Influence of bacteria on menaquinone concentrations in fermented dairy products

by

Ivan Grant Hartling

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Abstract

Menaquinones (MK's) are a form of vitamin K, supplementation with which has been shown to provide health benefits by reducing arterial calcification and improving bone density. Fortifying MK's in foods may provide the health benefits associated with MK's to the general population. This study aimed to increase the amount of MK's in yogurt, which typically contains very little MK's, by co-fermenting milk with both typical yogurt bacteria and bacteria known to produce MK's in cheese and natto. A LC-MS/MS method was first developed to measure MK's as well as all other fat-soluble vitamins. The LC-MS/MS method was able to quantify phylloquinone, MK-4, MK-7, MK-9, cholecalciferol, α-tocopherol, and was able to detect MK-8, but was unable to quantify ergocalciferol or retinol due to ionization suppression from the sample matrix. The LC-MS/MS method was used to measure MK's in non-fat milk samples fermented with either one bacteria ssp. or a combination of *S. thermophilus*, *L. bulgaricus*, and one of *L. lactis*, *L. cremoris*, *P. shermanii*, or *B. subtilis*. Fermentations were carried out at 30 – 45 °C for 36 h to determine the optimal incubation temperature at which to carry out replicate fermentations. The pH was measured throughout fermentation as an indicator of overall growth and the relative amount of each bacteria ssp. after fermentation was measured using qPCR. Only *L. cremoris* produced MK-8 and MK-9, while all samples, including negative controls, contained high concentrations of MK-7. The qPCR results showed co-fermentations contained over 99 % *S. thermophilus*, except in co-fermentations with *L. lactis* which contained 59 % *L. lactis*, 41 % *S. thermophilus*, and < 0.1 % *L. bulgaricus*. Delaying inoculation of *S. thermophilus* and *L. bulgaricus* resulted in almost exclusive growth of the first inoculant. Overall, *L. cremoris* showed the greatest potential for enhancing the MK content of yogurt as it produced up to 234.4 ng/g MK-9, 174.9 ng/g MK-7, and produced MK-8, but further experiments are required to improve the growth of yogurt and MK producing bacteria.

Lay Summary

Vitamin K_2 is a group of similar molecules called menaquinones (MK's), supplementation with which improves both bone and cardiovascular health. Here we show that the addition of MK producing bacteria can be used to increase the MK concentration in yogurt. We first developed an analytical chemistry method using liquid chromatography-tandem mass spectrometry to quantify MK's, vitamin K_1, vitamin D_3, and vitamin E. Out of four MK producing bacteria subspecies investigated, *L. cremoris* produced large quantities of MK-7, MK-8, and MK-9 when fermented on its own, while the others only produced MK-7 in lesser amounts. However, when added simultaneously with yogurt bacteria, MK producing bacteria did not grow well. By delaying the addition of typical yogurt bacteria, growth of MK producing bacteria increased significantly, but growth of typical yogurt bacteria diminished to near zero. This study demonstrated that MK producing bacteria can be used to increase MK's in yogurt.

Preface

I was lead investigator and responsible for project design, all lab work and experimentation, data processing and analysis, and thesis writing. R.L.A. Cerri and D.M. Veira supervised and provided help for project design, as well as editing and providing feedback on thesis drafts. S. Wang helped design and interpret results of Chapter 3, and provided editing and feedback on thesis drafts. K.K. Donkor provided help regarding project design and analysis of Chapter 2, and provided editing and feedback on Chapter 2.

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To my parents and sisters, thank you for your support and patience. And thanks for listening to all my complaints and frustrations. Katrina, when I arrived at the UBC Dairy Centre I never expected to meet someone who would become so important to me. Thank you for all the adventures, whether it was a short trip to the lake to get out of the lab or a holiday in Europe, you made the time spent on this project immensely more enjoyable.

Chapter 1: Introduction

1.1 Introduction to vitamin K

Vitamin K refers to a group of fat-soluble vitamins consisting of vitamin K_1 (phylloquinone; PK), vitamin K_2 (menaquinone; MK), and vitamin K_3 (menadione). They are essential for the body to function properly and like all vitamins, apart from vitamin D, cannot be synthesized within the body. Phylloquinone (PK) has long been known for its essential role in blood clotting, and the literature on PK is well established. Menaquinones (MK) on the other hand have recently generated significant interest since it was found that MK's may possess their own unique biological functions, and supplementation with MK's has been shown to have multiple health benefits, including increased bone density (Knapen et al., 2013; Koitaya et al., 2013), and reduced arterial calcification (Knapen et al., 2015a). These health benefits, which are believed to be unique to MK and not phylloquinone, have generated significant research into metabolism and additional functions of MK's, quantitation of MK's in foods, and a re-examination of dietary requirements.

To gain a better understanding of MK's this introduction will begin by looking at the different forms of vitamin K, their associated terminology, and metabolism. It will then review health benefits attributed to menaquinones, followed by an overview of dietary requirements and food sources. It will conclude by reviewing methods of quantifying fat-soluble vitamins including MK's in dairy products.

1.1.1 Vitamin K chemical structure and nomenclature

All forms of vitamin K contain a methylated naphthoquinone ring system, which in the case of phylloquinone and all MK's is bound to an aliphatic side chain consisting of repeating isoprenoid units with varying degrees of saturation. PK contains four isoprenoid residues of which only one is unsaturated (Figure 1.1). All isoprenoid residues in MK's are typically

unsaturated and can vary in length from four to thirteen residues. MK's are generally termed MK-n where the "n" refers to the number of isoprenoid residues (Figure 1.1). While the aliphatic side chain varies between the many forms of vitamin K, it is the naphthoquinone ring system, which on its own is called menadione (Figure 1.1), that is responsible for the primary biological role of K vitamins (Shearer and Newman, 2008). Menadione itself does not exist as a naturally occurring dietary form of vitamin K, rather it is an intermediate in the biological conversion of phylloquinone to MK-4 in mammals (Hirota et al., 2013).

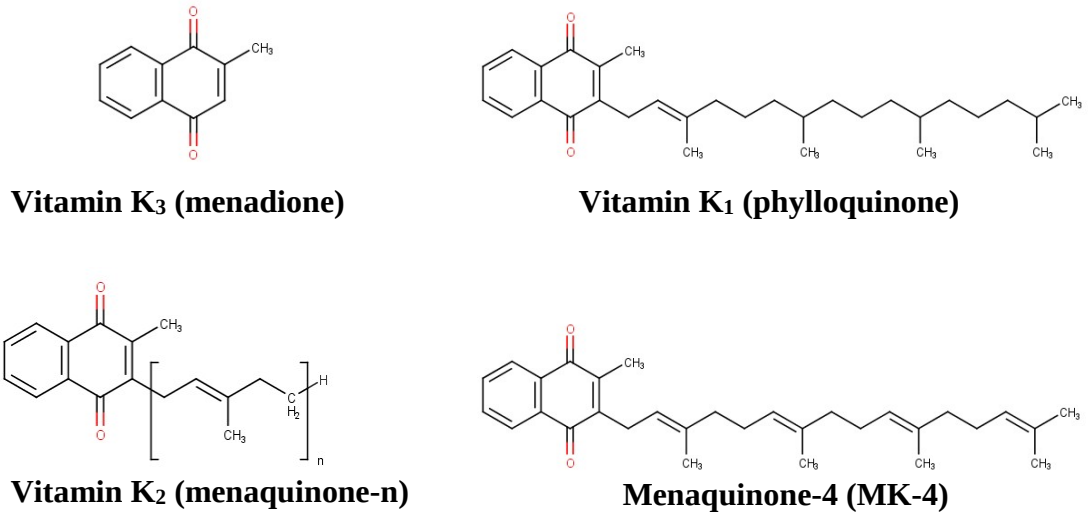

Figure 1.1. Chemical structures of K vitamins. Menadione contains only a naphthoquinone ring system while phylloquinone and all menaquinones contain an aliphatic side chain. The side chain for menaquinone consists of repeating isoprene units.

1.1.2 Vitamin K metabolism

Studies investigating vitamin K metabolism have, until recently, focused primarily on the metabolism of phylloquinone and its role in blood clotting. It was assumed that the metabolism and function of MK's was the same as phylloquinone, but over the last few decades, evidence has been accumulating which suggest MK's possess metabolic differences to PK, resulting in more effective carboxylation of extra-hepatic proteins and greater bioavailability (Beulens et al., 2013).

Being fat-soluble, digestion of K vitamins is comparable to digestion of lipids and the other fat-soluble vitamins in humans and other monogastric species. K vitamins enter the stomach bound to proteins in the food. The acidity of the stomach, combined with proteolytic enzymes, releases vitamins from their associated proteins. Once freed, the hydrophobic vitamins mix with fat-globules in the foods oily phase. The fat-globules are emulsified by bile salts which results in the formation of micelles. Micelles contain a lipophilic core which is a much more hospitable environment for K vitamins than the aqueous phase surrounding them, and K vitamins remain in the micellar core until absorption in the intestines (Ji et al., 2009; Shearer et al., 2012).

As early as the 1970's, work by Hollander et al., suggested that K vitamins were absorbed via carrier-dependent proteins. However, it was not until 2015 that Niemann-Pick C1-like 1 (NPC1L1) was identified as one of the transporters of phylloquinone (Takada et al., 2015). Other transporters, including scavenger receptor class B type I (SR-BI) and cluster determinant 36 (CD36) have also been identified as transporters of K vitamins in intestinal absorption (Goncalves et al., 2014). Identifying carrier proteins is an important step in understanding absorption of K vitamins.

Before identifying carrier proteins, studies were already investigating absorption kinetics and bioavailability using stable isotope labeled PK. Absorption studies showed PK is poorly absorbed (5-15 % absorption) and meal composition can greatly effect absorption with higher fat content aiding in absorption (Schurgers and Vermeer, 2000; Jones et al., 2008, 2009; Novotny et al., 2010). Also, vitamins A, D, and E have been shown to further reduce PK absorption suggesting common uptake routes (Goncalves et al., 2015). Compared to PK, MK's are much more readily absorbed, and MK-7 has a significantly longer serum half-life time than PK (Schurgers and Vermeer, 2000; Schurgers et al., 2007). The long half-life of MK-7 generated interest in the bioavailability of other MK's which showed MK-4 had a shorter serum half-life

than MK-7 (Sato et al., 2012). Because of their greater bioavailability, MK's likely contribute significantly to overall vitamin K intake even though dietary intake of MK's is less than that of PK (Beulens et al., 2013). Because of its greater bioavailability, studies investigating health benefits of MK supplementation primarily use MK-7.

In the body, PK and all MK's perform the same biochemical function. They both act as cofactors for γ-glutamyl carboxylase, an enzyme responsible for the carboxylation of glutamic acid residues to form γ-carboxyglutamic acid (GLA) residues capable of binding calcium (Buitenhuis et al., 1990). Due to the indispensable role of vitamin K in this process, proteins containing GLA residues are referred to as vitamin K dependant proteins (VKDP). The most well-known VKDP's are involved in blood clotting and include blood clotting factors VII, IX, X, as well as prothrombin, protein S, and protein C. Other VKDPs include matrix GLA protein (MGP), osteocalcin, protein Z, Gas6, PRGP1, and PRGP2 (Furie et al., 2016). While clotting factors, the function of GLA domains in these proteins, and the effects of vitamin K on clotting factors are well understood, the function of other VKDP's are still being researched (Furie et al., 2016). The emerging functions of other VKDP's such as MGP and osteocalcin have led to several health benefits being attributed to vitamin K supplementation.

1.2 Health benefits of vitamin K

Vitamin K was discovered almost 100 years ago based on its vital role in blood coagulation. In 1934, Henrik Dam found that chicks fed diets deficient in cereals and seeds exhibited subcutaneous and intramuscular haemorrhages (Dam, 1934). After further study, Dam determined the antihemorrhagic factor was fat-soluble and termed the factor vitamin K for "Koagulation" in the German and Scandinavian languages (Dam, 1935). Since its initial discovery in blood clotting, the role of vitamin K in coagulation has been studied extensively; however, more recently, new biological functions have been attributed to vitamin K as new

VKDPs are discovered, and it has been proposed that PK and MK's are not equally effective at inducing carboxylation of these proteins (Schurgers et al., 2007).

1.2.1 Cardiovascular health

MGP is a VKDP vital for vascular health. Its importance was demonstrated by Luo et al. (1997) who found mice lacking MGP died within two months of birth due to arterial calcification and subsequent rupture. Their findings indicated MGP is responsible for regulating extracellular matrix calcification and since then the role of MGP and vitamin K in vascular calcification has been studied extensively.

MGP must be both phosphorylated and carboxylated for its active form to function properly. The carboxylation step is dependent on vitamin K, which has been demonstrated to increase the active form of MGP in a dose dependant manner (Dalmeijer et al., 2012). The relationship is strong enough that the ratio of undercarboxylated MGP to carboxylated MGP is often used as a measure of vitamin K status in individuals (Delanaye et al., 2014). The active form of MGP in turn has been shown to be correlated with reduced vascular calcification, and reduced areas of calcification (Dalmeijer et al., 2013; Delanaye et al., 2014).

Since MGP regulates vascular calcification and is dependent on vitamin K, it is logical that vitamin K would also influence vascular calcification by increasing the active form of MGP. However, the first study to look at the link between dietary vitamin K intake and arterial calcification failed to find any relationship between the two (Villines et al., 2005). The negative results of the study were possibly due to examining PK instead of MK as several studies since have found associations between MK intake and arterial calcification (Maas et al., 2007; Beulens et al., 2009; Gast et al., 2009; Knapen et al., 2015a; Caluwé et al., 2016). Most notably, Knapen et al. (2015) found that supplementation with 180 µg/day of MK-7 for three years reduced arterial stiffness. These results indicated MK supplementation has potential for reducing vascular

calcification and several randomized controlled trials are currently being carried out to confirm the effectiveness of MK's for reducing vascular calcification (Caluwé et al., 2016).

1.2.2 Skeletal health

In addition to the cardiovascular health benefits, benefits regarding bone health have also been attributed to vitamin K. These health benefits include increased bone density and reduced fracture risk, but studies investigating the effects of vitamin K on bone health have had mixed results. Population and cohort based studies have found some associations between vitamin K intake and bone density or fracture risk in the United States (Booth et al., 2003), Korea (Kim et al., 2015), Japan (Yaegashi et al., 2008), and Norway (Apalset et al., 2011). However, one study found no association between vitamin K intake and fracture risk (Chan et al., 2012).

The increased bone density and reduced risk of fracture associated with vitamin K intake is attributed to the VKDP osteocalcin. Osteocalcin is a calcium transport protein which has been shown to be an important regulator of bone matrix deposition (Ducy et al., 1996). Since vitamin K is required for the carboxylation of undercarboxylated osteocalcin (ucOC) to the active carboxylated form, increased vitamin K intake via diet or supplementation has been hypothesized to reduce the amount of ucOC which should in turn improve bone health.

Multiple studies have investigated the effect of MK supplementation on ucOC levels and found that supplementation with MK-4 or MK-7 improves γ-carboxylation of osteocalcin and reduces ucOC levels in a dose dependant manner (Nakamura et al., 2014; Inaba et al., 2015). Others have demonstrated that low serum ucOC is linked to increased bone density and lower risk of hip fracture (Szulc et al., 1996; Booth et al., 2004). While the link between MK's and osteocalcin, and between osteocalcin and bone health seem well established, the effects of MK supplementation of bone health have been inconsistent.

Most studies investigating the effects of MK supplementation on bone health use MK-7 due to its higher bioavailability (Sato et al., 2012) and have trial lengths of one year or greater. Of studies lasting one year, Emaus et al., (2010) found MK-7 supplementation (360 µg/day) did not reduce bone loss in early menopausal women, but Koitaya et al., (2013) found MK-4 (1.5 mg/day) supplementation prevents forearm bone loss in postmenopausal women. While one year studies have had conflicting results, the only three-year study to date found low dose MK-7 supplementation (180 µg/day) reduced bone loss in postmenopausal women (Knapen et al., 2013). The differences in results between studies are most likely due to the different study lengths as well as the different doses and MK's used.

1.2.3 Other extrahepatic health benefits

Apart from bone and cardiovascular health benefits, several other health benefits and biological roles have been attributed to MK's. It is becoming apparent that MK's are important for neuron and brain health. MK-4 has been reported to accumulate in brain tissues and lifelong low PK intake impairs cognitive function in mice (Carrié et al., 2011). Vitamin K's effects on the brain appears to be mediated by the VKDP's Gas6 and protein S which also have roles in regulating cell proliferation in the subventricular zone of the brain (Gely-Pernot et al., 2012). Along with its function in the brain, MK's also play a role in estrogen metabolism (Otsuka et al., 2005). Due to their role as a cofactor, it is likely that more functions will arise in the future as we identify and gain a better understanding of more VKDP's.

1.3 Menaquinones in food

1.3.1 Biosynthesis

Plants and bacteria are the only organisms capable of synthesizing vitamin K. Plants produce exclusively PK, which is used in the photosynthetic membrane of photosystem I (Bouvier et al., 2005), and bacteria produce MK-5 to MK-13 depending on the strain and growing conditions.

While humans cannot synthesize any form of vitamin K, they can, along with all mammals, convert PK to MK-4 (Okano et al., 2008; Nakagawa et al., 2010). The conversion of PK to MK-4 was originally thought to take place in the gut until it was shown that germ-free mice could still convert PK to MK-4 (Ronden et al., 1998). Later it was revealed that humans possess an enzyme, UBIAD1, which is capable of converting PK to MK-4 within tissues (Nakagawa et al., 2010).

1.3.2 Bacteria which produce MK's in food

Bacteria use MK's to shuttle electrons between membrane proteins in the electron transport chain (Hiratsuka et al., 2008). The amount and type of MK's produced by bacteria varies greatly. Many species of bacteria do not produce any MK's and others only produce MK's under certain conditions or produce different MK's depending on the growth conditions (Rezaïki et al., 2008; Berenjian et al., 2011). Bacteria produce MK's using one of two metabolic pathways. The traditional pathway converts chorismate to MK using a series of enzymes (MenA – MenG). This pathway exists in many gram-positive microbes, but an alternative pathway using futalosine was found in some gram-negative bacteria and Archaea (Hiratsuka et al., 2008).

Regarding food production, gram-positive bacteria are commonly used to ferment food. A list of bacteria commonly used for food production and their ability to produce MK's is provided in Table 1.1. Some bacteria used in food production such as *Bacillus subtilis* subsp. *natto* produce large amounts of MK's, while others, such as all *Lactobacilli* do not produce any MK's. Notably, many genus' used in cheese production, such as *Lactococcus, Leuconostoc,* and *Propionibacterium* produce MK's, while those used in yogurt, *Streptococcus* and *Lactobacillus,* do not. Of the probiotics commonly added to yogurt, *Enterococcus* produces MK's while, *Lactobacillus* and *Bifidobacterium* do not.

Table 1.1. Ability of common food bacteria to produce MK's.

Genus	Species	Subspecies	MK biosynthesis	Reference
Bacillus	*subtilis*	*natto*	+	(Sato et al., 2001)
Propionibacterium	*freudenreichii*		+	(Furuichi et al., 2006)
Lactococcus	*lactis*	*lactis*	+	(Morishita et al., 1999; Parker et al., 2003)
Lactococcus	*lactis*	*cremoris*	+	(Morishita et al., 1999; Parker et al., 2003)
Lactococcus	*plantarum*		+	(Morishita et al., 1999)
Lactococcus	*raffinolactis*		+	(Morishita et al., 1999)
Leuconostoc	*lactis*		+	(Morishita et al., 1999)
Leuconostoc	*mesenteroides*	*cremoris*	+	(Morishita et al., 1999; Parker et al., 2003)
Leuconostoc	*mesenteroides*	*dextranicum*	+	(Morishita et al., 1999; Parker et al., 2003)
Enterococcus	*durans*		+	(Parker et al., 2003)
Enterococcus	*faecalis*		+	(Morishita et al., 1999; Parker et al., 2003)
Streptococcus	*thermophilus*	*salivarius*	-	(Morishita et al., 1999; Parker et al., 2003)
Lactobacillus	*acidophilus*		-	(Morishita et al., 1999; Parker et al., 2003)
Lactobacillus	*casei*		-	(Morishita et al., 1999; Parker et al., 2003)
Bifidobacterium	*bifidum*		-	(Morishita et al., 1999; Parker et al., 2003)
Bifidobacterium	*breve*		-	(Morishita et al., 1999; Parker et al., 2003)

1.3.3 Dietary requirements and sources

Current government recommended daily intakes (RDI's) for MKs do not exist. Instead, MK's are grouped with phylloquinone to form a general RDI for vitamin K, which in Canada is 80 µg per day for those above 2 years of age (Canadian Food Inspection Agency, 2016). The problem with a combined RDI is that current RDI's are based solely off phylloquinone requirements for blood clotting. New biological functions specifically associated with MK's have not yet been

addressed by government RDI's, but many scientists agree that the RDI for vitamin K should be increased to account for these functions or a separate RDI for phylloquinone and MK should be created (Beulens et al., 2013; Walther et al., 2013). There are several factors which have made creating an updated RDI for MK very challenging. Firstly, the unique biological functions attributed to MK's (e.g., reduced arterial calcification) do not have any associated acute symptoms resulting from MK deficiency. The only acute symptom of vitamin K deficiency is impaired blood clotting, which is what the current RDI accounts for. The second challenge is that there is a significant lack of knowledge regarding MK's in food nutrient databases.

The primary dietary source of PK is green leafy vegetables as PK is found inside chloroplasts. Unlike PK, MK's are not found in vegetables and can only be obtained from animal products and some fermented foods. Animal products, apart from the liver which stores some ingested PK and MK's, only contain MK-4 which as previously mentioned can be converted from PK by mammals. All other forms of MK produced by bacteria can be found in various fermented foods. Not all fermented foods contain MK's and the amount can vary greatly depending on which species and strain of bacteria is used for fermentation (Manoury et al., 2013). A list of common sources of vitamin K is provided in Table 1.2. As the focus of this study is MK's, sources of PK, such as spinach and other plants, are not listed. The amount of MK's in many foods is still largely unknown. Most nutrition databases include PK and some have MK-4, but long chain MK's have only been measured in several studies looking at select dairy and meat products (Koivu-Tikkanen et al., 2000; Elder et al., 2006; Kamao et al., 2007a; Manoury et al., 2013).

Table 1.2. Concentrations of K vitamins in common dietary sources.

	Vitamin K concentration (ng/g)*						
Food item	PK	MK-4	MK-6	MK-7	MK-8	MK-9	MK-10
Chicken	0	221-600	-	0	0	0	-
Pork	0	9-60	-	0	0	0	-
Beef	6-24	17-150	-	1	4	0	-
Liver	0-58	4-141	-	16-26	14-25	6-10	-
Eggs	3-6	56-70	-	-	-	-	-
Cheese, cheddar	30	102	9-30	0-23	11-62	0-253	0-65
Cheese, hard	19-30	33-78	0-20	0-22	0-105	0-324	0-45
Cheese, soft	13	36	4-26	0-17	21-140	66-940	0-57
Yogurt	2-3	4-10	-	0	0	0	-
Milk (whole)	3-10	10-20	-	-	-	-	-
Natto	23-50	0	-	7960-9390	-	-	-

*values are a summary of ranges reproduced from (Koivu-Tikkanen et al., 2000; Elder et al., 2006; Kamao et al., 2007a; Manoury et al., 2013). Where no range is reported, all papers reported 0 ng/g or only one paper measured the vitamin in that food.

Natto, a fermented soybean dish, is the best source of MK-7, but is not commonly consumed outside of Japan. Some of the best sources of MK's in North American diets are chicken and cheeses. Chicken are commonly fed menadione which is converted to MK-4 and is the reason chicken contain far more MK-4 than pork or beef (Booth, 2012). Cheese generally has higher overall MK concentrations than meats although they vary greatly depending on the type of cheese and which bacteria are used to produce it (Manoury et al., 2013). The high MK concentrations found in cheese are a stark contrast to those in yogurt. Even though both cheese and yogurt are fermented dairy products, yogurt contains none of the long chain MK's found in cheeses as the bacteria used to produce yogurt (*S. thermophilus* and *L. bulgaricus*) do not produce MK's.

As previously mentioned, increased MK intake improves both cardiovascular and skeletal health. Increasing MK intake in the general population could provide significant health benefits. One way of increasing MK intake is by increasing the MK content of commonly consumed foods. Yogurt is a perhaps the best target for increasing the MK content as it contains very low MK

concentrations, but cheese, which is produced similarly, is one of the best sources of MK's. Additionally, yogurt fortified with MK's has been shown to be more effective at increasing plasma MK concentrations than MK capsules (Knapen et al., 2015b, 2016). Yogurt may be fortified with MK's by adding MK's to the yogurt or by producing yogurt with MK producing bacteria. Bacteria have already been used to fortify vitamin B_{12} in soy yogurt (Gu et al., 2015) and *B. subtilis* has been used to create a novel MK-7 rich fermented dairy product (Southee et al., 2016). Based on yogurts suitability as a matrix for MK delivery, further investigation into fortifying yogurt using MK producing bacteria is needed.

1.4 Quantification of fat-soluble vitamins in dairy products

As the body of evidence showing health benefits associated with MK's grows, the need for a complete understanding of the MK content of foods also grows. One factor contributing to our lack of knowledge regarding the MK content of foods is the absence of a standard method for quantifying MK's in foods. Internationally recognized standard methods for the quantification of vitamins A, D, E, and PK already exist, but not for MK's (Blake, 2007). Ideally, one method could be used to quantify all forms of fat-soluble vitamins, but while some methods have come close, they did not quantify the long chain MK's (Blanco et al., 2000; Gomis et al., 2000; Gentili et al., 2013).

Quantitation of fat-soluble vitamins is typically carried out using high performance liquid chromatography (HPLC) to separate analytes followed by fluorometric, UV-Vis, or mass spectrometry (MS) detection (Blake, 2007). Before analysis by HPLC, vitamins must be extracted from the food sample using one of several extraction techniques. The two main challenges in developing a method to quantify all fat-soluble vitamins are the development of an extraction method suitable for all vitamins and the development of a chromatography method which provides good separation without excessively long elution times.

1.4.1 Extraction of K Vitamins from Dairy

Fat-soluble vitamins in food are often bound to lipoproteins or encapsulated in fat globules. Releasing the vitamins from these complexes is an essential step in any extraction method. For vitamins A, D, and E saponification with sodium hydroxide is the standard and well-established method of releasing the vitamins. However, both PK and all MK's are unstable in the alkaline conditions created during saponification which results in significant degradation of these vitamins and low recovery (Blake, 2007). Other methods developed for releasing PK and MK's from lipoproteins and fat globules may be more suitable for simultaneous extraction of all fat-soluble vitamins.

One of the first methods of releasing K vitamins from fat globules was to use enzymatic hydrolysis along with sodium hydroxide (Lambert et al., 1992). This caused degradation similar to the use of saponification, but the use of lipase hydrolysis without sodium hydroxide break down fat globules and release K vitamins in a non-alkaline environment. Lipase hydrolysis is used by many groups when extracting K vitamins from dairy products, particularly for dairy products with high fat content (<10%) such as cheese and butter (Koivu-Tikkanen et al., 2000).

One alternative to lipase hydrolysis is to use heat to melt fat-globules or a combination of heat with acid to perform an acid hydrolysis. Koivu-Tikkanen et al., (2000) compared an extraction method using isopropanol/hexane and heat with one using acid hydrolysis. The acid hydrolysis method consisted of adding water and HCl to the sample before heating in a boiling water bath and then extracting with ether. They found slightly higher recovery for phylloquinone and MK-4 using acid hydrolysis, but higher MK-8 and MK-9 recovery using heat and isopropanol/hexane. This demonstrated that different methods work better for different forms of vitamin K making it difficult to develop one method which is best for every form. Other groups have also used or modified this acid hydrolysis method such as Manoury et al., (2013) who increased the heating

time for the acid hydrolysis from 10 to 30 minutes, and also used isopropanol/hexane for the extraction instead of ether. They also compared acid hydrolysis to a lipase treatment and found similar recovery of MK-9 for spiked milk samples, but much higher recovery using acid hydrolysis for cheese samples. This demonstrated that acid hydrolysis could be used as an alternative to lipase treatment.

Whether using acid hydrolysis, lipase treatment, or saponification, a liquid-liquid extraction is required following the chosen treatment. During liquid-liquid extraction, a nonpolar solvent (e.g., hexane, isopropanol, ether) is added to extract all fat-soluble compounds from the mixture. The liquid-liquid extraction is often repeated multiple times after which the extracts are combined and concentrated, typically by evaporation under nitrogen (Blake, 2007).

Another technique occasionally used to aid in extraction is sonication. Sonication can be used to disrupt the fat-globule membrane and increase extraction efficiency. Sonication can be used on its own to disrupt fat-globule membranes as part of a hexane extraction (Fournier et al., 1987; Peterson et al., 2002; Elder et al., 2006; Ferreira et al., 2006) or it can be used in conjunction with another method such as lipase hydrolysis to increase the effectiveness of the treatment (Lambert et al., 1992).

1.4.2 Sample cleanup

Food samples can be very complex, consisting of many different types of molecules with different chemical properties and many orders of magnitude different concentrations. This makes food challenging to analyze as there can be many interfering compounds extracted along with the analytes of interest. Fat-soluble vitamins are often found in extremely small quantities in food, which adds to the difficulty of accurately quantifying them. To accurately quantify fat-soluble vitamins in food, it is important to remove as many possible interfering molecules as possible. This is particularly important when analyzing dairy products due to the large amount of lipids

and other fat-soluble compounds which are often extracted alongside fat-soluble vitamins when using liquid-liquid extraction.

The most common method of removing interfering compounds coextracted with analytes is solid phase extraction (SPE). SPE is essentially a form of preparatory chromatography. The sample is applied to a cartridge containing a stationary phase which retains the analytes of interest while the contaminants pass through. After washing to remove more contaminants, the analytes are eluted using a solvent which disrupts the interaction between stationary phase and analyte. SPE using both silica and C18 cartridges have been used to purify fat-soluble vitamin extracts, but C18 cartridges have been found to remove some MK's resulting in decreased recovery (Koivu-Tikkanen et al., 2000).

1.4.3 HPLC

To accurately quantify the many types and forms of fat-soluble vitamins in a sample, most methods require the individual analytes to be separated from each other in addition to being separated from other chemically similar compounds present in the sample. Gas chromatography and liquid chromatography are the two most common separation techniques, and since fat-soluble vitamins are not volatile, liquid chromatography is the standard method used for quantitation of fat-soluble vitamins.

High performance liquid chromatography (HPLC) is an analytical chemistry method which uses high pressure to force liquids through a column packed with silica beads to which different compounds can be bound. Most commonly, the compounds in the column are carbon chains (e.g., C8, C18, C30) which separate analytes in the sample based on their polarity, but other column packing is also available which can separate analytes based on other chemical properties (e.g., electrical charge, size, and structure).

Depending on the polarity of the analyte(s) of interest, either normal phase HPLC or reversed phase HPLC can be used. In normal phase HPLC, the mobile phase is non-polar and the column contains a polar stationary phase. This is useful for separating polar compounds, but since all fat-soluble vitamins are non-polar, reverse phase HPLC is used. In reversed phase HPLC, the stationary phase is non-polar and the mobile phase is polar. The non-polar vitamins will interact with the non-polar stationary phase and be retained longer on the column while more polar molecules pass through quicker in the polar mobile phase. Reversed phase HPLC using a C18 column is the most commonly used technique for separating analytes when quantifying fat-soluble vitamins in samples. Since K vitamins are very non-polar, they interact very strongly with reverse phase columns and have long elution times. Because of this, some studies have used non-aqueous reversed phase HPLC to separate fat-soluble vitamins (Gentili et al., 2013). By using a non-polar mobile phase with reversed phase HPLC, MK's elute much quicker.

1.4.4 Detection methods

HPLC separates analytes, but alone does not measure concentrations. Following separation, a detector is required to measure the concentration of analyte(s). The most common detectors used for analysis of fat-soluble vitamins use UV-Vis light, fluorescence detection after post-column reduction, or mass spectrometry detection (Karaźniewicz-Łada and Głõwka, 2016). All detection methods have their own benefits and drawbacks. UV-Vis is very simple and straightforward, but has higher detection limits than fluorescence detection. Fluorescence detection in turn has higher detection limits than MS detection (Karaźniewicz-Łada and Głõwka, 2016). Mass spectrometry is generally seen as the most sensitive method of detection and performs better when quantifying large numbers of analytes. One study comparing tandem-MS (MS/MS) detection to UV-Vis diode array detection found that only MS/MS allowed for the detection of PK while both performed similarly for detection of vitamins A and E (Kopec et al., 2013). MS/MS detection

has become common for analysis of PK and MK's in blood (Karaźniewicz-Łada and Głõwka, 2016) and has also been used successfully to simultaneously quantify retinol, cholecalciferol, ergocalciferol, α-tocopherol, γ-tocopherol, δ-tocopherol, PK, and MK-4 in milk (Gentili et al., 2013). One of the only drawbacks of mass spectrometry is its susceptibility to matrix effects which can either enhance or suppress signals. However, multiple techniques, including standard addition, matrix-matched calibration, and echo-peaks have been developed to compensate for matrix effects (Zrostlíková et al., 2002; Alder et al., 2004).

1.5 Objectives and hypothesis

This thesis reports the development and validation of a LC-MS/MS method for measuring fat-soluble vitamins in dairy products and the use of this method for the measurement of PK, MK-4, MK-7, and MK-9 in yogurt fermented with MK producing bacteria. We hypothesized that co-fermenting milk with traditional yogurt bacteria (*S. thermophilus* and *L. bulgaricus*) and MK producing bacteria (*L. lactis, L. cremoris, P. shermanii,* and *B. subtilis*) would produce a yogurt like product with enhanced MK content.

Chapter 2: Development of a liquid chromatography–tandem mass spectrometry method for the quantification of fat-soluble vitamins in dairy products

2.1 Introduction

Fat-soluble vitamins include all forms of vitamins A, D, E, and K. They are vital nutrients which except for vitamin D, humans must obtain from their diets. Fat-soluble vitamins are commonly quantified in food for both nutrition information (food packaging, nutrient databases) as well as for research purposes. Dairy products are a good source of fat-soluble vitamins, but to date there is no standard method developed for the simultaneous measurement of all fat-soluble vitamins including the long chain MK's in dairy products (Blake, 2007). Development of such a method would be beneficial as it would reduce analysis time, costs, and chemicals.

HPLC is by far the most effective method of analysis for quantifying fat-soluble vitamins in food samples. Current methods for quantifying fat-soluble vitamins in food rely on HPLC coupled with fluorescence or mass spectrometry detection. Several groups have measured fat-soluble vitamins using these methods (Koivu-Tikkanen et al., 2000; Gentili et al., 2013; Manoury et al., 2013). Notably, Gentili et al. (2013) quantified forms of vitamins A, D, E, as well as PK, and MK-4 using LC-MS/MS. However, they did not quantify longer chain MK's such as MK-7 or MK-9 and their method used saponification during extraction which can destroy MK's, so a different extraction would be better for quantifying long chain MK's.

Typically, methods for quantifying K vitamins (PK, MK's) use a hexane extraction. If the sample has lots of fat, a lipase digestion, acid hydrolysis, or ultrasound bath is used to disrupt fat globules prior to the hexane extraction (Koivu-Tikkanen et al., 2000; Manoury et al., 2013). Following hexane extraction, SPE is commonly used to remove interfering or co-eluting compounds from the extract before the extract is blown down under nitrogen and reconstituted to

concentrate the sample. Most methods which only measure PK and MK's use fluorescence detection following a post-column reduction with metallic zinc (Lambert et al., 1992; Indyk and Woollard, 2000; Koivu-Tikkanen et al., 2000; Elder et al., 2006; Ferreira et al., 2006; Hojo et al., 2007; Manoury et al., 2013; Qureshi et al., 2013). Fluorescence detection is effective for quantifying MK's and PK, but when also quantifying other fat-soluble vitamins LC-MS is more effective as used by Gentili et al. (2013). The objective of this study was to develop a LC-MS/MS method for the simultaneous quantification of retinol, ergocalciferol, cholecalciferol, α-tocopherol, PK, MK-4, MK-7, and MK-9.

2.2 Materials and Methods

2.2.1 Chemicals and materials

The following standards of fat-soluble vitamins were purchased from Sigma-Aldrich (Oakville, ON, Canada): retinol, α-tocopherol, ergocalciferol, cholecalciferol, phylloquinone, and menaquinone-4. The other standards, menaquinone-7 and menaquinone-9, were purchased from Chromadex (Irvine, CA, U.S.A.) and Santa Cruz Biotechnology (Dallas, TX, U.S.A.) respectively. All standards had a purity grade of > 95%.

Hexane and tetrahydrofuran (THF) were HPLC grade and purchased from Sigma-Aldrich, methanol and water were LC/MS grade and purchased from Thermo Fisher Scientific (Ottawa, ON, Canada), isopropanol was reagent grade and purchased from VWR International (Mississauga, ON, Canada). Butylated hydroxytoluene (BHT) was purchased from

Sigma-Aldrich and used as an antioxidant in both standard solutions and in extractions.

2.2.2 Samples

Full fat (3.25 %) yogurt used for method development was purchased from a local grocery store. Non-fat yogurt used for method validation was made from skim milk (Dairyland, Burnaby, BC,

Canada) plus 10 % skim milk powder (Pacific Dairy, Beijing, China) fermented with *Streptococcus salivarius* subsp. *thermophilus* (DSM 20617) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (DSM 20081) for 36 h at 42 °C.

2.2.3 Standards

Stock solutions of each standard in isopropanol/hexane (90:10) with 0.1% BHT were prepared monthly. Working standards and a multistandard solution for method development and standard curves were prepared weekly at varying concentrations depending on their use. All standards were stored in the dark at -18 °C.

2.2.4 Extraction of fat-soluble vitamins

Since most published extraction methods fail to extract all fat-soluble vitamins, four extraction methods were tested for their ability to extract retinol, α-tocopherol, ergocalciferol, cholecalciferol, PK, MK-4, MK-7, and MK-9. The methods tested included an acid hydrolysis method modified from Manoury et al., (2013), a simple hexane extraction based on Schurgers and Vermeer, (2000), a dispersive SPE method, and a lipase treatment. All methods were carried out in reduced light and all reagents contained 0.1% BHT to prevent oxidation of photosensitive vitamins. All extraction methods were tested on full fat yogurt (3.25% fat).

The acid hydrolysis method was modified from Manoury et al., (2013). Ten grams of yogurt was weighed into a 50-mL polytetrafluoroethylene (PTFE) centrifuge tube with 10 mL isopropanol and mixed thoroughly. An internal standard or spike (for testing recovery) was added, after which 5 mL of 1 M HCl was added and the mixture was heated in a boiling water bath for 30 min. After cooling in an ice bath, 8 mL of hexane was added and the mixture was shaken for 2 min before being centrifuged for 10 min at 3000 g. The upper hexane layer was transferred to a glass conical tube and blown down under Nitrogen in a 55 °C water bath. The residue was reconstituted in 1 mL isopropanol/hexane (80:20).

A simple hexane extraction, similar to the acid hydrolysis method, was modified from Qureshi et al. (2009). Ten grams of yogurt was weighed into a 50-mL PTFE centrifuge tube along with 5 mL of water and 10 mL isopropanol. The internal standard was added and the solution was warmed for 5 min in a 60 °C water bath. Vitamins were then extracted with 8 mL hexane as described in the acid hydrolysis method.

Both the acid hydrolysis method and the simple hexane extraction were tested with and without using SPE to cleanup the sample following reconstitution. Two SPE methods were tested using Silica Sep-Pak® SPE cartridges (Waters, Mississauga, ON, Canada). In the first method, the column was conditioned with 6 mL isopropanol, and equilibrated with 6 mL hexane before loading the sample. The sample was then eluted with 6 mL isopropanol/hexane (20:80) followed by 6 mL isopropanol. For the second method, from Ferreira et al., (2006), the column was conditioned with 4 mL hexane/diethyl ether (96.5:3.5) and equilibrated with 4 mL hexane. The sample was loaded and washed with 4 mL hexane before being eluted in 4 mL hexane/diethyl ether (96.5:3.5). In both cases, the eluate was blown down and reconstituted in 1 mL isopropanol/hexane (80:20).

A QuEChERS method using Enhanced Matrix Removal-Lipid (EMR-Lipid; Agilent Technologies, Santa Clara, CA, USA) was tested as an alternative to traditional hexane extractions. The extraction was carried out per manufacture recommendations with some modifications. Briefly; 5 g of yogurt (3.25% fat) was mixed with either 5 mL acetonitrile or 5 mL acetone and the internal standard. Then, 5 mL of water was added to the EMR-Lipid tube followed by 5 mL of the acetonitrile or acetone extract. The mixture was immediately vortexed for 2 min, then centrifuged for 5 min at 5000 rpm. The supernatant was transferred to a separate tube and 1.5 g $MgSO_4$ was added. The vortex and centrifuge steps were repeated and the supernatant was blown down under nitrogen and reconstituted in 1 mL isopropanol/hexane

(80:20). A modified method was also tested in which 1 mL of hexane was added after the first vortex step. The mixture was then vortexed a second time, centrifuged, and the hexane layer was used for injection.

Due to the large amount of fat in the samples, a lipase treatment based on Manoury et al. (2013) was also tested. Ten grams of yogurt was incubated with 10 mL PBS buffer, the internal standard, and 0.5 g lipase at 45 °C for 90 min. This was then followed by extraction using the simple hexane extraction method described previously.

2.2.5 Method Validation

Method validation was carried out on non-fat yogurt. Standard curves were prepared from measurements of 3 replicates of 6 concentrations representing expected concentrations. Recovery was measured using samples spiked before and after extraction (n = 3). Intraday and interday precision were measured using three spiked replicates either measured three times the same day (intraday) or three consecutive days (interday). The limit of detection (LOD) and limit of quantitation (LOQ) were determined by measuring a series of samples with decreasing concentrations of internal standards. The LOD was the concentration at which the signal-to-noise ratio of the peak area was > 3 and > 10 for LOQ.

2.2.6 Instrumentation

The LC-MS system consisted of an Agilent 1200 HPLC coupled to an Agilent 6530 MSD with electrospray ionization (Agilent Technologies, Santa Clara, CA, USA). The separation abilities of a reversed phase C30 column (Accucore 2.6 µm, 150 mm x 2.1 mm; Thermo Fisher Scientific) and a C18 column (Eclipse Plus 1.8 µm, 100 mm x 2.1 mm; Agilent) were compared. The column temperature was optimized by looking at the separation of standard solutions run at 20 - 50 °C. Two mobile phase compositions were tested. The first consisted of methanol/H_2O (90/10, v/v, phase A) and methanol/THF (70/30, v/v, phase B) while the second was a non-

aqueous mobile phase consisting of methanol (phase A) and isopropanol/hexane (50/50, v/v; phase B). Each mobile phase also contained 0.3% formic acid to increase ionization. The flow rate was optimized between 0.15 mL/min and 0.5 mL/min and the mobile phase gradient was as follows (t in min): t_0, B = 0%; t_1, B = 0%; $t_{1.1}$, B = 20 %; t_{25}, B = 20%; $t_{25.1}$, B = 0%; t_{30}, B = 0%. Detection was performed in positive ion mode and the other MSD parameters were as follows: gas temperature, 300 °C; drying gas, 8 L/min; nebulizer pressure, 8 psig; sheath gas temperature, 350 °C; sheath gas, 10 L/min. The nozzle, skimmer, capillary, and fragmentor voltages were optimized according to Table 2.1.

Table 2.1. Optimization of Q-TOF parameters.

Parameter	Range tested (V)	Optimum voltage (V)
Nozzle voltage	800 – 1200	900
Skimmer voltage	60 – 100	80
Vcap	2600 – 3400	2600
Fragmentor voltage	50 – 250	150

Analysis was carried out in targeted MS/MS mode using the mass transitions and retention times listed in Table 2.2 to identify peaks for each of the fat-soluble vitamins used in this study. A solution of vitamin standards injected at 1.5 min (echo-peak) was used to compensate for matrix effects and instrument variation. The peak area ratio of vitamin to echo-peak standard was used for quantification.

Table 2.2. Retention times and mass transitions used to identify fat-soluble vitamins.

Peak	RT (min)	Vitamin	Transition (m/z)
1	4.13	Cholecalciferol	385.3/259.2
2	4.90	α-tocopherol	431.4/165.1
3	5.16	menaquinone-4	445.3/187.1
4	8.23	phylloquinone	451.3/187.1
5	13.64	menaquinone-7	649.5/187.1
6	15.19	menaquinone-8	717.5/187.1
7	24.93	menaquinone-9	785.6/187.1

2.3 Results

2.3.1 Optimization of chromatography conditions

Two HPLC columns were tested for their ability to separate fat-soluble vitamins. Both the C18 and C30 columns achieved baseline separation of MK-9, MK-7, PK, and the C30 column also baseline resolved cholecalciferol. Neither column could fully resolve MK-4 from α-tocopherol and the C18 column also failed to fully resolve cholecalciferol. Theoretical plates calculations for each column showed that the C30 column had higher theoretical plates for both MK-7 and MK-9 (Table 2.3). Due to the importance of MK-7 and MK-9 to analysis in later sections of this study, the C30 column was selected for routine analysis. The C30 column also reduced ionization suppression of MK's from lipids which could not be removed during the extraction procedure. However, the C30 column was not able to be used to measure retinol (eluted in initial solvent front and was suppressed) or ergocalciferol (detection limit too high).

Table 2.3. Comparison of theoretical plates between C18 and C30 columns for the separation of fat-soluble vitamins.

	Theoretical plates					
Column	cholecalciferol	α-tocopherol	PK	MK-4	MK-7	MK-9
C18	2360	6801	5361	5468	9015	12932
C30	1366	1644	6702	2074	17578	22685

Two mobile phase compositions were investigated and optimized to provide rapid separation of all analytes. Both mobile phase compositions provided adequate separation of all vitamins except MK-4 and α-tocopherol which never achieved baseline separation with either composition or any gradients used. Analytes eluted quicker in the methanol (phase A) and isopropanol/hexane (phase B) without loss of separation so it was chosen as the mobile phase for validation and routine analysis.

Other chromatography conditions optimized included the flow rate (0.15 – 0.5 mL/min) and column temp (20 – 50 °C). The column temp was selected to reduce run times without loss of analyte separation. Low temperatures provided good separation, but long runtimes. A temperature of 40 °C significantly reduced run times without negatively affecting analyte separation. Flow rate was similarly optimized to reduce run time while maintaining maximum separation and good peak shape. A relatively low flow rate of 0.2 mL/min was found to provide the best peak shape and good separation. Higher flow rates decreased retention times, but peak shape suffered dramatically (Figure 2.1).

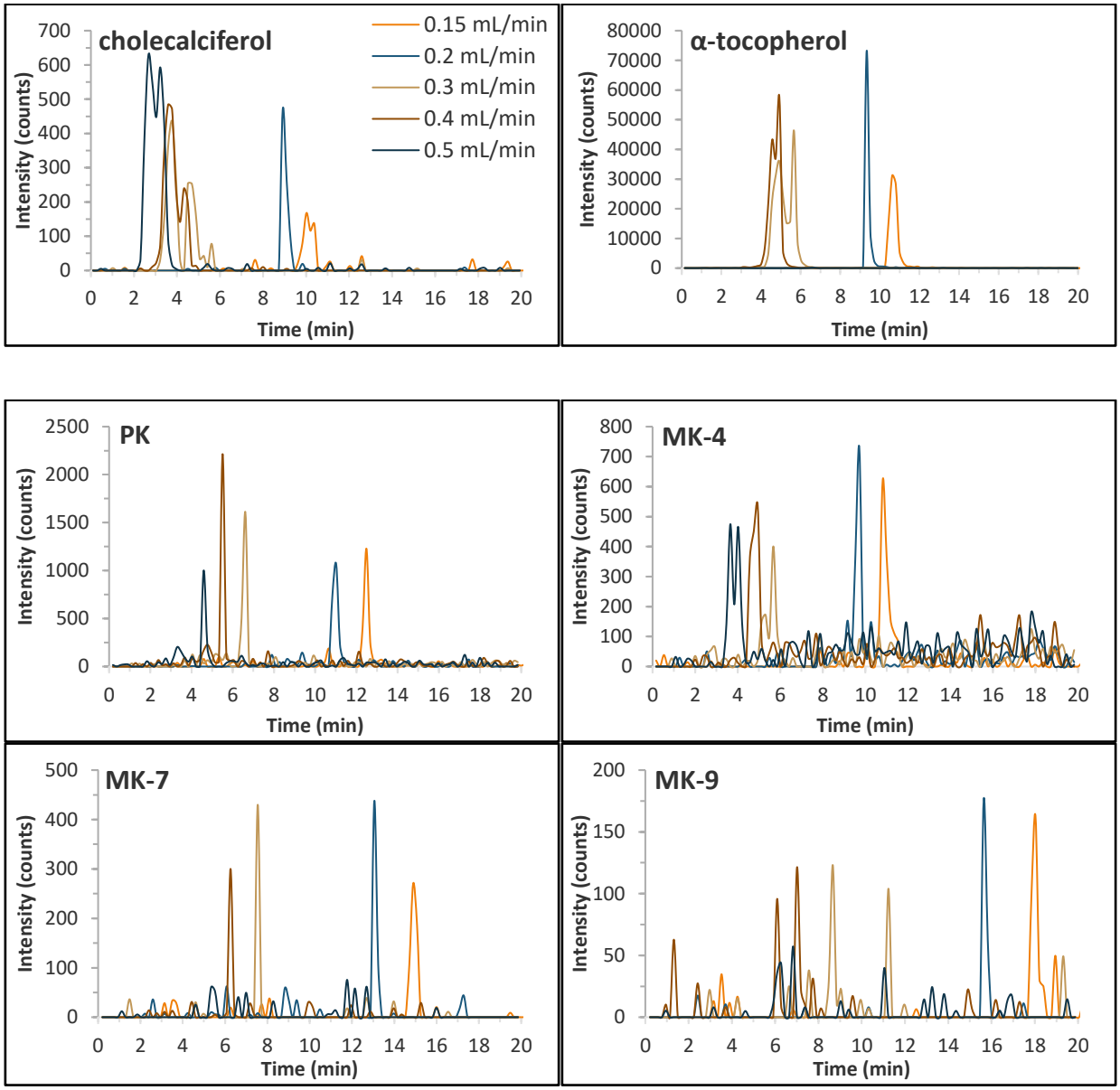

Figure 2.1. Extracted-ion chromatograms demonstrating the effect of flow rate on peak shape and separation. Standards of cholecalciferol (top left), α-tocopherol (top right), phylloquinone (PK; middle left), menaquinone-4 (MK-4; middle right), menaquinone-7 (MK-7; bottom left), and menaquinone-9 (MK-9; bottom right) were analyzed at flow rates between 0.15 mL/min and 0.5 mL/min.

2.3.2 Optimization of mass spectrometry parameters

Detection of analytes by the Q-TOF mass detector was optimized to maximize analyte peak areas and detection limits. The collision energy, used to fragment analytes during tandem MS was optimised for each analyte. The collision energies tested and resulting peak areas of each vitamin are shown in Table 2.4. The best collision energy was lowest for vitamin D_3 (15 V) and

highest for MK-9 (45 V). The optimal collision energy for MK's increased with their size (MK-4, 25 V; MK-7, 35 V; MK-9, 45 V). In the case of MK-7, the largest peak area was not selected as the optimum collision energy as the noise was significantly higher at 40 V than at 35 V.

Table 2.4. Effect of collision energy on MS/MS peak areas of analytes. Peak areas at each collision energy are shown with the optimum value highlighted.

Col energy (V)	delta toc	D3	E	K1	MK-4	MK-7	MK-9
10	37449	8944	230966	5650	2807	1632	1247
15	31172	**10510**	432719	7806	4172	1990	2145
20	**57255**	5045	374863	11082	6003	4450	2335
25	37402	620	598091	**17728**	**7410**	2227	2466
30	27077	1761	**603308**	11397	5304	3688	2920
35	26375	236	544609	5515	2739	**4601**	3571
40	21075	0	549374	7583	3102	5532	4676
45	21773	0	387629	5178	2120	3576	**5848**
50	11635	0	317174	2482	2014	4600	3837

2.3.3 Extraction methods

During development of the extraction method, it became apparent that all extraction methods suffered from incomplete removal of other fat-soluble molecules such as lipids which caused ionization suppression. The effect of ionization suppression was great enough to completely mask spiked samples from multiple extraction methods tested. Extraction methods were tested and optimized using 3.25% fat milk, but due to the effects of ionization suppression, skim milk was used for method validation following selection of the best extraction procedure.

The acid hydrolysis method effectively extracted all fat-soluble components of the sample. Extraction of a 10-mL milk sample resulted in approximately 300 mg of oily residue following nitrogen blow down. Reconstitution of the oily residue and injection into the LC-MS system resulted in significant ionization suppression. The simple hexane extraction performed comparably to the acid hydrolysis method with the advantage of being faster and using slightly

less reagents. Following nitrogen blowdown there was also approximately 300 mg of oily residue which caused ionization suppression.

The QuEChERS method used dispersive SPE to target and bind the long, straight, aliphatic side chains of lipids creating a precipitate which could then be removed through centrifugation. Unfortunately, fat-soluble vitamins also possess aliphatic side chains, but as they are branched there was a possibility they would not be targeted. We tested the method using both acetonitrile and acetone as solvents per manufacturer recommendations, and found it effectively removed the lipids, but recovery of the fat-soluble vitamins, especially the longer chain MK's, dropped significantly. To ensure it was not an issue with solubility, we tried extracting with hexane from the sample after it was mixed with the EMR-Lipid. This provided a small increase in recovery of vitamins, but the recovery was still too low for accurate analysis.

To remove the interfering lipids and reduce ionization suppression, enzymatic lipase digestion of lipids and two SPE methods were examined. They were tested on extracts from the acid hydrolysis method and the simple hexane extraction. The lipase digestion resulted in no noticeable reduction in ionization suppression. Both SPE methods resulted in all MK's eluting in the wash step with the remaining vitamins eluted in the elution. When run separately, the wash and elution had slightly reduced ionization suppression, but when recombined to have all vitamins in one run, there was no improvement over samples with no SPE. As none of the tested methods performed better than the simple hexane extraction, that method was chosen for method validation as it was the fastest method and used the least amount of chemicals and consumables. Ultimately, we were unable to remove the ionization suppression so we used non-fat yogurt for method validation which when not concentrated during extraction reduced ionization suppression and the echo-peak technique was used to compensate for matrix effects.

2.3.4 Method validation

Standard curves for all vitamins shown in Table 2.5 showed excellent linearity within the range of expected concentrations (PK, MK-4, MK-7, and cholecalciferol: 20 – 700 ng/g; MK-9: 20 – 1500 ng/g; α-tocopherol: 100 – 7500 ng/g). At concentrations, higher than those listed, linearity was lost. The squared linear regression coefficients (r^2) were >0.99 for all vitamins except cholecalciferol which was 0.98. Standard curve y-intercepts were not forced through 0, but were no more than 0.031 (Table 2.5). Slopes of the standard curves were similar for all forms of vitamin K as well as α-tocopherol. While a standard for MK-8 was unavailable, the slopes for MK-4, MK-7, and MK-9 ranged from 0.890 to 1.116 so it is likely a standard curve for MK-8 would fall in this range.

Table 2.5. Slope and regression coefficients of standard curves for all analytes measured.

Vitamin	Slope + y-intercept	Correlation coefficient
α-tocopherol	1.075 – 0.005	0.9919
Cholecalciferol	0.411 + 0.031	0.9840
Phylloquinone	0.965 + 0.029	0.9908
Menaquinone-4	1.116 – 0.005	0.9944
Menaquinone-7	0.993 + 0.004	0.9979
Menaquinone-9	0.890 + 0.016	0.9982

Recovery for all vitamins ranged from 72% for PK and MK-9 to 96% for MK-7 (Table 2.6). Intraday variation was very good apart from cholecalciferol which had an intraday variation of 18.7%. Interday variation was generally higher than intraday variation, but variation for all vitamins remained below 20%. The limits of detection and quantitation were adequate for measuring vitamins in food. The detection limit for α-tocopherol and cholecalciferol may have been lower than reported, but 4 ng/g was the lowest concentration of spiked cholecalciferol and 20 ng/g was the lowest concentration of spiked α-tocopherol tested.

Table 2.6. Recovery and precision of fat-soluble vitamins measured by LC-MS/MS.

Vitamin	Recovery (%)	Method Limits (ng/g)		Method Precision (% RSD)	
		LOD	LOQ	Intraday	Interday
α-tocopherol	88	20	20	7.9	4.7
Cholecalciferol	95	4	4	18.7	17.4
Phylloquinone	72	4	40	1.3	11.8
Menaquinone-4	86	4	20	0.2	13.5
Menaquinone-7	96	4	20	9.5	8.8
Menaquinone-9	72	4	40	4.7	15.6

2.4 Discussion

The literature describes several methods for quantifying multiple fat-soluble vitamins, but only one which quantifies forms of vitamin A, D, E, and K (Gentili et al., 2013). However, this study, by Gentili et al. (2013) did not quantify any of the longer chain MK's found in fermented foods. As their method came closest to quantifying all forms of the vitamins of interest in this study, we used their method as a starting point.

While vitamins A, D, E, and K are all fat-soluble vitamins, their solubility and chemical properties vary significantly. This makes simultaneous quantification challenging as the long chain MK's are extremely hydrophilic causing them to have much longer elution times on C18 and C30 columns than retinol and ergocalciferol. The use of a non-aqueous mobile phase allowed for faster elution of the MK's, but caused retinol and ergocalciferol to co-elute with other compounds causing ionization suppression.

Ionization suppression occurs during MS analysis when the analyte is not fully ionized, typically due to high concentrations of co-eluting compounds (Taylor, 2005). Ionization suppression occurs at the ionization source of the mass spectrometer and ESI, such as was used in this study, is known to be particularly vulnerable to ionization suppression (King et al., 2000). The loss of detection of retinol and high detection limit detection for ergocalciferol can be partially

attributed to ionization suppression. Also, lower flow rates reduce ionization suppression (Kloepfer et al., 2005) which explains why the optimal flow rate was so low (0.2 mL/min). For the column used, a higher flow rate would have been expected to produce better results as it should increase separation efficiency, while decreasing elution times.

There are a few methods to reduce ionization suppression by either removing co-eluting compounds during extraction or separating analytes from co-eluting compounds on column. Ideally, analytes are separated from ion suppressing species during extraction which completely eliminates ionization suppression. SPE is often used to remove interfering compounds from samples which would in turn reduce ionization suppression. However, both SPE methods we tried were unable to remove interfering compounds without also losing some of the vitamins of interest. Further optimization of SPE conditions and perhaps using other types of SPE besides silica may have yielded better results, but we decided to instead try a QuEChERS method for removing lipids from food samples. Unfortunately, the QuEChERS method removed all analytes along with the lipids so non-fat dairy was used for method validation.

Method validation confirmed the performance of the method was adequate for quantifying α-tocopherol, cholecalciferol, PK, MK-4, MK-7, and MK-9 in non-fat yogurt. The recoveries for PK and MK-4 were higher than those reported by Gentili et al. (2013), but the recoveries for retinol, α-tocopherol, and cholecalciferol were lower. The PK, MK-4 and MK-7 recoveries were also lower than those reported by Kamao et al. (2007) (PK, 97.5%; MK-4, 99.4%; MK-7, 97.1%) who used a lipase treatment before extracting with hexane followed by SPE. The lower recoveries were likely due to the use of a single hexane extraction instead of repeated extraction as done by others (Koivu-Tikkanen et al., 2000; Kamao et al., 2007b; Gentili et al., 2013; Manoury et al., 2013).

Intraday and interday variation were also comparable to the method by Gentili et al. (2013). The intraday variation was higher than the interday variation for several vitamins which was unusual. The LODs and LOQs were approximately three or more times higher than those reported by other LC-MS methods (Kamao et al., 2007c; Gentili et al., 2013). The lower sensitivity and the unusually large intraday variation may have been partially due to differences in instrumentation and methodology, but also likely resulted from ionization suppression.

Overall the developed LC-MS/MS method provides accurate and sensitive quantitation of α-tocopherol, cholecalciferol, PK, MK-4, MK-7, and MK-9. However, the method suffered from matrix effects which hampered the ability to measure retinol and ergocalciferol and made quantitation of vitamins in high fat samples unfeasible. Further development of a method for analysis of the vitamins we targeted should use an ionization source other than ESI, and an extraction procedure which better removes lipids and other co-eluting compounds.

Chapter 3: Biofortification of MK's in yogurt using MK producing lactic acid bacteria

3.1 Introduction

Recent studies have suggested that increased intake or supplementation of MK's in human diets can provide health benefits in terms of increased bone density and reduced arterial calcification (Knapen et al., 2013, 2015a). Despite these findings, there remains inadequate nutritional information on MK's in foods which leaves consumers with few options, apart from supplementation, for increasing their dietary MK intake. As an alternative to supplementation, fortification of commonly consumed foods could increase dietary MK intake and provide health benefits to a much broader portion of the population. In addition, due to matrix effects, fortification of some foods such as yogurt may be more effective at increasing plasma MK levels than capsules (Knapen et al., 2016).

Yogurt typically contains only small amounts of MK-4 from milk and no longer chain MK's (Koivu-Tikkanen et al., 2000; Kamao et al., 2007a). This is due to the bacteria species typically used to produce yogurt which lack the ability to produce MK's. The two bacteria species required to produce and call a product yogurt are *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, neither of which is capable of producing MK's (Morishita et al., 1999). Except for *E. durans,* none of the typical bacteria species added as probiotics produce MK's either (Parker et al., 2003). However, other fermented foods such as cheese and natto contain very high concentrations of long chain MK's due to the bacteria used in fermentation.

Several bacterial species used for cheese fermentation are known to produce significant amounts of MK-7, MK-8, and MK-9. Of these, lactococcus species in particular are known for their MK production abilities. Both *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* are

very commonly used in cheese production and have been shown to produce up to 576 µg/g and 395 µg/g lyophilized cells respectively (Morishita et al., 1999; Parker et al., 2003). Another bacterium, well known for its use in Swiss cheese, is *Propionibacterium freudenreichii* ssp. *shermanii*. *P. shermanii* is known to produce MK's (Furuichi et al., 2006), as well as vitamin B_{12}, and has potential benefits as a probiotic (LeBlanc et al., 2011). One bacterium, which produces very high concentrations of MK-7, is *Bacillus subtilis* from natto. Natto has the highest natural MK concentration of any food and *B. subtilis* and under optimal conditions can produce over 60 mg/L MK-7 (Sato et al., 2001; Berenjian et al., 2011).

Despite the interest in increasing dietary MK intake, only one study has tried to fortify MK's in dairy using MK producing bacteria ssp. from other fermented foods (Southee et al., 2016). Southee et al. (2016) fermented milk with *B. subtilis* ssp. *natto* to produce a novel MK-7 enhanced fermented dairy product, but no studies have tried to fortify MK's in yogurt. The objective of this study was to use MK producing bacteria to increase the MK concentrations found in yogurt. To accomplish this, we added *L. lactis*, *L. cremoris*, *P. shermanii*, and *B. subtilis* to milk both individually and together with standard yogurt bacteria (*S. thermophilus* and *L. bulgaricus*). We measured MK's in the finished product using liquid chromatography tandem-mass spectrometry (LC-MS/MS) and measured the relative amount of bacteria using qPCR.

3.2 Materials and methods

3.2.1 Microorganisms and culture conditions

Bacterial species were selected which have previously been shown to produce menaquinones, and are commonly used for food production. Four species were selected and are listed in Table 3.1. along with traditional yogurt bacteria used in this study.

Table 3.1. Bacterial strains used for biofortification of menaquinones in yogurt.

Genus	Species	Subspecies	MK production*	Common use	Source
Streptococcus	*salivarius*	*thermophilus*	None	Yogurt	DSM 20617
Lactobacillus	*delbrueckii*	*bulgaricus*	None	Yogurt	DSM 20081
Lactococcus	*lactis*	*lactis*	MK-8, 9, 10	Cheese	DSM 20481
Lactococcus	*lactis*	*cremoris*	MK-7, 8, 9, 10	Cheese	DSM 20069
Propionibacterium	*freudenreichii*	*shermanii*	MK-7	Cheese	DSM 4902
Bacillus	*subtilis*		MK-7	Natto	DSM 1092

*MK's known to be produced by each bacteria ssp. according to (Morishita et al., 1999; Sato et al., 2001; Furuichi et al., 2006)

3.2.2 Preparation of stock bacteria cultures

Each bacterial strain was grown overnight at the recommended temperature for each strain in skim milk media consisting of 25 g/L nutrient broth (Sigma-Aldrich, Oakville, ON, Canada) and 28 g/L skim milk powder (Pacific Dairy, Beijing, China). Cells were counted by microscope and once a concentration greater than 10^9 cells/mL was reached the fermentation was stopped, and 0.5 mL portions of culture were mixed with 0.5 mL of 50% glycerol and frozen at -80 °C until use.

3.2.3 Yogurt production and determination of optimal growth temperature

To produce yogurt, skim milk (Dairyland, Burnaby, BC, Canada) containing an additional 17 g/L skim milk powder was heated to 95 °C for 10 min to re-pasteurize the milk and denature the proteins before cooling to the experimental temperature. Individual fermentations were prepared by adding 45 mL of cooled milk to a 50-mL centrifuge tube and inoculating with ~10^8 bacterial cells of one of the six study strains or ~10^8 cells each of *S. thermophilus*, *L. bulgaricus* and one of the other four strains (Table 3.2).

Table 3.2. Sample ID and bacterial composition of experimental fermentations.

Sample ID	Strain 1	Strain 2	Strain 3
ST	*S. thermophilus*	-	-
LB	*L. bulgaricus*	-	-
LL	*L. lactis*	-	-
LC	*L. cremoris*	-	-
PF	*P. shermanii*	-	-
BS	*B. subtilis*	-	-
ST+LB+LL	*S. thermophilus*	*L. bulgaricus*	*L. lactis*
ST+LB+LC	*S. thermophilus*	*L. bulgaricus*	*L. cremoris*
ST+LB+PF	*S. thermophilus*	*L. bulgaricus*	*P. shermanii*
ST+LB+BS	*S. thermophilus*	*L. bulgaricus*	*B. subtilis*
Con	*S. thermophilus*	*L. bulgaricus*	-

To determine the optimal incubation temperature for each bacterial strain, all samples listed in Table 3.2 were incubated at 30, 33, 36, 39, 42, and 45 °C for 36 hours. Two replicates of each sample were made for each temperature. One sample was used for vitamin analysis, the other for pH measurements. Following determination of the optimal temperature, replicates of each sample at their respective optimum temperature were prepared (n = 3) following the same procedures. Samples to be used for vitamin and/or qPCR analysis were stored at -20 °C until use.

Optimal growth temperature was determined based on pH curves. The pH was measured at the time of inoculation and every six hours subsequently. The pH measurements were plotted on a graph from which the optimal temperature was determined. The optimal temperature was selected as the temperature closest to the standard yogurt production temperature of 42 °C which also had a significant drop in pH. In the case of *B. subtilis* and *P. shermanii* which did not change pH significantly, the optimal temperature was the temperature with the highest concentration of MK-7 and MK-9 in the preliminary temperature screen.

3.2.4 Delayed inoculation of *S. thermophilus* and *L. bulgaricus*

To alter the yogurt composition with the aim of increasing the proportion of MK producing bacteria, fermentations were carried out in which the MK producing bacteria were inoculated prior to inoculation with *S. thermophilus* and *L. bulgaricus*. The yogurt was prepared as previously described, but only inoculated with *L. lactis* or *L. cremoris*. The samples were incubated at the optimal temperature and *S. thermophilus* and *L. bulgaricus* inoculations were added at one of three time points. The first inoculation time was when the pH first began to drop (*L. lactis* = 16 h, *L. cremoris* = 18 h), the second when the pH fell below 5.0 (*L. lactis* = 19 h, *L. cremoris* = 24 h), and the third when the pH fell below 4.5 (*L. lactis* = 24 h, *L. cremoris* = 26 h). Three replicates were made for each inoculation time point for *L. lactis* and *L. cremoris*. As *P. shermanii* and *B. subtilis* did not change pH they were not used for this experiment.

3.2.5 LC-MS/MS quantification of MK's

MK's in fermented samples were measured using the LC-MS/MS method developed in Chapter 2. All reagents contained 0.1% BHT as an antioxidant and were carried out in reduced light. Samples were thawed in a warm water bath before weighing 5 g of sample into a 50-mL PTFE centrifuge tube to which 5 mL of water, and 10 mL isopropanol were added. The mixture was shaken and placed in a 60 °C water bath for 5 min., after which 8 mL of hexane was added and the mixture was vortexed for 30 s. The mixture was then centrifuged for 10 min at 3000 g. The upper hexane layer was transferred to a glass conical tube and blown down under Nitrogen in a 55 °C water bath. The residue was reconstituted in 1 mL isopropanol/hexane (80:20) and transferred to an LC auto sampler vial.

LC-MS/MS was carried out using an Agilent 1200 HPLC coupled to an Agilent 6530 MSD with electrospray ionization (Agilent Technologies). A 5 µL injection was used followed by a 5 µL injection of a standard solution 1.5 min after sample injection. The standard solution injection

creates echo peaks to serve as internal standards. A reversed phase C30 column (Accucore 2.6 µm, 150 mm x 2.1 mm; Thermo Fisher Scientific) thermostated at 40 °C was used. The mobile phase consisted of methanol (Phase A) and isopropanol/hexane (50/50, phase B) at a flow rate of 0.2 mL/min. The mobile phase gradient was as follows (t in min): t_0, B = 0%; t_1, B = 0%; $t_{1.1}$, B = 20 %; t_{25}, B = 20%; $t_{25.1}$, B = 0%; t_{30}, B = 0%. Detection was performed in positive ion mode using a nozzle voltage of 900 V, a fragmentor voltage of 150 V, a skimmer voltage of 80 V and the capillary voltage was 2600 V. The other MSD parameters were as follows: gas temperature, 300 °C; drying gas, 8 L/min; nebulizer pressure, 8 psig; sheath gas temperature, 350 °C; and sheath gas, 10 L/min. A collision energy of 25 V was used for fragmenting MK-4, 35 V for MK-7, 40 V for MK-8, and 45 V for MK-9.

3.2.6 qPCR analysis of relative bacterial content

Samples used for qPCR were thawed before gDNA was extracted from 250 mg of sample using a QIAGEN DNeasy Blood and Tissue kit (QIAGEN, Toronto, ON, Canada) following manufacturer's instructions. Prior to extraction, samples were treated with an enzymatic lysis buffer according to the pre-treatment for gram-positive bacteria protocol described in the kits handbook. DNA purity and concentration were checked using the A_{260}/A_{280} ratio.

Specific primers for *L. bulgaricus*, *L. lactis*, and *P. shermanii* were designed using sequences previously reported in literature and are listed in Table 3.3. The primer for *L. lactis* was also used for *L. cremoris.* Primers for *S. thermophilus* and *B. subtilis* were designed using Primer3 software version 4.0.0 (http://primer3.ut.ee/) targeting sequences from the Genbank database (https://www.ncbi.nlm.nih.gov). Primer specificity was checked using the BLAST search tool (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were chosen and designed to have as similar melting temperatures as possible so all three reactions for each sample could be carried out on the same PCR plate.

Table 3.3. Targeted sequences of primers used in this study.

Target species	Primer name	Primer sequence (5'→ 3')	Length	(T_m) °C	Amp. size	Ref
S. thermophilus	STpheSF	TCCTGTCCAAGCTCGTACAC	20	59.7	111	
	STpheSR	TGGCTGTGAGTCGCATCATC	20	60.8		
L. bulgaricus	LBpheSF	ACGTTGACGCTGACCACC	18	61.0	50	(Bottari et al., 2013)
	LBpheSR	GGCTTGAACTGGTGAAGTCTG	21	59.3		
L. lactis/L. cremoris	LLTufF	TGAAGAATTGATGGAACTCG	20	53.7	126	(Achilleos and Berthier, 2013)
	LLTufR	CATTGTGGTTCACCGTTC	18	54.7		
P. shermanii	PSTufF	GCAACATCGGCACCATCGGACAC	23	66.5	102	(Falentin et al., 2010)
	PSTufR	CGAACGAGTTCCACTGCGGGTAC	23	65.4		
B. subtilis	BSsspEF	AGCTGCTGGTCAAGGTCAAT	20	59.9	63	
	BSsspER	CTGACTTGCTGAGCGTTTGT	20	59.4		

Species specific primers were combined with gDNA from pure cultures of *S. thermophilus, L. bulgaricus, L. lactis, L. cremoris, P. shermanii,* and *B. subtilis* to develop the real-time PCR assay. Primer efficiency was measured by preparing standard curves for each primer using 5-fold serial dilutions of genomic DNA. Melting curve analysis was used to ensure the absence of primer dimer formations. All PCR reactions contained 10 µL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Mississauga, ON, Canada), 1 µL forward and reverse primers (1x concentration), and 8 µL nuclease-free water, to which 1 µL of extracted genomic DNA was added. The qPCR was performed on a C1000 Touch Thermocycler with a CFX96 Real-Time System (Bio-Rad) used to detect SYBR Green fluorescence. The reactions were carried out using an initial denaturation at 98 °C for 3 min, followed by 35 amplification cycles (10 s at 95 °C, 30 s at 57.5 °C). This was followed by a melting curve analysis from 65 °C to 95°C in 40 min before cooling to room temperature.

Bacteria were quantified in each sample relative to *S. thermophilus* using the Pfaffl equation: $ratio = (E_{target})^{\Delta CP_{target}(control-sample)}$(Pfaffl, 2001). The shortened equation without a reference gene was used since relative amounts were only compared within samples and no samples were spread over multiple PCR plates. The ratio of target to *S. thermophilus* was converted to a percent composition for the sample. Standard deviations were calculated as the standard deviation between the three biological replicates of each sample.

3.2.7 Statistical analysis

MK concentrations in samples and controls were compared using Student's T-test. Pairwise correlation between bacteria composition and MK concentrations were performed using Pearson correlation. All statistical analysis were performed using R (R Core Team, 2016).

3.3 Results

3.3.1 Bacterial growth and temperature screen

The selected bacterial strains ability to grow in milk was assessed by pH measurements, and observations of milk properties. Incubation with *S. thermophilus* caused the milk to coagulate into a gel consistent with the texture of yogurt with a layer of liquid on top. The amount of liquid varied between samples incubated at different temperatures with more liquid found in samples incubated at higher temperatures. Individual fermentations with *L. lactis* and *L. cremoris* also produced a yogurt like gel, but individual fermentations with *L. bulgaricus*, *P. shermanii*, and *B. subtilis* remained liquid throughout the entire fermentation.

To determine the optimal growth temperature for each bacterial species, pH measurements were taken as an indicator of growth and plotted in Figure 1.1. The pH measurements for individual fermentations of *S. thermophilus* revealed a significant lag period of approximately 6-12 h, depending on the temperature, before the pH began to drop. Fermentations with all other bacterial strains also contained a lag period of several hours (*L. lactis* and *L.* cremoris) to more than 24 h (*L. bulgaricus* and *P. shermanii*). The pH for *B. subtilis* was not expected to drop, but at 39 °C, 42 °C, and 45 °C there was a small drop in pH late in fermentation (Figure 3.1 F).

To select the optimal growth temperature, we looked for a large drop in pH as well as an inverse sigmoidal curve representing lag, exponential, and stationary phases of growth. The typical yogurt bacteria, *S. thermophilus* and *L. bulgaricus*, both grew at 33 – 45 °C with little differences between temperatures so the optimal temperature for each of the MK producing bacteria was chosen as the highest temperature between 33 °C and 45 °C which met the above criteria. Based upon these criteria, the optimal growth temperature for *L. lactis* was 36 °C and for *L. cremoris* 33 °C. Due to the small pH change for both *P. shermanii* and *B. subtilis*, menaquinone concentrations were used to select the optimal temperature.

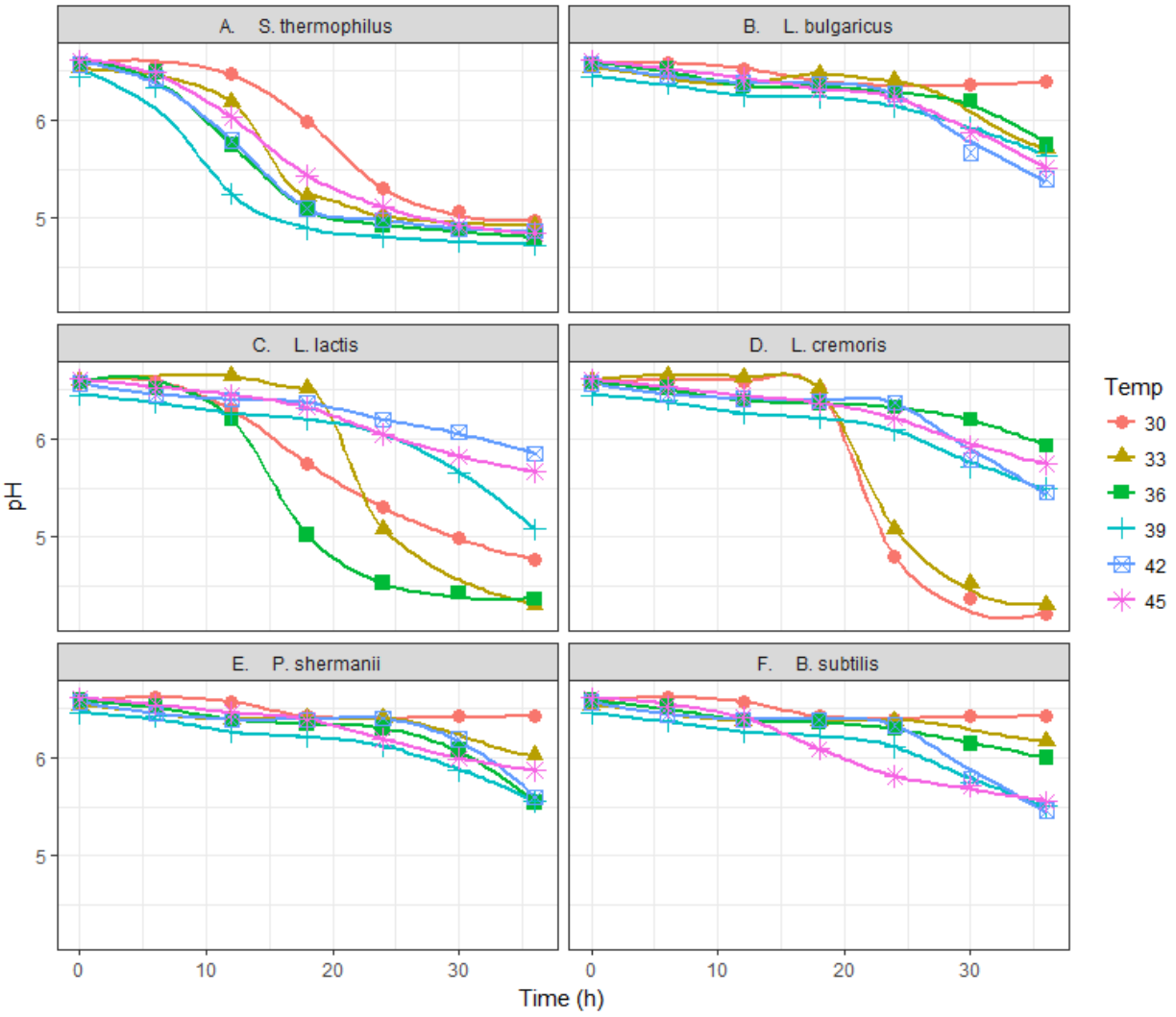

Figure 3.1. pH curves for each bacterial ssp. studied incubated at each experimental temperature.

MK's were measured in samples from the temperature screen to ensure MK's were produced and assist in choosing the optimal growth temperature for *P. shermanii* and *B. subtilis*. Measurement of MK's revealed large differences in MK concentrations depending on the temperature and bacterial species used for fermentation. MK-4 concentrations were below quantitation or detection limits in most samples and no samples contained high concentrations of MK-4. The only samples in which MK-9 was present were individual fermentations of *L. cremoris* at 30 °C and 33 °C. All samples contained high concentrations of MK-7 including *S. thermophilus* and *L. bulgaricus* fermentations.

3.3.2 Menaquinones at optimal growth temperature

Following selection of the optimal growth temperature for each MK producing bacterial species, triplicate fermentations were produced at the optimal temperatures. MK concentrations in the replicates shown in Table 3.4 were consistent with the initial temperature screen. It was apparent that there were relatively high concentrations of MK-7 in all samples including the negative controls at both 36 °C and 33 °C. Only the BS sample had significantly higher MK-7 concentrations than the control ($p = 0.037$), and the PF and LC showed a tendency to be greater than the control ($p = 0.051$; $p = 0.064$).

Quantitation of MK-8 was not possible as no standard was available, but the presence of MK-8 was determined based on the MS/MS transition. The only bacterial ssp. which produced MK-8 and MK-9 was *L. cremoris* and while the MK-9 concentration was high when *L. cremoris* was fermented on its own, in a mixed culture the MK-9 concentration was below the quantitation limit. MK-7 levels were also lower in all mixed cultures indicating the MK producing bacteria may not grow well in combination with typical yogurt bacteria.

Table 3.4. Menaquinone concentrations of samples fermented at optimal temperatures.

Sample	Temp (°C)	Vitamin K Concentrations (ng/g)[a] MK-9	MK-7	Mk-4	Presence of MK-8 (+/-)[b]
LL	36	LOQ	153.0 ± 87.8	24.1 ± 21.8	-
ST+LB+LL	36	LOQ	85.5 ± 18.1	ND	-
PF	36	LOQ	448.2 ± 185.1*	LOQ	-
ST+LB+PF	36	ND	88.4 ± 26.6	LOQ	-
BS	36	LOQ	305.5 ± 81.6**	LOQ	-
ST+LB+BS	36	LOQ	126.8 ± 30.7	ND	-
Con	36	ND	159.4 ± 61.7	27.7 ± 26.1	-
LC	33	171.0 ± 71.7**	244.9 ± 109.4*	LOQ	+
ST+LB+LC	33	LOQ	112.0 ± 38.2	LOQ	+
Con	33	LOQ	89.6 ± 20.8	LOQ	-

[a]Mean and standard deviation of 3 replicates. [b]MK-8 was either detected (+) or not detected (-) in each sample, but was not quantified. ND: not detected. LOQ: below limit of quantitation.
**greater than control ($p < 0.05$). *greater than control ($p < 0.1$)
BS: *B. subtilis*, LB: *L. bulgaricus*, LC: *L. cremoris*, LL: *L. lactis*, PF: *P. shermanii*, ST: *S. thermophilus*

Real-time quantitative PCR was used to determine the relative amount of each bacteria ssp. in mixed fermentations. The results, shown in Table 3.5 confirmed that in mixed fermentation, the typical yogurt bacteria were outgrowing the MK producing bacteria except for *L. lactis* which outgrew both *S. thermophilus* and *L. bulgaricus*. Of the typical yogurt bacteria, *S. thermophilus* vastly outgrew *L. bulgaricus* and comprised more than 99% of the bacteria in fermentations with *L. cremoris*, *P. shermanii*, and *B. subtilis*.

Table 3.5. Relative percent of bacteria determined by qPCR in mixed fermentations.

Sample	Relative percentage[a] of bacterial ssp. S. thermophilus	L. bulgaricus	L. lactis	L. cremoris	P. shermanii	B. subtilis
ST+LB+LL	41.23 ± 8.26	0.02 ± 0.00	58.75 ± 8.27	-	-	-
ST+LB+LC	99.96 ± 0.00	0.03 ± 0.01	-	0.01 ± 0.00	-	-
ST+LB+PF	99.95 ± 0.01	0.05 ± 0.01	-	-	< 0.01	-
ST+LB+BS	99.76 ± 0.11	0.05 ± 0.02	-	-	-	0.19 ± 0.10

[a]all data are mean ± s.d. (n = 3)
BS: *B. subtilis*, LB: *L. bulgaricus*, LC: *L. cremoris*, LL: *L. lactis*, PF: *P. shermanii*, ST: *S. thermophilus*

3.3.3 Delayed inoculation of *S. thermophilus* and *L. bulgaricus*

To obtain more similar growth between the three bacterial ssp. within each sample, we tried inoculating the milk with *L. lactis* or *L. cremoris* several hours before *S. thermophilus* and *L. bulgaricus*. Menaquinone concentrations from these samples, shown in Table 3.6, were similar to individual fermentations of *L. lactis* and *L. cremoris* used as positive controls. The *L. cremoris* mixed culture in which *S. thermophilus* and *L. bulgaricus* were added after 24 hours had significantly higher concentrations of MK-9 than the individual fermentation of *L. cremoris* ($p = 0.001$).

Table 3.6. Menaquinone concentrations of fermentations with delayed inoculation of typical yogurt bacteria.

	Time (h) before	Vitamin K Concentrations (ng/g)[a]			MK-8 (+/-)
Sample	inoculation	MK-9	MK-7	Mk-4	
ST+LB+LL	16	LOQ	59.8 ± 8.6	21.6 ± 13.2	-
ST+LB+LL	19	LOQ	91.2 ± 46.7	LOQ	-
ST+LB+LL	24	LOQ	89.3 ± 51.2	LOQ	-
ST+LB+LC	18	76.2 ± 18.5	168.3 ± 32.1	LOQ	+
ST+LB+LC	24	234.4 ± 6.1*	155.9 ± 50.6	LOQ	+
ST+LB+LC	26	139.9 ± 33.0	174.9 ± 56.4	26.1 ± 10.6	+
LL	-	3.7	60.0	LOQ	-
LC	-	167.1	145.4	LOQ	+

[a]Data are mean ± s.d. (n = 3)
LOQ: below limit of quantitation
*greater than individual fermentation of LC or LL ($p < 0.05$)
BS: *B. subtilis*, LB: *L. bulgaricus*, LC: *L. cremoris*, LL: *L. lactis*, PF: *P. shermanii*, ST: *S. thermophilus*

Again, qPCR was used to determine the relative amounts of each bacterial ssp. in the samples. The results, shown in Table 3.7, show that inoculating milk with *L. lactis* or *L. cremoris* before inoculating with *S. thermophilus* and *L. bulgaricus* result in almost exclusive growth of *L. lactis* or *L. cremoris*. Inoculation at all three time points resulted in the bacterial composition being over 99% *L. lactis* or *L. cremoris* with almost no *L. bulgaricus* and very little *S. thermophilus*. As with the previous simultaneous inoculations, *L. lactis* and *S. thermophilus* grew mutually better than *L. cremoris* and *S. thermophilus*.

Table 3.7. Relative percent of bacteria determined by qPCR in fermentation with delayed *S. thermophilus* and *L. bulgaricus* inoculation.

Sample	Time (h) before inoculation	Relative percentage[a] of bacterial ssp.		
		S. thermophilus	L. bulgaricus	L. lactis
ST+LB+LL	16	0.12 ± 0.05	< 0.01	99.88 ± 0.05
ST+LB+LL	19	0.13 ± 0.03	< 0.01	99.87 ± 0.03
ST+LB+LL	24	0.15 ± 0.06	< 0.01	99.84 ± 0.06
		S. thermophilus	L. bulgaricus	L. cremoris
ST+LB+LC	18	0.06 ± 0.04	< 0.01	99.94 ± 0.04
ST+LB+LC	24	< 0.01	< 0.01	99.99 ± 0.00
ST+LB+LC	26	< 0.01	< 0.01	99.99 ± 0.00

[a]all data are mean ± s.d. (n = 3)
BS: *B. subtilis*, LB: *L. bulgaricus*, LC: *L. cremoris*, LL: *L. lactis*, PF: *P. shermanii*, ST: *S. thermophilus*

To identify significant correlations between the bacteria and MK concentrations, a pairwise Pearson correlation heatmap was prepared (Figure 3.2). The heatmap revealed very strong negative correlations between *L. lactis* and *S. thermophilus*, *L. lactis* and *L. bulgaricus*, *L. cremoris* and *S. thermophilus*, and *L. cremoris* and *L. bulgaricus*. The only significant correlation between a bacterial ssp. and MK concentration was a positive correlation between *L. cremoris* and MK-9.

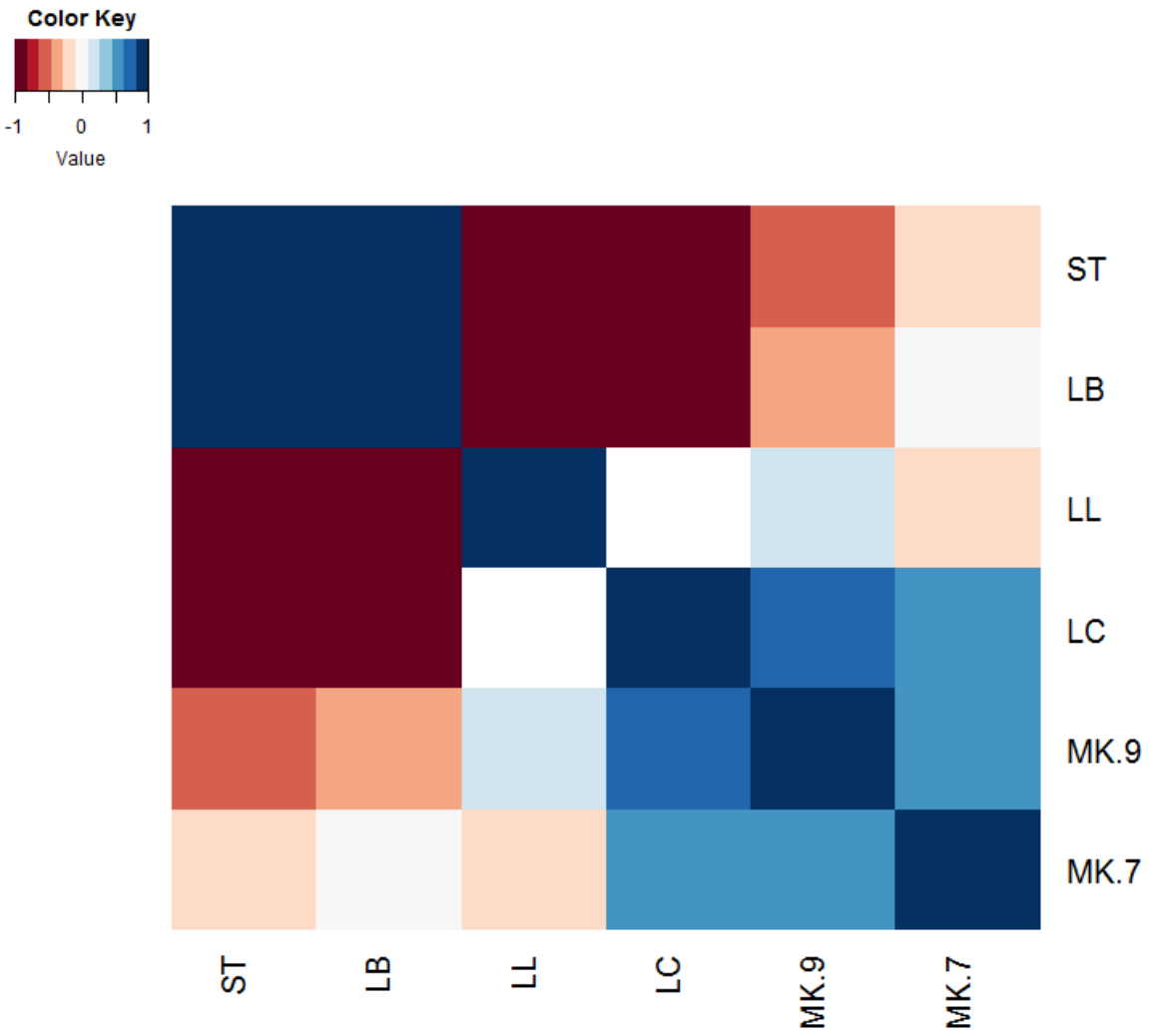

Figure 3.2. Heatmap of Pearson correlations between bacterial ssp. and MK concentrations.

3.4 Discussion

To the best of our knowledge, this is the first study which has attempted to fortify MK's in yogurt through the addition of MK producing bacteria. Others have found yogurt to be an excellent matrix for the delivery of supplementary MK's (Knapen et al., 2016), one study used bacteria to successfully biofortify vitamin B_{12} in soy yogurt (Gu et al., 2015), and another increased MK's by fermenting milk with *B. subtilis* (Southee et al., 2016), so it is logical that bacteria could be used to naturally fortify MK's in yogurt. In this study, we demonstrated that fermentation with *S. thermophilus* and *L. bulgaricus* in conjunction with *L. cremoris* produces a

yogurt-like product with up to 234 ng/g MK-9 and 155 ng/g MK-7. This represents a large increase over concentrations typically found in yogurt such as Kamao et al. (2007) who reported a mere 1 ng/g MK-7 in yogurt, and Schurgers and Vermeer (2000) who reported 2 ng/g MK-9 in yogurt. The concentrations of MK's reported here are more similar to those reported for cheeses (Schurgers and Vermeer, 2000; Manoury et al., 2013).

Manoury et al. (2013) measured MK's in perhaps the greatest variety of cheeses of any study to date, and found MK-7 concentrations from 0 - 223.3 ng/g and MK-9 concentrations from 0 - 939.7 ng/g. While those values are higher than the maximum MK concentrations found here, the MK concentrations in samples containing *L. cremoris* were higher than many cheeses reported by Manoury et al. (2013). In fact, the total MK concentration for the ST+LB+LC sample with 24 h delayed *S. thermophilus* and *L. bulgaricus* inoculation would be a significant source of dietary MK's; a 100-g serving of which would represent more than 49 % of the current RDI for vitamin K in Canada (39 µg plus unknown amount of MK-8; RDI = 80 µg, (Canadian Food Inspection Agency, 2016)).

Unfortunately, due to the unavailability of MK-8 standards, it was not possible to quantify MK-8 in this study. Significant amounts of MK-8 was detected in *L. cremoris* fermentations, but surprisingly no MK-8 or MK-9 was detected in *L. lactis* fermentations. It is known that some strains of *L. lactis* can produce MK-8, along with MK-9, and MK-10 (Morishita et al., 1999), but in both the initial temperature screen and replicates at optimal temperature, fermentations of *L. lactis* only contained MK-7 in concentrations above the limit of quantitation. The lack of MK-8 and MK-9 production by *L. lactis* may have been due to the growth conditions or the strain of *L. lactis* used. Multiple reports have shown that the MK profiles of bacteria are dependent on growth conditions such as carbon source, nitrogen source, and aerobic/anaerobic environment (Rezaïki et al., 2008; Berenjian et al., 2011). Growth conditions in this study may have caused *L.*

lactis to produce more MK-10 which was not measured or caused it to produce MK-7 which was produced by *L. lactis* in this study, but not in previous reports (Morishita et al., 1999). Also, not every strain of *L. lactis* produces all three forms of MK (Morishita et al., 1999), so another strain of *L. lactis* may produce more MK's.

We managed to produce fermented milk products with high concentrations of MK's; however, growing all three ssp. of bacteria in the mixed cultures proved difficult. The qPCR results showed that *S. thermophilus* grew almost exclusively if all bacterial ssp. were inoculated simultaneously, and *L. lactis* or *L. cremoris* grew almost exclusively when inoculated first. The inability to grow concurrently may be due to a couple factors. The first is that based on the pH curves in Figure 3.1, *S. thermophilus* started growing much faster than the other bacteria, so during simultaneous inoculation it would have grown quickly, using up most of the nutrients before the other bacteria entered exponential phase. This is supported by the fact that *L. lactis*, which also started growing quickly was able to grow concurrently with *S. thermophilus*. Based on this idea, delaying the inoculation of *S. thermophilus* should have resulted in greater concurrent growth; however, this was not seen. Instead, whichever bacterial ssp. was added first grew almost exclusively. This may indicate the *S. thermophilus* and *L. bulgaricus* inoculations were too late. It is also possible that antibacterial compounds such as nisin and multiple lactococcins produced by *L. lactis* and *L. cremoris* inhibited growth of *S. thermophilus* and *L. bulgaricus* (Jack et al., 1995). However, Vinderola et al. (2002) found that *S. thermophilus* is capable of growing in the presence of nisin, so it is more likely that the inoculation was too late.

As for *P. shermanii* and *B. subtilis*, their lack of growth could also be attributed to several factors. Ideally, *P. shermanii* should be grown under anaerobic conditions and while Furuichi et al., (2006) found increased growth of propionibacteria by switching from anaerobic to aerobic conditions, they still started the culture in anaerobic conditions which our fermentations were

not. As for *B. subtilis*, it is typically used for the production of natto, a fermented soybean dish, so it may not be ideal for growing in milk. Sato et al. (2001) and Berenjian et al., (2011) both investigated the optimal growth conditions for *B. subtilis* to produce MK-7 and while they both found glycerol was the best carbon source, Sato et al. (2001) also found that *B. subtilis* can grow on lactose, galactose, and glucose, the main sugars present in milk. Sato et al. (2001) also tested the nitrogen source and found skim milk to be a poor nitrogen source for *B. subtilis* growth, a factor that likely contributed to the poor growth of *B. subtilis* seen here. A recent report by Southee et al., (2016) demonstrated that *B. subtilis* can be used to enrich MK's in milk, although they added glycerol to their fermentations, a strategy which, if used, may have benefited *B. subtilis* fermentations in this study. Also, the pH for *B. subtilis* was not expected to drop as was seen when incubated at 38 °C or above, as previous studies have shown an increase in pH during *B. subtilis* fermentation (Berenjian et al., 2011).

The fermentations were carried out in a biosafety cabinet and all glassware was rinsed with ethanol to prevent contamination, but there were signs of possible contamination in some fermentations. Most notably, the negative controls with *S. thermophilus* and *L. bulgaricus* contained significant amounts of MK-7. As neither *S. thermophilus* nor *L. bulgaricus* contain the full pathway to produce MK's (Morishita et al., 1999; Hiratsuka et al., 2008), this likely indicates another bacterial ssp. was present in the fermentation which was capable of producing MK-7. Skim milk powder was the most likely source of contamination as it is known to contain as many as 10^7 bacterial cells per gram of powder, of which, 10^5 may be viable (Rueckert et al., 2005). Additionally, one of the most ubiquitous species of bacteria in milk powders is *Bacillus lichenformis* (Buehner et al., 2015; Gopal et al., 2015) which is known to produce significant amounts of MK-7 (Goodman et al., 1976). Since *Bacillus lichenformis* is heat resistant and spore forming (Buehner et al., 2015), it, along with other Bacillus species may survive the heat

treatment used prior to fermentation and could grow during fermentation. This would further increase the base level of MK-7 in all samples. Growth of contaminants could also have caused the small unexpected drop in pH in *B. subtilis* fermentations. While contamination of dairy products with *Bacillus lichenformis* is undesirable due to its potential to cause spoilage (Gopal et al., 2015), it may also be contributing a small to moderate amount of MK-7 to products made with skim milk powder. This study demonstrated that it is possible to use MK producing bacteria to produce yogurt with high MK concentrations. The MK-7, MK-9, and MK-8 concentrations produced by *L. cremoris* in yogurt fermentations in particular are nutritionally significant. However, obtaining relatively equal growth of all three bacterial ssp. in a sample proved difficult and delaying inoculations did not result in more equal growth. Perhaps using different time points for the delayed inoculations or altering the number of cells used to inoculate the milk would be more successful. Also, further optimization of growth conditions is necessary to reduce production time and produce a product ready for human consumption.

Chapter 4: General discussion

4.1 Summary

There is an ever-growing body of research demonstrating numerous health benefits associated with MK intake. As more and more studies investigate the role of MK's in vascular calcification (Geleijnse et al., 2004; Knapen et al., 2015a) and bone health (Knapen et al., 2013), there is a greater need for studies investigating MK nutrition. Two areas of MK nutrition requiring further study are methods of quantifying MK's in foods, and fortification of MK's in foods.

With all the evidence supporting health benefits around increased MK intake, it is more important than ever that consumers be well informed regarding the MK content of the food they eat. Unfortunately, there is little information regarding MK's in many foods. Other fat-soluble vitamins are commonly measured and included in nutrient information on food packaging, but standard methods for quantifying these vitamins differ from methods used for quantifying MK's (Blake, 2007). A method capable of quantifying all fat-soluble vitamins, would make it much easier to provide nutrient information on foods as it would decrease analysis time, costs, and consumables. One of the main goals of this study were to develop a LC-MS/MS method for quantifying all fat-soluble vitamins in dairy products.

Low dose supplementation with MK's has been shown to be effective at reducing vascular calcification (Knapen et al., 2015a) and increasing bone density (Knapen et al., 2013); however, few studies have investigated fortification of foods as an alternative to supplementation. Yogurt presents one of the best targets for fortification of MK's in a commonly consumed food. Present MK concentrations in yogurt are insignificant due to the bacteria used in yogurt production, but bacteria used to produce other foods, such as cheese and natto, produce large amounts of MK's. Therefore, addition of bacteria from these foods to milk during yogurt production may increase the MK concentration in yogurt. Additionally, yogurt to which MK had been added has been

shown to be an effective matrix for the delivery of MK's in humans (Knapen et al., 2016). A second goal of this study was to investigate the potential of using MK producing bacteria to biofortify MK's in yogurt.

Development of the LC-MS/MS method for quantifying retinol, ergocalciferol, cholecalciferol, α-tocopherol, PK, MK-4, MK-7, and MK-9 involved testing and optimising chromatographic conditions, mass spectrometry parameters, and extraction methods. The acid hydrolysis and simple hexane extraction methods both effectively extracted all fat-soluble vitamins, but also resulted in ~300 mg of oily residue remaining after nitrogen blow down. Reconstitution of the oily residue in hexane/isopropanol (90:10) and injection into the LC-MS system resulted in significant ionization suppression. To try to remove the suppressing compounds, two silica SPE methods were investigated. Both methods slightly reduced ionization suppression, but we were unable to elute all analytes in one fraction. When fractions were recombined to measure all analytes, the benefits to ionization suppression disappeared. The lipase and EMR-Lipid extraction techniques performed far worse than the acid hydrolysis or simple hexane extraction so the simple hexane extraction was used as it was fastest and used the least reagents.

When developing the LC method, two HPLC columns were tested, a C18 and C30, of which the C30 performed better in terms of separation efficiency and reduced ionization suppression of MK-7 and MK-9, but was unable to quantify retinol or ergocalciferol at concentrations found in foods. The C30 column was chosen for routine analysis even though it meant losing the ability to measure retinol and ergocalciferol as the ability to measure MK's was more important for Chapter 3 of this thesis. A non-aqueous mobile phase using a gradient of methanol (phase A) and isopropanol/hexane (phase B) was optimized and reduced elution times of long chain MK's compared to a mobile phase of methanol/H_2O (90:10, phase A) and methanol/THF (70:30, phase B). The optimal flow rate of 0.2 mL/min was very low for a 2.1 mm i.d. column. The greater

peak area and improved peak shape seen at low flow rates was indicative of ionization suppression as lower flow rates are known to reduce ionization suppression.

Due to the inability to remove ionization suppression, validation of the method was carried out using non-fat yogurt. The method performed well using non-fat yogurt with LOD's between 4 and 20 ng/g and LOQ's between 4 and 40 ng/g. The LOQ's were low enough for concentrations typically seen in foods, but lower LOQ's would be desirable as some vitamins which have very low concentrations in yogurt were very close to or below the LOQ. Recovery of vitamins ranged from 72% for PK and MK-9 to 96% for MK-7. Intraday precision was 0.2 – 18.7% and interday variation was 4.7 – 17.4%. The validation parameters compared well with similar methods which measured multiple fat-soluble vitamins.

Our study using bacteria to fortify MK's in yogurt demonstrated that fermentation with MK producing bacteria can produce significant amounts of MK's. All four ssp. of MK producing bacteria selected grew in individual fermentations of skim milk. The optimal growing temperature for *L. lactis*, *B. subtilis*, and *P. shermanii* was 36 °C and the optimum temperature for *L. cremoris* was 33 °C. At these temperatures, *L. cremoris* produced significantly more MK-9 ($p < 0.05$) and tended to contain more MK-7 ($p < 0.1$) than the negative control. Fermentation with *B. subtilis* also resulted in higher MK-7 concentrations than the control ($p < 0.05$) and *P. shermanii* showed a trend for higher MK-7 concentrations ($p < 0.1$). When MK producing bacteria were co-fermented with *S. thermophilus* and *L. bulgaricus* the increase in MK production was lost indicating the MK producing bacteria were likely not growing in co-fermentations. This was confirmed with qPCR which showed all co-fermentations contained over 99 % *S. thermophilus* except for the fermentation with *L. lactis* which contained 59 % *L. lactis*.

To produce fermentations with more equal growth of all ssp. of bacteria inoculated, we tried delaying the inoculation of *S. thermophilus* and *L. bulgaricus*. By delaying the inoculation until the beginning, middle, and end of the pH drop caused by fermentation with *L. lactis* or *L. cremoris*, MK concentrations similar to those found in individual fermentations of *L. lactis* and *L. cremoris* were obtained. Delaying the inoculation resulted in a complete switch in the dominant ssp. with *L. lactis* or *L. cremoris* comprising over 99 % of the total bacteria. Additionally, there was no significant correlation between bacterial composition and time of inoculation.

4.2 Limitations

The greatest limitation of this study was the inability to quantify fat-soluble vitamins in high-fat samples. This greatly limits the potential application of the LC-MS/MS method developed and required us to produce non-fat yogurt using skim milk with skim milk powder for thickening. The use of skim milk powder introduced further limitations as it often contains significant amounts of bacteria which we believe was responsible for MK-7 being present in the controls. As some of the MK-7 in each sample was likely coming from the skim milk powder, concentrations of MK-7 in full fat fermented dairy products would likely be lower those reported here.

Since isotope-labelled internal standards for some analytes were unavailable, we used the echo peak technique which is known to compensate for matrix effects (Zrostlíková et al., 2002; Alder et al., 2004). Injecting a standard solution shortly after the sample creates peaks which can compensate for matrix effects, but there are certain limitations inherent in this technique. Primarily, the technique assumes that peaks which elute very close together are subject to the same matrix effects (Alder et al., 2004). This is not always the case and if one peak is effected by the matrix more than the other the concentration may appear artificially higher or lower,

reducing the accuracy of the method. If all samples are identical and matrix-matched standards are used, this would not be an issue, but creating matrix-matched standards for vitamins in foods is not possible. Since matrix-matched standards were not used, different conditions for the sample and echo peak in samples and standard solutions could cause all measurements to be slightly higher or lower than their actual concentration.

The lack of isotope-labelled internal standards also limits the ability to compensate for variation in extraction. When using internal standards, the recovery is in effect accounted for in each sample, but when using the echo peak technique, internal standards are not used. Instead recovery was calculated by spiking a sample before and after extraction during each batch of extractions and the calculated recovery was applied to the whole batch. The recovery of analytes in each sample in the same batch should be similar, but not identical, so this technique introduces an additional source of variation to the method.

There are also certain assumptions made when using PCR that limit interpretation of qPCR results. First, when extracting gDNA from samples, it is assumed that cell lysis and extraction is the same for all species of bacteria. However, if one bacterial ssp. is more resistant to lysis than another it will appear that there is less of that bacteria ssp. than there actually is.

4.3 Future directions

Our method failed to quantify all fat-soluble vitamins, but development of such a method is still desirable. Future attempts at method development should compare the use of fluorescence detection with mass spectrometry. We encountered difficulties with ionization suppression which the use of fluorescence detection would avoid. If mass spectrometry is used for detection, ESI should be avoided due to its susceptibility to ionization suppression. Atmospheric pressure chemical ionization (APCI) may be more suitable. Also, further development of extraction procedures should focus more on development of SPE for removal of interfering compounds.

Perhaps using C18 SPE or both silica and C18 SPE as some groups have done would be more effective at removing ion suppressing interferences although they also reported C18 SPE reduced recovery of PK and MK's (Koivu-Tikkanen et al., 2000; Jones et al., 2009).

This study showed MK producing bacteria can produce nutritionally significant concentrations of MK's. However, growth of the three bacterial ssp. in each sample was imbalanced. To obtain more equal growth of all ssp. there are a couple options. One would be to continue investigating effects of varying the time of inoculation, but a better method may be to alter the inoculant size. Optimizing inoculant size to provide more even growth would prevent opening and disturbing the fermentation and would be better for commercial production. While *L. cremoris* produced significant amounts of MK's in this study, further optimization of growth conditions may increase the amount of MK's produced. Also, since there are strain differences in MK production, using different strains of *L. cremoris* may produce higher MK concentrations than reported here. Additionally, while *L. lactis* did not produce significant amounts of MK's, others have shown some strains of *L. lactis* do produce MK's (Morishita et al., 1999; Parker et al., 2003), so it's use for enhancing MK's in yogurt should not be ruled out.

4.4 Conclusions

The LC-MS/MS method developed was effective at quantifying MK-4, MK-7, and MK-9 in MK enhanced non-fat yogurt and could detect MK-8. Additionally, the method was capable of quantifying cholecalciferol, α-tocopherol, and PK, but fell short of being able to quantify all fat-soluble vitamins. Overall, LC-MS/MS is a very effective method for quantifying fat-soluble vitamins, but significant effort is required to reduce ionization suppression, particularly when using ESI.

Co-fermenting milk with a combination of typical yogurt bacteria (*S. thermophilus* and *L. bulgaricus*) and MK producing bacteria (*L. lactis*, *L. cremoris*, *P. shermanii*, *B. subtilis*)

produced a yogurt like product with nutritionally significant concentrations of MK's. In the case of *L. cremoris*, concentrations of up to 234.4 ng/g MK-9, 174.9 ng/g MK-7, and an unspecified amount of MK-8 were quantified in co-fermentations. The other three bacteria ssp. tested did not produce any MK-9 or MK-8, but *B. subtilis* and *P. shermanii* produced marginally more MK-7 than controls, when fermented individually. While all bacterial species grew individually in milk, they struggled to grow in conjunction with *S. thermophilus* and *L. bulgaricus.* Offsetting the time of inoculations of the different bacteria ssp. to obtain more equal growth proved effective at altering the bacterial composition, but resulted in almost exclusive growth of the first inoculant. Because of its high MK production, *L. cremoris* shows the greatest promise for fortifying MK's in yogurt.

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Appendices

Table 0.1. Concentrations of fat-soluble vitamins in samples from initial temperature screen.

Bacteria	Temp (°C)	MK-7	MK-9	MK-4	K1	E	D3
BS	30	145.6	ND	LOQ	ND	37.2	13.3
BS	33	148.7	LOQ	39.6	LOQ	66.3	32.8
BS	36	400.6	45.2	21.3	ND	65.4	22.0
BS	39	521.9	ND	52.1	LOQ	40.0	8.5
BS	42	431.8	LOQ	20.2	LOQ	53.7	16.7
BS	45	529.7	ND	LOQ	ND	LOQ	ND
ST+LB+BS	30	81.1	ND	ND	ND	25.7	LOQ
ST+LB+BS	33	80.9	ND	ND	ND	20.2	30.8
ST+LB+BS	36	124.5	ND	ND	ND	33.7	9.1
ST+LB+BS	39	60.7	ND	ND	ND	24.5	LOQ
ST+LB+BS	42	153.0	ND	ND	ND	29.3	10.5
ST+LB+BS	45	150.5	ND	ND	ND	LOQ	85.1
ST+LB+LC	30	71.1	LOQ	LOQ	ND	37.1	8.7
ST+LB+LC	33	97.3	ND	ND	ND	20.4	11.2
ST+LB+LC	36	85.8	ND	ND	ND	30.1	LOQ
ST+LB+LC	39	79.2	ND	ND	ND	LOQ	4.0
ST+LB+LC	42	138.8	ND	ND	ND	21.2	9.3
ST+LB+LC	45	212.0	ND	LOQ	ND	36.8	9.7
ST+LB+LL	30	LOQ	LOQ	ND	LOQ	22.8	LOQ
ST+LB+LL	33	28.0	LOQ	ND	ND	21.7	11.8
ST+LB+LL	36	94.5	ND	ND	ND	52.1	12.4
ST+LB+LL	39	76.5	ND	ND	ND	LOQ	LOQ
ST+LB+LL	42	129.7	ND	ND	ND	34.9	5.8
ST+LB+LL	45	222.7	LOQ	39.0	97.3	248.3	ND
ST+LB+PF	30	72.2	ND	ND	ND	24.8	ND
ST+LB+PF	33	90.2	ND	ND	ND	23.2	5.3
ST+LB+PF	36	147.4	ND	LOQ	ND	38.4	19.8
ST+LB+PF	39	52.6	ND	ND	ND	43.8	16.1
ST+LB+PF	42	133.4	ND	ND	ND	21.2	4.4
ST+LB+PF	45	157.3	ND	ND	ND	LOQ	LOQ
LB	30	206.5	ND	LOQ	ND	35.0	24.4
LB	33	361.7	ND	60.5	LOQ	65.2	20.9
LB	36	672.4	LOQ	26.3	LOQ	68.2	24.2
LB	39	493.0	ND	34.6	LOQ	57.1	12.5
LB	42	542.6	ND	22.1	LOQ	42.2	7.7
LB	45	455.6	ND	LOQ	ND	36.2	5.5
LC	30	121.1	145.1	ND	ND	LOQ	6.5
LC	33	224.8	117.2	25.3	ND	35.6	33.8
LC	36	312.5	LOQ	58.5	LOQ	45.6	5.3

LC	39	428.2	LOQ	20.7	LOQ	66.4	25.3
LC	42	509.6	ND	LOQ	LOQ	23.6	ND
LC	45	427.1	ND	LOQ	ND	29.2	7.3
LL	30	21.5	LOQ	ND	ND	LOQ	ND
LL	33	LOQ	LOQ	48.0	LOQ	40.2	46.2
LL	36	309.0	LOQ	48.4	ND	80.4	46.3
LL	39	257.8	LOQ	30.3	LOQ	46.0	5.7
LL	42	389.2	ND	33.9	LOQ	52.8	15.7
LL	45	279.3	ND	ND	ND	32.6	3.4
PF	30	173.4	ND	LOQ	ND	41.1	15.5
PF	33	207.5	LOQ	LOQ	LOQ	34.2	47.3
PF	36	1572.0	LOQ	20.6	LOQ	82.5	46.7
PF	39	399.3	LOQ	32.8	LOQ	60.5	12.6
PF	42	363.2	ND	LOQ	LOQ	31.7	22.0
PF	45	338.8	ND	ND	ND	51.2	8.0
ST	30	56.4	LOQ	ND	ND	LOQ	ND
ST	33	68.9	ND	ND	102.9	23.6	39.0
ST	36	156.7	LOQ	34.9	LOQ	36.4	20.9
ST	39	42.6	ND	ND	ND	33.7	5.9
ST	42	278.3	ND	36.5	ND	28.2	27.2
ST	45	210.5	LOQ	LOQ	LOQ	44.8	17.3
Neg	30	72.8	ND	LOQ	ND	29.9	ND
Neg	33	91.6	ND	ND	LOQ	30.2	35.2
Neg	36	213.6	ND	57.6	LOQ	30.7	22.1
Neg	39	43.3	LOQ	ND	LOQ	32.1	9.9
Neg	42	94.8	ND	ND	ND	20.5	6.4
Neg	45	235.9	ND	ND	ND	LOQ	ND