based VK form, phylloquinone (PK).

b. Determine if accumulation of dietary MK forms in tissues are independent of gut microbiota production of MK.

Materials & Methods:

Ten-week-old male and female C57BL6 mice were fed a VK-insufficient diet for 4 weeks, and then were randomized into the following groups (n=20 per group, 10 male and 10 female): (1) Control group (VK-insufficient diet); (2) PK group (5 µmol/kg diet); (3) shorter chain MK group (MK4 group: 5 µmol /kg diet); (4) longer chain MK group (MK9 group: 5 µmol/kg diet) and (5) Combo Group (PK: 1.6 µmol/kg; MK4: 1.6 µmol/kg; MK9: 1.6 µmol/kg). VK doses across all groups were equimolar and were 2-fold the minimum recommended intake of vitamin K. We chose these doses to mimic intakes of a healthy diet. In addition, we included a 6th group (n=20) fed a diet containing freeze-dried pork (Pork Group, 24% of the diet, **Figure 1**).

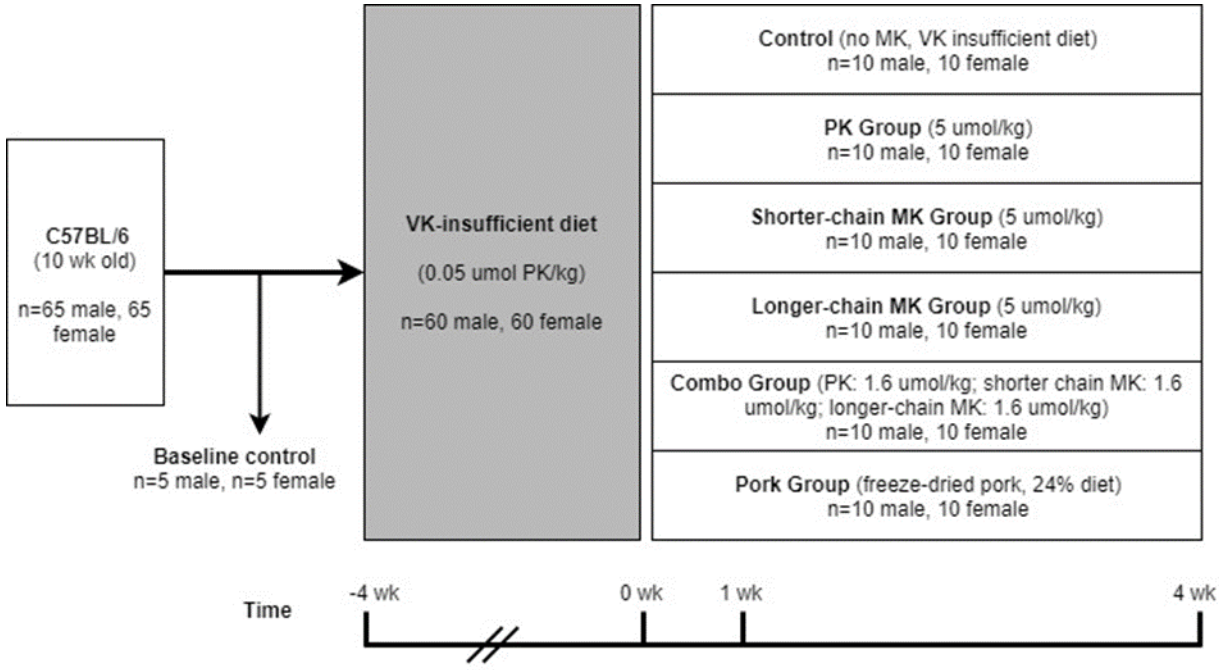

Figure 1: Study design

a. Procedures:

Body weight was measured every week. After 4-week intake of the experimental diets, mice were fasted for 12h and anesthetized to measure bleeding time by tail cut. After that, mice were sacrificed using secondary thoracotomy while still under anesthetic. At the time of sacrifice, multiple tissues including blood, liver, kidney, heart, small intestine, and feces were collected and frozen in liquid nitrogen, and then kept in a deep freezer (-80^{o}C) until analysis.

b. Measurements:

1) VK Analysis by LC/MS (Measures of VK diet and VK metabolism): PK and MK4-13 concentrations in blood, feces, tissues and rodent diets (total 850 samples) were measured by LC/MS according to our standard procedure. We have previously shown that this method provides an expedient and sensitive method for measuring PK, MK4 through MK13 in mouse tissues, diet samples and feces.

2) Blood clotting time (Measure of VK function): Eighteen-week-old mice (n=120) were anesthetized with CO_2 asphyxiation. Their tails were cut to yield the same wound diameters. To evaluate bleeding time, filter paper were applied to the edge of the wound every minute, taking care not to dislodge the clot.

3) High-throughput microbial community analysis on the Illumina HiSeq platforms (Measure of gut bacteria, which produce MK; conducted in B Wolfe's laboratory): Cecal samples (total 120 samples) were collected and stored at -80°C in accordance with published methods. DNA were extracted from cecal samples using a MoBio PowerSoil DNA extraction kit and barcoded 16S rDNA amplicon libraries were constructed to characterize bacteria diversity as we have previously described. Amplicons were sequenced on an Illumina HiSeq at the Michigan State University Research Technology Support Facility Core using 100 base-pair, paired-end reads. Demultiplexing, sequence filtering, operational taxonomic unit (OTU) assignment, and analysis were performed in QIIME.

c. Statistical Analysis Approach

Three male mice in the vitamin K deficient group died prior to the conclusion of the study and are therefore not included in the statistical analyses (n=7 for the vitamin K insufficient group, all other groups experienced no losses, n=10).
Vitamin K data are displayed as geometric means ± SEM, unless stated otherwise. Vitamin K concentrations were log-transformed to meet normality assumptions prior to statistical testing. Within each tissue, vitamin K concentrations were compared by diet group and sex using a 2-way ANOVA. If a diet group*sex interaction was observed, analyses were stratified by sex.
Principal coordinates analysis was used to examine bacterial community composition based on Bray-Curtis similarity, and differences by sex and diet group were determined using non-parametric PERMANOVA. Bacterial richness was compared by sex and by diet group using Welch's t-test. Changes in abundance of menaquinone biosynthesis genes were assessed by fitting negative binomial regressions using the DESeq-2 package.

Results:

a. Diets

By design, with the exception of the control diet, the diets containing purified VK forms were targeted to be equimolar in concentration. The control diet was intentionally formulated to contain less VK, to act as a negative (deficient) control in this experiment. The pork diet is included to demonstrate MK absorption from a real-food matrix, but by nature contains multiple forms of vitamin K and is lower in total vitamin K than the purified vitamin K diets (with the exception of the control diet) (**Table 1**). This should be kept in mind in the following analyses, as the tissue concentrations of any one form of vitamin K in a tissue the pork group are generally lower than the supplemented groups (as would be expected) and the comparison to the deficient group is the most relevant for the pork group. Additionally, the background diet of all the purified vitamin K diets is the same, while the pork diet has a different macronutrient composition due to inclusion of whole pork meat, which should be kept in mind when evaluating the microbiome data as macronutrient composition is known to affect gut

microbiome composition. The measured concentrations of vitamin K in the study diets (mean ± SD) are as follows (**Table 1**):

Table 1. Vitamin K concentrations in experimental diets (μg/kg diet).

VK forms	Control	PK	MK4	MK9	Combo	Pork
PK	20.6 ± 2.0	3200 ± 932	10.2 ± 0.4	9.2 ± 1.6	925 ± 173	11.1 ± 0.6
MK4	ND	ND	2150 ± 184	ND	821 ± 130	38.5 ± 7.8
MK9	ND	ND	ND	3820 ± 598	1310 ± 214	34.7 ± 9.8
MK10	ND	ND	ND	ND	ND	33.2 ± 10.5

ND – not detectable; the lower limit of detection: MK4:13.52; MK9:3.93; MK10:0.85 μg/kg.

b. Final body weight (g)

As expected, males had a significantly higher final body weight than females (**Table 2**), ($p<0.001$), but body weight did not differ by diet group ($p=0.7$)

Table 2: Final body weight of the animal (g).

	Control	PK	MK4	MK9	Combo	Pork
Males	34.9 ± 2.5	33.4 ± 3.4	34.9 ± 3.3	34.2 ± 3.0	34.2 ± 3.0	32.3 ± 3.1
Females	24.9 ± 3.0	25.1 ± 2.1	24.5 ± 2.8	25.0 ± 2.9	25.3 ± 3.1	26.6 ± 3.6

Mean ± SD.

c. Comparison of different forms of vitamin K absorption

1) Blood levels of vitamin K

For measurement of vitamin K in blood, we used serum. All serum vitamin K concentrations below limit of quantification; most also below the lower limit of detection (HPLC assay LLOD=0.01 pmol/g). This is not an unexpected finding as we used physiological relevant doses in the study. In contrast to humans, serum concentration of vitamin K in mice are low. Therefore, serum vitamin K concentrations are not considered statistically as part of this project.

2) Small intestine and liver

PK, MK4, MK9 and MK10 were detected in small intestine and liver samples (**Table 3**). PK and MK4 concentrations were significantly higher in females as compared to males in both the small intestine and liver.

PK in intestine and liver: As a significant sex*group interaction was observed, group analyses were conducted stratified by sex. However, the results were parallel: in both the small intestine and liver, PK was significantly higher in the PK group as compared to Control and Combo group (both $P<0.002$), and the Combo group was significantly higher than the Control group ($p<0.005$).

MK4 in intestine:

- In males, MK4 was significantly higher in the MK4 group as compared to all other groups (all $p<0.002$), but MK4 was not statistically different between PK, MK4, and Combo groups (all $p=1.0$). Male MK4 was significantly lower in the Control and Pork groups as compared to all other groups ($p<0.001$), but these groups did not statistically differ from each other ($p=1.0$).
- In females, MK4 did not differ between any of the supplemented groups (PK, MK4, MK9, or Combo; all $p=1.0$), but all supplemented groups were higher than the Control group and Pork group (both $p<0.001$), and the Control and Pork groups were not statistically different from each other ($p=0.58$).

MK4 in liver: similar results were found in liver, except MK4 in liver of the Pork group was significantly higher than the Control group in both male and female (both $p\leq0.02$).

MK9 in intestine: In males, the only group to contain MK9 in the intestine was the MK9 group, but the not all males in the group had detectable levels of MK9 and the average MK9 was not statistically different from zero ($p=0.07$). Therefore, males and females were not statistically compared. In females, MK9 was only found in the MK9, Combo, and Pork groups. MK9 was significantly higher in the MK9 group as compared to the Pork group ($p<0.001$), but not compared to the Combo group ($p=0.15$). The Combo group was not significantly higher than the Pork group ($p=0.07$).

MK9 in liver: Females had significantly higher liver MK9 as compared to males ($p<0.001$).

- In males, liver MK9 was higher in the MK9 group as compared to the Control and Pork groups ($p<0.03$), but was only marginally higher than the PK, MK4, and Combo groups (p ranging from 0.05 – 0.08). Liver MK9 in the PK, MK4, Control, and Pork groups were not significantly different from each other.
- In females, liver MK9 was greatest in the MK9 group as compared to all other groups (all $p<0.001$). Liver MK9 in the Combo group was significantly greater than the PK, MK4, Control, or Pork groups (all $p<0.001$), but these lesser groups were not significantly different from each other (all $p=1.0$).

MK10 in intestine: small intestine MK10 did not statistically differ by sex or diet group.

MK10 in liver: Liver MK10 was not significantly different by sex ($p=0.77$), but did differ by diet group ($p=0.006$). Liver MK10 was significantly higher in the Pork group as compared to all other groups (all $p<0.004$), but the lesser groups did not differ from each other (all $p=1.0$).

3) Extra-hepatic tissues

Vitamin K concentrations in extra-hepatic tissues are shown in **Table 4**. PK and MK4 were the only VK forms present in heart (no long-chain forms, confirmed via Q-TOF analysis). The kidney and brain only contained MK4.

PK: Heart PK was significantly higher in females as compared to males ($p<0.001$). The PK group had significantly higher heart PK as compared to all other groups (all $p<0.001$). The Combo group had significantly higher PK than the MK4, MK9, Control, and Pork groups (all $p<0.004$). The MK4, MK9, Pork, and Control diets did not differ from each other (all $p=1.0$).

MK4: Kidney and brain MK4 were significantly higher in females as compared to males (all $p<0.001$). In kidney and brain, MK4 did not differ between supplemented groups (PK, MK4, MK9, and Combo groups, all $P=1.0$). In kidney, the Pork group and Control group were lower in MK4 group as compared to all other groups (all $p<0.01$), but did not statistically differ from each other ($p=1.0$). This same pattern was seen in brain, but there was a trend towards higher brain MK4 in the Pork group as compared to Control group ($p=0.09$).

Heart MK4 was significantly higher in females as compared to males (all $p<0.001$). In males, there were no significant differences in heart MK4 by diet group ($p=0.14$).

In females, the heart MK4 in supplemented groups (PK, MK4, MK9, and Combo) did not statistically differ from one another (all P=1.0). The Control group has statistically less heart MK4 than all supplemented groups (all P<0.0001), but not statistically differ from the Pork group (P=1.0).

Table 3. Vitamin K concentrations in small intestine and liver (pmol/g tissue).

		Diet Group												2-way ANOVA p-values		
	pmol/g	Control		PK		MK4		MK9		Combo		Pork		Sex	Group	Sex* Group
		M n=7	*F* n=10	*M* n=10	*F* n=10	*M* n=10	*F* n=10	*M* n=10	*F* n=10	*M* n=10	*F* n=10	*M* n=10	*F* n=10	-	-	-
Small intestine	**PK**	1.1 ± 0.2	1.6 ± 0.4	21.4 ± 2.1	42.9 ± 5.9	ND	ND	ND	ND	2.8 ± 0.5	12.6 ± 2.3	ND	ND	<0.001	<0.001	0.005
	MK4	1.3 ± 0.5	1.5 ± 0.3	12.3 ± 1.2	45.0 ± 5.8	24.8 ± 3.7	48.0± 11.9	9.9 ± 1.7	32.5 ± 4.1	12.9 ± 1.4	43.7 ± 4.6	1.1 ± 0.1	2.4 ± 0.7	<0.001	<0.001	<0.001
	MK9	ND	ND	ND	ND	ND	ND	2.5 ± 2.8	9.7 ± 2.9	ND	3.8 ± 1.0	ND	1.3 ± 1.3	-	<0.001	-
	MK10	6.9 ± 14.2	2.3 ± 1.0	3.8 ± 6.9	3.2 ± 4.3	6.0 ± 3.9	1.9 ± 1.1	2.9 ± 1.7	2.1 ± 8.0	2.8 ± 1.8	1.8 ± 1.0	4.5 ± 3.4	5.2 ± 9.2	0.13	0.42	0.55
Liver	**PK**	4.4± 0.2	5.4 ± 0.37	30.0± 3.4	87.1 ± 7.6	4.7 ± 0.2	5.6 ± 0.3	4.8 ± 0.3	5.5± 0.2	9.7 ± 0.6	27.5 ± 2.8	4.3 ± 0.1	4.9 ± 0.2	<0.001	<0.001	<0.001
	MK4	2.0 ± 0.3	3.5 ± 0.4	9.9 ± 0.9	28.4 ± 2.2	15.1 ± 1.6	48.7 ± 4.9	7.5 ± 0.6	28.0 ± 3.1	9.5 ± 0.7	34.5± 2.3	3.2 ± 0.3	6.6 ± 0.7	<0.001	<0.001	<0.001
	MK9	4.5 ± 1.2	2.4± 3.1	3.6± 2.2	2.2± 2.0	3.5± 1.6	1.5± 0.7	16.4± 3.1	192± 25.9	6.0± 1.5	46.9± 4.2	3.6± 6.7	1.7± 3.0	<0.001	<0.001	<0.001
	MK10	7.7± 1.4	6.2± 4.1	7.9± 2.3	6.6± 3.5	7.6± 1.9	6.3± 4.2	8.5± 5.5	5.6± 3.4	7.5± 2.5	6.7± 2.6	22.9± 8.0	19.6± 5.0	0.77	0.006	0.97

Geometric mean ± SEM. Data were ln-transformed for normality prior to ANOVA.*For the small intestine, because entire groups lacked detectable levels of PK or MK9, ANOVA could not be conducted across all groups (cannot compare variances when variance is zero). Instead, did one-way *t*-tests for groups that possessed that form (*eg*, for PK - Control, PK, and Combo Groups) compared to zero. Then, if group was significantly different than zero, the group was included in a data subset and p-values for sex, group, and sex*group are from analysis of the data subset.

Table 4: Vitamin K contents in extra-hepatic tissues (pmol/g).

		Diet Group												2-way ANOVA p-values		
		Control		PK		MK4		MK9		Combo		Pork		Sex	Group	Sex* Group
	pmol/g	*M* n=7	*F* n=10	*M* n=10	*F* n=10	*M* n=10	*F* n=10	*M* n=10	*F* n=10	*M* n=10	*F* n=10	*M* n=10	*F* n=10	-	-	-
Kidney	**PK**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	<0.001	<0.001	<0.001
	MK4	6.8 ± 6.1	34.5 ± 6.3	48.1 ± 2.6	240 ± 17	50.4 ± 2.3	233 ± 11.8	32.3 ± 5.3	227 ± 9.7	44.4 ± 2.7	231 ± 9.4	8.6 ± 5.4	72.0 ± 4.1	<0.001	<0.001	0.84
Brain	**PK**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
	MK4	3.9 ± 0.3	15.6 ± 0.9	77.3 ± 5.9	342 ± 15.3	66.2 ± 4.8	277 ± 12.3	64.7 ± 3.8	295± 10.4	67.0 ± 5.0	323± 17.8	9.8 ± 1.3	33.4 ± 2.6	<0.001	<0.001	0.16
Heart	**PK**	ND	ND	11.7 ± 1.9	29.3 ± 2.5	ND	ND	ND	ND	2.0 ± 0.6	7.2 ± 0.9	ND	ND	<0.001	<0.001	0.45
	MK4	66.1 ± 5.2	83.5 ± 18	84.9 ± 7.5	180 ± 16	93.7 ± 9.8	188 ± 12.3	81.7 ± 7.0	174 ± 8.3	85.5 ± 7.8	197 ± 4.6	71.9 ± 6.8	96.0 ± 6.7	<0.001	<0.001	0.001

Geometric mean ± SEM. Data were ln-transformed for normality prior to ANOVA. *ND – not detectable

Table 5. Vitamin K contents in fecal samples (pmol/g)

	Diet Group												2-way ANOVA p-values		
pmol/g	Control		PK		MK4		MK9		Combo		Pork		Sex	Group	Sex* Group
Vitamer	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*	-	-	-
PK	ND	ND	2071 ± 146	2724 ± 121	ND	ND	ND	ND	691 ± 40.0	915 ± 48.8	ND	ND	0.002	<0.001	0.96*
MK4	1374 ± 122	1575 ± 107	1617 ± 176	1616 ± 128	2054 ± 109	2161 ± 126	1844 ± 194	1694 ± 107	2024 ± 269	2055 ± 250	1773 ± 259	1418 ± 216	0.91	0.01	0.59
MK5	6.1 ± 0.8	7.9 ± 1.5	6.2 ± 1.0	10.4 ± 1.4	6.5 ± 1.3	8.6 ± 1.2	7.6 ± 1.0	8.7 ± 1.3	6.4 ± 0.8	8.8 ± 1.7	5.0 ± 0.0	5.2 ± 0.2	0.06	0.005	0.41
MK6	828 ± 210	679 ± 143	562 ± 107	798 ± 112	601 ± 105	590 ± 122	636 ± 103	535 ± 122	561 ± 113	624 ± 135	279 ± 48.5	404 ± 57.0	0.68	0.005	0.47
MK7	30.9 ± 11.7	70.2 ± 28.9	43.8 ± 16.2	89.5 ± 16.0	44.9 ± 35.9	91.2 ± 16.6	29.2 ± 6.7	63.4 ± 8.3	27.8 ± 11.5	91.9 ± 20.8	13.2 ± 10.0	65.9 ± 12.9	<0.001	0.02	0.26
MK8	174 ± 32.2	325 ± 55.6	221 ± 37.1	362 ± 44.2	224 ± 31.7	305 ± 48.3	238 ± 39.0	346 ± 45.2	190 ± 26.2	349 ± 57.1	144 ± 20.5	325 ± 55.6	0.009	<0.001	0.51
MK9	213 ± 56.4	344 ± 84.5	287 ± 37.0	421 ± 52.0	275 ± 53.8	310 ± 71.8	3459 ± 434	5355 ± 499	990 ± 117	1884 ± 187	220 ± 37.9	470 ± 40.0	0.009	<0.001	0.52
MK10	938 ± 172	1237 ± 196	1167 ± 201	1566 ± 161	1137 ± 181	1394 ± 240	1360 ± 332	1664 ± 208	1302 ± 146	1524 ± 198	883 ± 135	1539 ± 97.0	0.47	0.58	0.82
MK11	534 ± 101	716 ± 115	676 ± 93.6	883 ± 77.3	632 ± 106	763 ± 169	712 ± 137	856 ± 102	645 ± 56.1	786 ± 91.9	464 ± 63.2	792 ± 58.3	0.34	0.76	0.83
MK12	67.8 ± 24.6	61.4 ± 11.9	77.3 ± 12.1	89.3 ± 13.3	92.2 ± 19.7	93.1 ± 28.5	97.4 ± 19.4	98.5 ± 17.6	87.3 ± 9.8	88.0 ± 11.1	66.8 ± 8.2	93.5 ± 10.0	0.97	0.49	0.85
MK13	196 ± 84.7	59.9 ± 62.6	150 ± 34.1	99.6 ± 50.9	255 ± 83.6	107 ± 57.0	204 ± 79.2	130 ± 39.8	179 ± 54.3	91.2 ± 37.6	228 ± 35.3	184 ± 63.8	0.13	0.53	0.68

Geometric mean ± SEM. ND, nondetectable or below lower limit of detection. n= 20 per diet group (10M, 10F) *except* control group (9M, 10F). Data were cleaned to change values <LOD to LOD, and were ln-transformed prior to ANOVA.

*Because of nondetectable levels of PK in MK4, MK9, VK deficient, and Pork Groups, ANOVA could not be conducted across all groups (cannot compare variances when variance is zero). Instead, did one-way *t*-test for PK and Combo Groups compared to zero. Both significantly different than zero. P-values for sex, group, and sex*group are from analysis of a data subset with data from PK and Combo Groups only.

4) Feces

PK: Fecal PK was significantly higher in the PK group as compared to the Combo group ($p<0.001$), higher in females as compared to males ($p=0.002$).

MK4: Fecal MK4 did not differ by sex ($p=0.91$). Fecal MK4 was significantly lower in the Control group as compared to the MK4 and Combo groups (both $p<0.02$), but did not differ from the MK9, PK, or Pork groups (all $p=1.0$). Fecal MK4 was not significantly different between the MK4 and Combo groups ($p=1.0$).

MK5: Fecal MK5 did not differ by sex ($p=0.06$). Fecal MK5 was significantly lower in the Pork group as compared to all other groups (all $p<0.02$), but the other groups were not different from each other (including Control, all $p=1.0$).

MK6: Fecal MK6 did not differ by sex ($p=0.68$). Fecal MK6 was significantly lower in the Pork group as compared to all other groups (all $p<0.05$), but the other groups were not different from each other (including Control, all $p=1.0$).

MK7: Fecal MK7 was significantly higher in female mice as compared to males ($p<0.001$), but no sex*group interaction was observed ($p=0.26$). Fecal MK7 differed by Group overall ($p=0.02$), but after correction for multiple comparisons in pairwise tests, groups were not statistically different in fecal MK7 (likely driven by Pork group; after correction for multiple comparisons the Pork group was only marginally lower in fecal MK7 than the PK, Combo, and MK4 groups (p ranging from 0.07-0.1)).

MK8: Fecal MK8 was significantly higher in female mice as compared to males ($p=0.009$), but no sex*group interaction was observed ($p=0.51$). Fecal MK8 was higher in the MK9 group as compared to all other groups (all $p<0.001$). The Combo group was higher in MK8 as compared to the PK, MK4, Pork, and Control groups (all $p<0.001$). PK, MK4, Pork, and Vitamin K groups were not significantly different from each other (all $p=1.0$).

MK9: Fecal MK9 was significantly higher in female mice as compared to males ($p=0.009$), but no sex*group interaction was observed ($p=0.52$). Fecal MK9 was significantly higher in the MK9 group as compared to all other groups (all $p<0.001$). Fecal MK9 was higher in the Combo group as compared to the PK, MK4, Pork, or Control groups (all $p<0.001$). Fecal MK9 in the PK, MK4, Pork, and Control groups were not statistically different from each other (all $p=1.0$).

MK10, MK11, and MK12: Did not statistically differ by sex (all $p>0.33$) or group (all $p>0.48$).

MK13: Although there was no overall significant sex difference ($p=0.13$), there was a trend towards higher fecal MK13 in male mice.

d. Vitamin K function

VK function is defined as blood clotting time, which did not differ by sex ($p=0.2$) or diet group ($p=0.33$).

Table 6: Blood clotting time (s)

	Control	**PK**	**MK4**	**MK9**	**Combo**	**Pork**
Males	236.4 ± 87.0	178.7 ± 101.6	165.2 ± 93.1	175.3 ± 104.5	162.5 ± 102.1	175.2 ± 101.2
Females	161.9 ± 105.2	214.5 ± 92.6	196.2 ± 100.5	111.7 ± 69.7	123.9 ± 80.0	137.8 ± 93.8

Mean ± SD.

e. Vitamin K and the Microbiome

The principal coordinates analysis based on Bray-Curtis similarity of bacterial community composition was shown in **Figure 2**. Male and female mice displayed significantly different communities (non-parametric PERMANOVA, $F=25.43$, $p<0.001$). Pork fed mice harbored

significantly different microbial communities (non-parametric PERMANOVA, F=19.2, p=0.003) than other diets, but no difference in composition was observed between other treatments.

Female mice had significantly higher bacterial richness compared to male mice (Welch's t-test, t = 2.2699, df = 92.739, p-value = 0.02553) while no significant differences in bacterial richness were observed between dietary regiments (**Figure 3**). Heatmaps of the log10 abundance of vitamin K biosynthetic genes are shown in **Figure 4**. Within sex, females (left panel, "F") on the MK9 group appeared to have more gene copies of menaquinone biosynthetic genes relative to the other diet groups, while males (right panel, "M") in the Combo and Pork diets appeared to have the most copies of menaquinone biosynthetic genes relative to the other diet groups. However, after adjustment for multiple comparisons, there were no differences in abundance among diet groups in either males or females (all p>0.9).

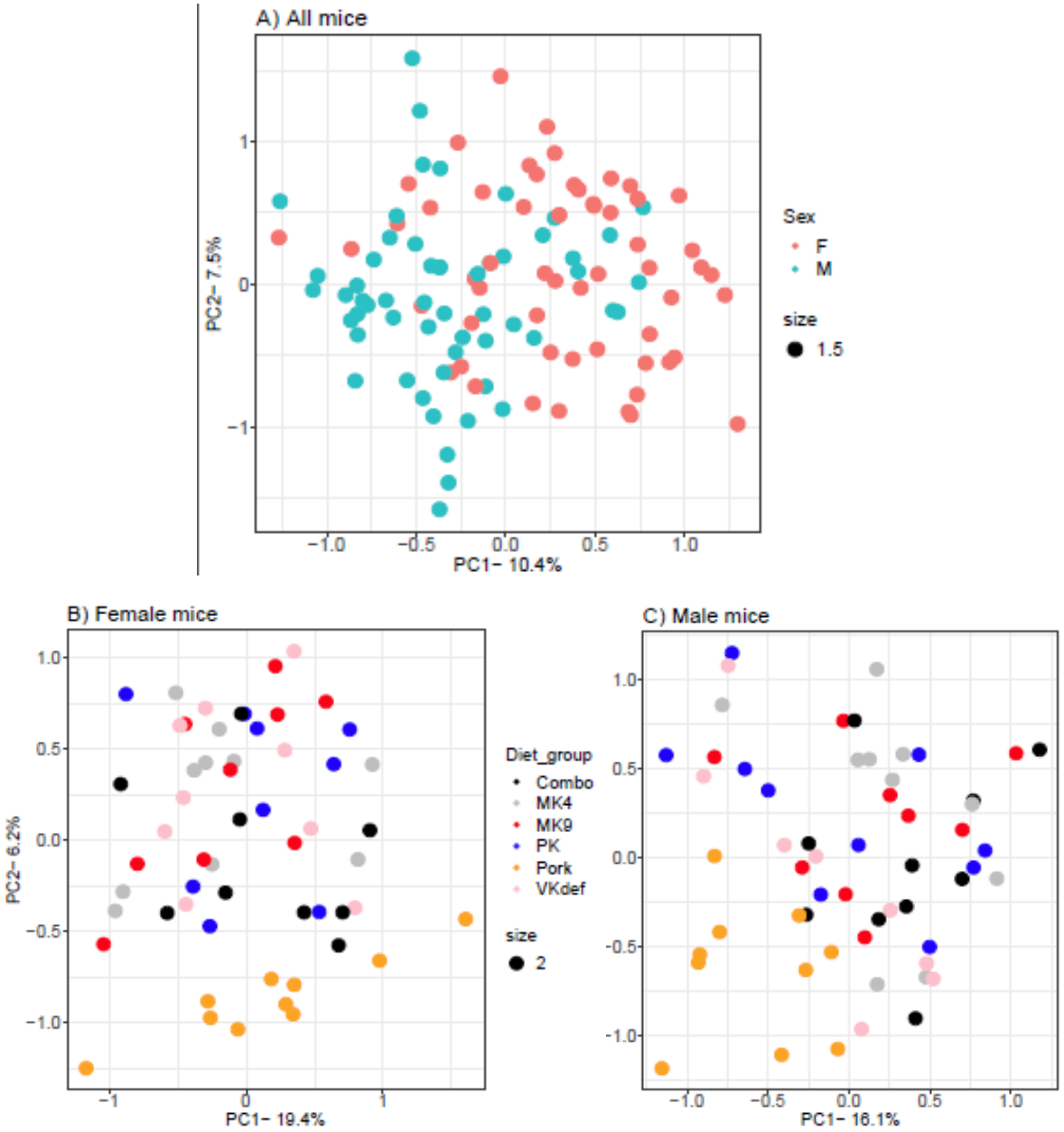

Figure 2: Principal coordinates analysis based on Bray-Curtis similarity of bacterial community composition for all mice (A), female mice (B), and male mice (C). Points in (A) are coloured by mouse gender and points in (B, C) are coloured by the diet the mice received.

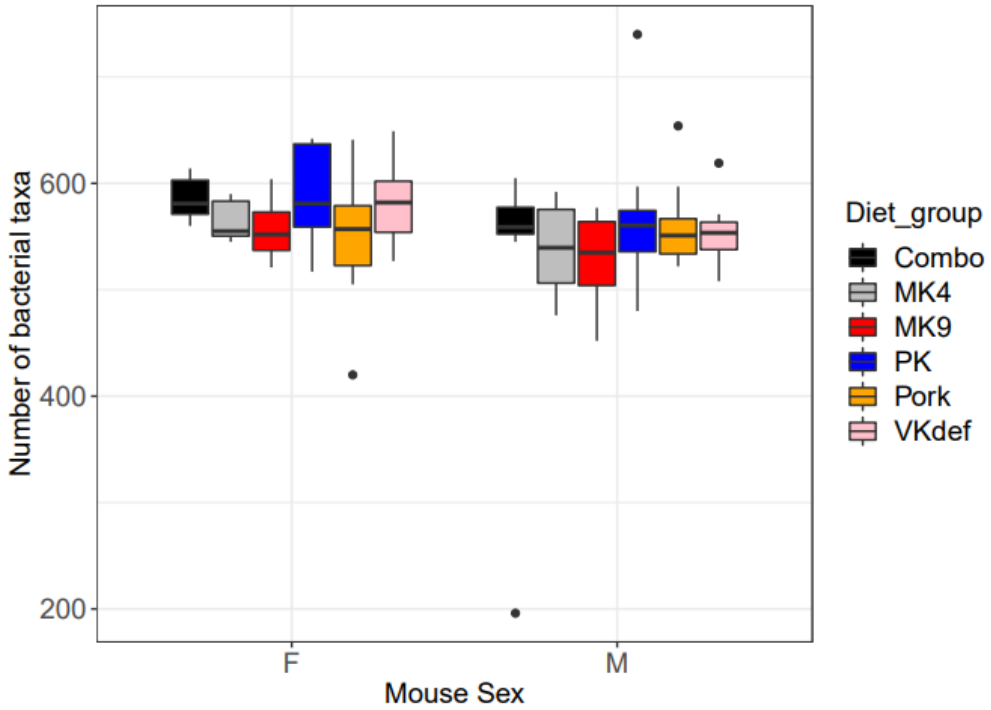

Figure 3: Box-and-whisker plot of observed bacterial richness (total number of taxa/species) of male and female mice. Boxes are coloured by feeding regiment. Boxes are the 25th and 75th quartiles, the black line is the median, and dots are outliers.

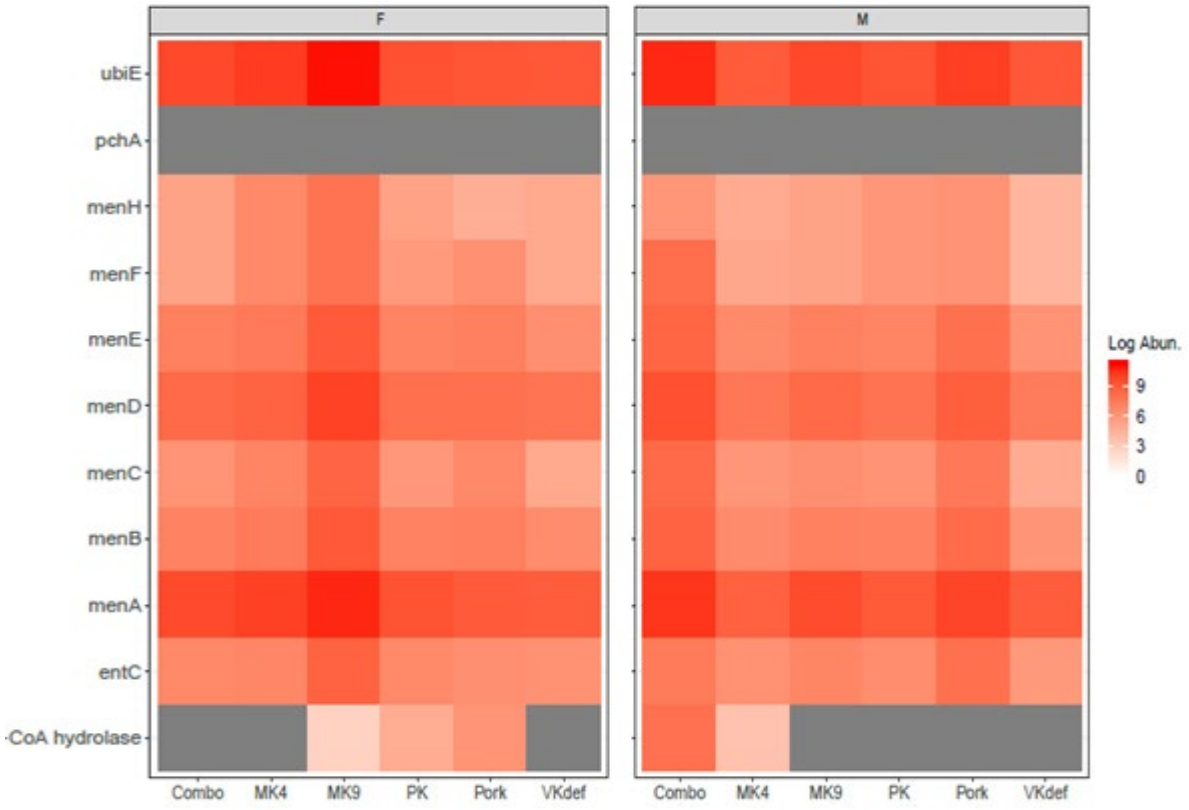

Figure 4: Heatmaps of the log10 abundance of vitamin K biosynthetic genes as determined by PICRUSt. Grey tiles indicate the absence of the gene from a group. *CoA hydrolase: 1,4-dihydrzy-2-naphthoyl- CoA hydrolase.

IX. Discussion:

We have demonstrated that menaquinones in pork (PK, MK4, MK9 and MK10) are absorbed and stored in liver. Significant sex differences of VK contents were detected in most tissues and even fecal samples, which is consistent with our previous findings. The different forms of vitamin K consumed were detectable in the liver following consumption. Pork, which contained MK4, MK9, and MK10, had concomitant increases in the liver. As the pork did not have equimolar amounts of vitamin K compared to the purified forms, we cannot comment on the relative absorption of individual menaquinone forms. However, this proof of principle study does establish absorption of vitamin K forms from intake of pork. There was no MK9 found in analyzed extra-hepatic tissues, except very small amount of MK9 in small intestine of MK9 supplemented groups.

The origin of these MK forms has long been a source of speculation because prior to our analysis of pork products, these longer-chain MK forms were not considered dietary forms. The prevailing theory was that these MK forms were exclusively absorbed from the large intestine where multiple MK forms are produced by gut bacteria, and although it has been stated that approximately 50% of the daily requirement for VK is supplied by the gut microbiota, there is little evidence to support this estimate. Our research team was the first to directly demonstrated tissue accumulation of vitamin K forms are independent of gut microbiota. Among the supplemented groups, fecal MK5-8 and MK10-13 did not differ by group, which suggests gut microbiota-produced MK were not altered by supplementation of dietary vitamin K. There was also no significant differences in bacterial richness were observed between dietary regiments.

Unfortunately, average blood clotting time were not significantly different by groups, due to the sensitivity of the test method. Should future research demonstrate that MK forms have biological function, fresh pork cuts and certain processed pork products will be considered a rich dietary source of VK, and can be used in various diet plans to increase overall VK intake.