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# Polyunsaturated fatty acids influence differential biosynthesis of oxylipids and other lipid mediators during bovine coliform mastitis

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## ABSTRACT

Coliform mastitis is a severe and sometimes fatal disease characterized by an unregulated inflammatory response. The initiation, progression, and resolution of inflammatory responses are regulated, in part, by potent oxylipid metabolites derived from polyunsaturated fatty acids. The purpose of this study was to characterize the biosynthesis and diversity of oxylipid metabolites during acute bovine coliform mastitis. Eleven cows diagnosed with naturally occurring acute systemic coliform mastitis and 13 healthy control cows, matched for lactation number and days in milk, were selected for comparison of oxylipid and free fatty acid concentrations in both milk and plasma. Oxylipids and free fatty acids were quantified using liquid chromatography-tandem mass spectrometry. All polyunsaturated fatty acids quantified in milk were elevated during coliform mastitis with linoleic acid being the most abundant. Oxylipids synthesized through the lipoxygenase and cytochrome P450 pathways accounted for the majority of the oxylipid biosynthesis. This study demonstrated a complex and diverse oxylipid network, most pronounced at the level of the mammary gland. Substrate availability, biosynthetic pathways, and degree of metabolism influence the biosynthesis of oxylipids during bovine coliform mastitis. Further studies are required to identify targets for novel interventions that modulate oxylipid biosynthesis during coliform mastitis to optimize inflammation.
**Key words:** inflammation, mastitis, oxylipid, lipids

## INTRODUCTION

Gram-negative bacteria are a predominant cause of clinical mastitis infections in dairy cows (Erskine et al., 1988; Oliveira et al., 2013). Whereas some infections are subclinical and self-limiting, a proportion of affected cows develop an acute and severe systemic disease resulting in significant decrease in milk production and sometimes death (Erskine et al., 1988; Hogan and Smith, 2003). Subclinical and self-limiting infections are associated with a controlled inflammatory response with a prompt return to normal tissue homeostasis and milk production (Vangroenweghe et al., 2005). Acute gram-negative mammary infections, however, are characterized by dysregulated inflammatory responses elicited by LPS (Sordillo et al., 2009). Binding of LPS to toll-like receptor 4 (**TLR4**) results in activation of nuclear factor κB (**NF-κB**) with subsequent transcription and translation of proteins important for inflammatory mediator biosynthesis. Potent phospholipid mediators crucial for onset and progression of inflammation following LPS exposure also are released including platelet activating factor and oxidized lipids known as oxylipids (Corl et al., 2008).

The biosynthesis of oxylipids is preceded by release of their PUFA precursors, including linoleic acid (**LA**), arachidonic acid (**ArA**), eicosapentaenoic acid (**EPA**), and docosahexaenoic acid (**DHA**) from the *sn*-2 esterification site in the phospholipid glycerol backbone (Rosenthal et al., 1995). The abundance of PUFA in phospholipids is dependent on and can be modified by dietary FA composition in several species (Calder, 2008; Childs et al., 2008). Metabolism of PUFA may occur in situ on esterified forms in phospholipids or, more commonly, following their release and availability as FFA in the cytosol (Kuhn et al., 2015). The release of PUFA as free or pre-oxygenated fatty acids from the *sn*-2 ester linkage is mediated by phospholipase enzymes (Murakami et al., 2011). Several classes of phospholipase enzymes are known, but the cytosolic $PLA_2$ (**cPLA$_2$**) is the predominant isoform class responsible for *sn*-2 ester linkage hydrolysis (Buczynski et al., 2009). The activation of $cPLA_2$ is mediated by an increase in intracellular calcium in response to several stimuli, including LPS from gram-negative bacteria (Rosenthal et al., 1995). Activation of other classes of

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lipases releases other bioactive lipid mediators from cell membrane phospholipids, including endocannabinoids (Zubrzycki et al., 2014a).

Regardless of PUFA type, oxygenation can occur through enzymatic pathways such as cyclooxygenase (**COX**), lipoxygenase (**LOX**), or cytochrome P450 (**CYP**) and nonenzymatic auto-oxidation mediated by some reactive metabolites (Buczynski et al., 2009). The COX enzymes are bifunctional, initially abstracting a hydrogen atom mediated by a tyrosine radical at the oxygenase site and followed by addition of dioxygen to generate prostaglandin (**PG**) $G_2$ (Wu et al., 2011). Then, $PGG_2$ is reduced at the peroxidase site of COX into the more stable $PGH_2$, which undergoes further metabolism by various prostaglandin synthases to a variety of bioactive prostanoids. Lipoxygenases utilize their non-heme $Fe^{2+}$ to form ferrous hydroxide, which subsequently abstracts a hydrogen atom followed by insertion of molecular oxygen, forming a peroxy radical that is further converted to peroxy fatty acids (Kuhn et al., 2015). Similarly, cytochrome enzymes use their non-heme $Fe^{2+}$ in mediating the oxidation, peroxidation, and hydroxylation of PUFA and some of the COX-derived prostaglandins including $PGE_2$ and $PGD_2$ (Spector et al., 2004).

The nonenzymatic oxidation of PUFA is primarily orchestrated by pro-oxidant metabolites such as reactive oxygen species (**ROS**) that include superoxide and hydrogen peroxide. Although physiologically important at low levels, ROS concentrations exceeding the cellular antioxidant capacity can induce oxidative stress where there is peroxidation of cell membrane PUFA (Sordillo et al., 2009). In dairy cows, the transition period is associated with oxidative stress related to increased ROS production by enhanced mitochondrial metabolism (Bernabucci et al., 2005). The induction of the oxidative burst mechanism used by leukocytes in killing bacteria is another major source of ROS during infections such as mastitis (Sordillo et al., 2009). The highly reactive unpaired electrons of ROS free radicals target the double bonds in PUFA and generate oxylipids, including hydroperoxy fatty acids and isoprostanes. Such auto-oxidation of PUFA differs from the enzymatic-derived pathways, by producing an assortment of both positional and enantiomeric isomeric products because of lack of specificity as exhibited by enzymes (Milne et al., 2011).

The initial oxygenation FA products from either enzymatic or nonenzymatic oxidation are subjected to further downstream metabolism to form a diverse network of oxylipids. Prostaglandin $H_2$ metabolites are converted to downstream prostaglandins by specific prostaglandin synthases. For example, specific prostaglandin synthases metabolize $PGH_2$ to prostaglandins $D_2$, $E_2$, $F_{2\alpha}$, and $I_2$. The LOX-derived peroxy FA are further converted into leukotrienes, lipoxins, resolvins, and protectins (Kuhn et al., 2015), whereas CYP-derived fatty acid epoxides are further converted to fatty acid diols by soluble epoxide hydrolases. At least 140 oxylipids have been characterized to date, most with potent regulatory effects on inflammation (Tam, 2013).

Oxylipid biosynthesis is complex and is regulated by available PUFA substrates, activation of oxygenation pathways, and the extent to which initial oxygenation products are metabolized. A broader understanding of oxylipid diversity, changes in patterns of oxylipid accumulation, and their direct effects on inflammatory responses during bovine mastitis is required to enable evaluation of efficacious intervention modalities that promote resolution of inflammation. The purpose of this study, therefore, was to investigate the biosynthesis of oxylipid metabolites during acute naturally occurring coliform mastitis infection in dairy cows. Based on previous studies documenting increased FFA during mastitis (Atroshi et al., 1989a,b), the hypothesis for this study was that oxylipid biosynthesis would be correlated with their FFA substrates in both milk and plasma during naturally occurring bovine coliform mastitis.

## MATERIALS AND METHODS

### *Chemicals*

Acetonitrile, methanol, and formic acid of liquid chromatography-mass spectrometry grade were purchased from Sigma-Aldrich (St. Louis, MO). Deuterated and nondeuterated oxylipid standards were purchased from Cayman Chemical (Ann Arbor, MI). Butylated hydroxy toluene was purchased from ACROS (Thermo Fisher, Fair Lawn, NJ), EDTA and triphenylphosphine were purchased from Sigma-Aldrich, and indomethacin was purchased from Cayman Chemical.

### *Animals*

This study was approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC) and cows were enrolled with client consent. The study was conducted at a commercial dairy operation in Michigan with about 3,300 lactating cows with an approximate rolling herd average milk production of 12,250 kg. Cows were housed in freestall barns and grouped according to lactation number, DIM, and milk yield. Cows were milked 2 times daily. Diets were formulated to meet the energy requirements based on production (Table 1) and feed was delivered 2 times/d as TMR.