# From Membrane Biophysics to the Farm: Applications of Fatty Acid and Monoglyceride Chemistry to Animal Health

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## Introduction

Antibiotics have long been used in the livestock industry for therapeutic purposes to prevent severe clinical disease and death [Boyd et al, 2019]. Antibiotics work against bacteria and different classes of antibiotics have distinct targeting spectrums and potencies [Gustafson and Bowen, 1997]. Certain antibiotics have also been and continue to be used for treating subclinical diseases [Dibner and Richards, 2005], and successful intervention can improve livestock growth and feed efficiency [Cromwell, 2002]. Mechanistically, antibiotics can affect bacterial cell integrity and reproduction, and thus exert growth-promoting effects by modulating the composition of bacterial populations within the gastrointestinal tract. They can also reduce bacterial pathogen levels throughout the body to prevent disease.

Despite these important capabilities, there are mounting concerns that antibiotic usage in food animals is contributing to the global problem of antibiotic-resistant bacteria across human and animal populations [Barton, 2014]. These concerns have led to calls to significantly reduce or stop the use of antibiotics in animals that are also used in human medicine, particularly in cases where they are used at sub-therapeutic levels to promote growth [Marshall and Levy, 2011]. Legislative bans on growth-promoting antibiotics have been passed in different parts of the world, such as the European Union [Cogliani, et al, 2011], while other regulatory actions, such as the revised Veterinary Feed Directive in the United States, are encouraging more judicious use of human medically relevant, therapeutic antibiotics in food animal production, including halting sub-therapeutic use of such antibiotics [Schulz, et al, 2017].

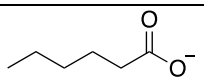
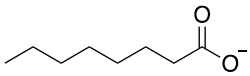
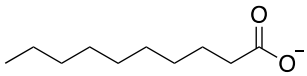
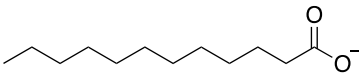
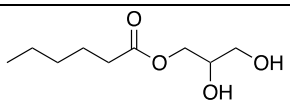
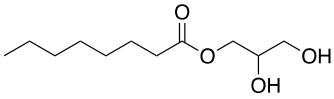
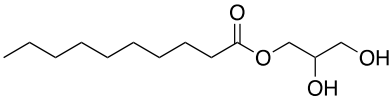
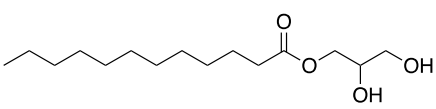
Concerns and legislative actions around antibiotic use in food animals are leading to an increase in the search for tools to counter pathogens. The increasing threat of viral pathogens that are foreign to North America, has also prompted the search for a means to directly destroy viruses. A recent addition to vaccines, therapeutic drugs, and immune enhancers is medium-chain fatty acids (MCFAs), especially saturated MCFAs with 6-12 carbon-long chains. They have proven benefits as feed additives by improving animal health, performance, and nutrient digestibility [Baltić, et al 2017]. Importantly, MCFAs and their monoglyceride derivatives (MCMGs) exhibit both antiviral and antibacterial activity [Thormar and Hilmarsson, 2997; Messens et al, 2010]. The combined health-promoting and pathogen-mitigating functions of MCFAs and MCMGs are particularly significant in light of the ongoing challenges of porcine epidemic diarrhea virus (PEDv), porcine reproductive and respiratory syndrome virus (PRRSv) and with the emerging threat of

African swine fever virus (ASFv) in pigs, as well as with the ongoing health issues of bovine coronavirus, bovine herpes virus 1 (BHV-1) responsible for Infectious bovine rhinotracheitis and bovine respiratory syncytial virus in calves. Formaldehyde is one common mitigant that is used to reduce the risk of these viruses and bacterial pathogens in feed, but it is under increasing regulatory pressure due to the hazards associated with its use and residue in feed. Over the past few years, there has been progress in understanding the mode of action of MCFAs and MCMGs as antivirals and antimicrobials and there is promise in both monogastric and ruminant production for their potential to replace antibiotics while improving animal health and productivity. In addition to multifunctional inhibitory activity against both viruses and bacteria, this class of antibiotic replacement candidates is additionally distinguished from the myriad of other candidates by an ability to be absorbed and transit to systemic sites of viral infection (Jackman et al., 2020). Replacement strategies must include this facet.

Physical Properties of MCFAs and MCMGs

MCFAs and MCMGs are single-chain lipid amphiphiles. An overview of the basic physical properties of important MCFAs and MCMGs is presented in Table 1.

Table 1. Overview of MCFAs and MCMGs

| | Compound Name (Molecular Formula) | Chemical Structure | Mol. Wt. (Da) | Melt. Point (°C) | CMC[1] (μM) | Smell[3] |
|---|---|---|---|---|---|---|
| Fatty Acids | Caproic Acid ($C_6H_{12}O_2$) |  | 116.2 | -3.4 | N.D.[2] | Strong |
| | Caprylic Acid ($C_8H_{16}O_2$) |  | 144.2 | 16.5 | N.D. | Mod. |
| | Capric Acid ($C_{10}H_{20}O_2$) |  | 172.3 | 31.6 | 3500 | Mild |
| | Lauric Acid ($C_{12}H_{24}O_2$) |  | 200.3 | 43.8 | 900 | Minor |
| Monoglycerides | Monocaproin ($C_9H_{18}O_4$) |  | 190.2 | 19.4 | N.D. | Minor |
| | Monocaprylin ($C_{11}H_{22}O_4$) |  | 218.3 | 35.6 | N.D. | Minor |
| | Monocaprin ($C_{13}H_{26}O_4$) |  | 246.3 | 51.4 | 600 | Minor |
| | Monolaurin ($C_{15}H_{30}O_4$) |  | 274.4 | 62.5 | 60 | Minor |

[1] CMC – Critical Micelle Concentration in μM units. [2]N.D. – Not Determined.
[3] Smell is qualitatively ranked on the order of strong, moderate, mild and minor.

Fatty acids are hydrocarbon chains and one end of the hydrocarbon chain has a carboxylic acid functional group with a $pK_a$ value around pH 5. Most fatty acid molecules are anionic (deprotonated) around neutral pH conditions, while they are mainly nonionic (protonated) in acidic pH environments such as the stomach. Fatty acids with saturated hydrocarbon chains are generally preferable to work with because saturated fatty acids are more chemically stable and less prone to oxidation-related rancidity. Comparatively, the hydroxyl groups of monoglycerides have very high $pK_a$ values (around 14), thereby remaining nonionic across physiologically relevant pH conditions and rendering them highly stable.

## Mechanisms of Action

MCFAs and MCMGs have unique mechanisms of disrupting phospholipid membranes and are principally active in the micellar state [Yoon et al, 2015; Kawakami et al, 2017; Yoon et al, 2019]. MCMGs form micelles at lower concentrations than MCFAs (see Table 1), which helps to explain why MCMGs are often more biologically potent than fatty acids and also why longer chain lengths exhibit more potent inhibitory activity than shorter ones within this group. For example, the $C_{12}$ monoglyceride (glycerol monolaurate, abbreviated as GML) has a lower critical micelle concentration (CMC) value (60 μM at pH 7.4) and typically greater potency than both the $C_{12}$ fatty acid (lauric acid; CMC of 900 μM at pH 7.4) and $C_{10}$ monoglyceride (glycerol monocaprate; CMC of 600 μM at pH 7.4) [Yoon et al, 2017; Valle-Gonzalez et al, 2018], see Figure 1 [Jackman et al, under review]. Another important consequence of MCFAs and MCMGs targeting pathogenic membranes is that it is more difficult for susceptible pathogens to develop resistance to these compounds. It is generally acknowledged that there is a very high barrier for pathogens to develop resistance to MCFAs and MCMGs [Desbois and Smith, 2010; Schlivert and Peterson, 2012].

MCFAs and MCMGs are antimicrobial agents that can disrupt the phospholipid membrane surrounding membrane-enclosed pathogens such as bacteria and enveloped viruses. In terms of antibacterial activity, the compounds can inhibit bacterial growth (“bacteriostatic”) through disruption of membrane electron transport and energy metabolism or through displacement of cell-surface membrane enzymes and receptors [Yoon et al, 2015]. They can also induce bacterial cell lysis and death (“bactericidal”) [Yoon et al, 2018]. In general, MCFAs and MCMGs exhibit more potent inhibitory activity against Gram-positive bacteria than Gram-negative bacteria. This can be partially explained by the fact that Gram-positive bacteria have simpler, single lipid bilayer cell membrane structures while Gram-negative ones typically have more complex inner and outer membrane structures.

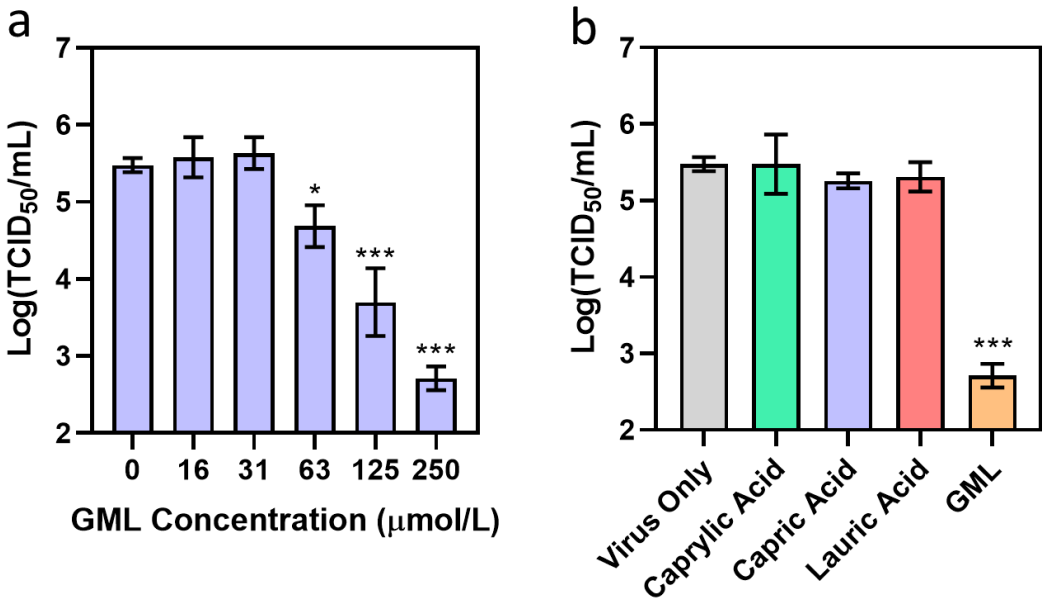



Figure 1. (a) Antiviral activity of GML against African swine fever virus at various concentrations above and below GML's critical micelle concentration of 60 μM. (b) Antiviral activity of selected MCFAs and GML at 250 μM. At 5 mM concentration (20x higher concentration), all MCFAs and GML demonstrated virucidal activity, yielding around 1.2 log reductions in viral infectivity (data not shown). [Jackman et al, 2020 under review]

Pioneering work completed by the Kabara group in the 1970's conducted detailed structure-function studies investigating how fatty acid chain length affects antimicrobial activity [Kabara et al, 172]. In general, capric ($C_{10}$) and lauric ($C_{12}$) acids exhibited the highest potencies among fatty acids while the corresponding monoglycerides with equivalent chain lengths were typically even more potent. Nevertheless, it is important to note that different MCFAs and MCMG inhibit different spectrums of pathogens with varying potencies, so appropriate selection is required depending on the pathogen(s) being targeted.

MCFAs and MCMGs can also disrupt a wide range of lipid bilayer-enveloped viruses by damaging and/or effectively destroying enveloped virus particles and compromising infectivity [Jackman et al, 2018]. MCFAs and MCMGs principally exhibit antiviral activity by lysing enveloped virus particles ("virucidal"). They can also disrupt viral protein structures which are required for fusion with host cells, viral replication and re-assembly and protection of the viral RNA or DNA, likely by destabilizing the lipid membranes which support these proteins. On the other hand, MCFAs and MCMGs are inactive against non-enveloped viruses. The list of viruses susceptible to MCFAs or MCMGs includes vesicular stomatitis, herpes simplex, visna, respiratory syncytial, parainfluenza type 2, avian influenza, and ASFv [Thormar et al, 1987; Hilmarsson et al, 2007; Hariastuti, 2011; Sola et al, 1986]. More recent studies demonstrated that they also exhibit strong antiviral activity against other swine-specific viral pathogens, such as

PRRSv and PEDv which contain lipid bilayer envelopes that are necessary for structural integrity and infectivity [Du et al, 2017; Lee, 2015].

## Membrane Biophysics

To date, the main scientific approach to study MCFAs and MCMGs has involved empirical testing in microbiology laboratories. The key questions asked by researchers have been whether certain MCFAs or MCMGs inhibit the virus or bacterium under investigation and, if so, how potent is the inhibitory activity? While researchers have long known that the inhibitory activity of MCFAs and MCMGs is associated with viral and bacterial membrane damage, it has proven far more challenging to understand why different MCFAs and MCMGs exhibit varying degrees of inhibitory activity.

One recent solution is an engineering platform called the supported lipid bilayer (SLB), which mimics the basic structural properties of biological membranes. Using SLB technology, researchers are now able to directly study the molecular-level interactions of MCFAs and MCMGs with lipid membranes [Kawakami, 2017]. SLB experimental capabilities revealed that MCFAs and MCMGs interact with lipid membranes in different ways depending on the molecular properties of the tested MCFAs or MCMGs, such as molecular length, shape, and charge. It is possible to conduct concentration-dependent experiments using SLB platforms and to rapidly determine the lowest concentration at which compounds exhibit membrane-disruptive activity. This is related to the CMC value of the compound and has further been shown to correlate with the minimum inhibitory concentration (MIC) value in antibacterial assays. These methods demonstrate the predictive power and efficiency of engineering technologies and will soon allow for the replication of the specific membrane properties from bacteria or viruses and testing of tailor-made mixtures of compounds to most effectively, and at the lowest concentrations, disrupt their functional properties.

## Anti-Inflammatory Activity

In addition to antimicrobial properties, certain MCFAs and MCMGs also exhibit immunomodulatory properties. For example, GML is known to affect immune cells, especially T cell lymphocytes, due to membrane interactions linked to cell signaling pathways [Zhang et al, 2018]. Zhang et al. demonstrated that GML treatment can also decrease cytokine production *in vitro* and thus GML exhibits immunosuppressive effects that can be useful for anti-inflammatory applications [Zhang et al 2016]. It was suggested that orally administered GML could be useful for reducing gut inflammation *in vivo*, while it has been demonstrated that vaginal applications of GML can reduce inflammation and infection in a Simian Immunodeficiency Virus (SIV) challenge study [Li et al, 2009]. Additional work that our team conducted with Dr. Barry Bradford's group demonstrated a common anti-inflammatory response among lauric acid and two of its derivatives, GML and lauric acid methyl ester. All forms demonstrated similar reductions in NFκβ expression, indicating lower inflammatory responses to a lipopolysaccharide (LPS) challenge in murine macrophages (Figure 2) ([Mamedova et at, 2019].

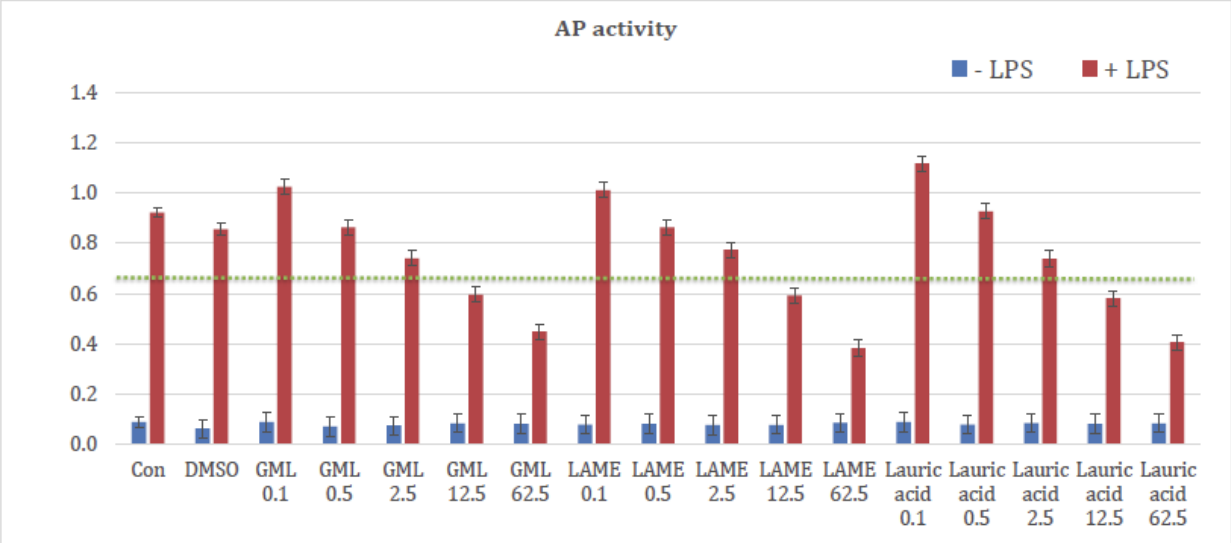



Figure 2. Response of NFκβ to increasing concentrations of lauric acid derivatives following an LPS challenge [Sivinski, et al. 2020].

## Growth Promotion

Concomitant with these various beneficial effects to reduce pathogen challenges and inflammation in animals, MCFAs and MCMGs have also been shown to consistently improve animal performance, presumably by preventing the endocrine constraint imposed by high immune stress (Spurlock, 1997) and influencing the balance of gut microbial populations. In a series of studies, Hanczakowska and colleagues investigated the effect of 0.2% caprylic acid and/or capric acid as antibiotic replacements and feed supplements in pigs. They evaluated how diet supplementation with caprylic and/or capric acids affects pig performance, apparent digestibility of nutrients, intestinal microflora, and structure of the ileum [ Hanczakowska et al, 2011]. Growth rate was higher ($P$<0.01) for pigs that received caprylic or capric acids or a combination thereof, along with decreased mortality and increased protein and fiber digestibility when compared to a control group without additive (neither MCFA nor antibiotic). In addition, *Clostridium perfringens* levels in the cecum and ileum were reduced by both fatty acids *(*$P$<0.01) along with increases in aerobic bacteria *(*$P$<0.05) and decreases in *Candida* spp. *(*$P$<0.05) and positive improvements in the structure of the mucosal epithelium in the ileum. Similar effects were observed when fumaric acid (1.5% of the diet) was mixed with either caprylic or capric acid (0.2% of the diet). Fumaric/caprylic acid supplementation led to the largest body weight gains; ADG was 276 g vs. 234 g in the untreated control group ($P$<0.01) [Hanczakowska et al, 2011]. All three fatty acid treatments led to significant decreases in *Escherichia coli* levels in the digesta collected from the small intestine relative to the untreated control group ($P$<0.01).

In more recent work, Gebhardt et al. evaluated MCFAs as a dietary additive in nursery pig diets [Gebhardt et al, 2020]. They tested a 1:1:1 blend of caproic, caprylic, and capric acids that were fed at 0, 0.25, 0.5, 1.0, and 1.5% of the diet. Linear dose-dependent improvements in ADG, ADFI, and FCR were noted ($P$<0.01). Cochrane et al.

also investigated whether MCFAs could be a useful alternative to the antibiotic chlortetracycline in nursery pig diets [Cochrane et al, 2018]. The pigs were challenged with enterotoxigenic *E. coli*, followed by a control diet without additive or one supplemented with (1) 400 g/ton chlortetracycline, or with 1% of the diet composed of an MCFA mixture that contained (2) a 1:1:1 blend of caproic, caprylic, and capric acids, (3) a 12:48:40 blend, or (4) a 4:54:38 blend of the same fatty acids. It was determined that *E. coli*-challenged pigs that received any of the MCFA-containing diets exhibited similar FCR values to those receiving the antibiotic-containing diet.

## Livestock Applications

### Feed Mitigation

When MCFAs and MCMGs are delivered as feed additives, they can also play an important role in feed pathogen mitigation by inhibiting infectious pathogens (viruses, bacteria), that might be present in the feed and remain viable in the feed matrix for extended periods of time [Dee et al, 2018]. In effect, MCFAs and MCMGs potentially decrease the concentration of infectious pathogens in feed and thereby reduce the probability that animals consuming such feed become infected.

A prominent example of a feed borne pathogen is PEDv. Dee et al. investigated the effectiveness of a 2% MCFA blend comprised of caproic, caprylic and capric acids (1:1:1 ratio) to inhibit PEDv contamination of various classes of swine feed ingredients [Dee et al, 2016]. It was determined that the MCFA blend reduced mean PEDv viral loads in the feed ingredients, as indicated by viral RNA concentrations (viral genome copies) relative to the levels found in the negative control groups treated only with saline solution ($P<0.05$). Subsequent inoculation of piglets with PEDv-contaminated ingredients caused infection, as indicated by detectable PEDv in the small intestine, viral shedding in feces, mild diarrhea, and anatomical changes. By contrast, all piglets inoculated with MCFA-treated, PEDV-contaminated feed ingredients showed no evidence of PEDv infection and the MCFA blend performed equally as well as formaldehyde in the piglet inoculation studies. In an *in vitro* study our team conducted with Dr. Lorin Warnick's group, in which pig feed contained varying levels of GML, 0 to 2% wt/wt, was inoculated with a multi-drug-resistant strain of *Salmonella typhimurium*, and demonstrated a dose-dependent reduction in viable Salmonella after 24 hours of incubation (Figure 3).

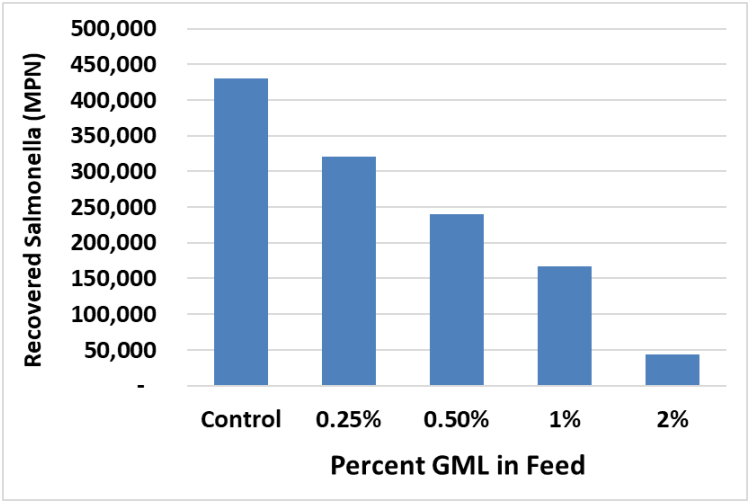



Figure 3. Salmonella (MPN) recovered from feed containing GML and spiked with *Salmonella*. (Warnick and Elrod, unpublished)

Cochrane and co-workers have also systematically studied the mitigating effects of individual MCFAs and combinations thereof on PEDv-contaminated feed samples [Cochrane, 2019]. Test samples included 1% MCFA blend [caproic, caprylic, and capric acids; 1:1:1 ratio] (aerosolized), 1% MCFA blend [caproic, caprylic, and capric acids; 1:1:1 ratio] (non-aerosolized), 0.66% caproic acid, 0.66% caprylic acid, 0.66% capric acid, 0.66% lauric acid, and 1% capric and lauric acid mixture (1:1 ratio). It was determined that the 1% MCFA blends inhibited PEDv to the greatest extent along with caproic, caprylic, and capric acids alone to varying extents ($P<0.05$). This feed pathogen mitigation strategy also protected pigs, who consumed PEDv-contaminated feed, against infection, as indicated by the lack of PEDv in fecal swabs and cecum content.

In related work, which we conducted with collaborators at the Armenian National Academy of Sciences, varying levels of an MCFA mixture and GML (from 0 to 2% wt/wt) were added to pig feed and then spiked with ASFv. At 30 minutes and 24 hours post-inoculation, samples were taken and assayed for viral infectivity, viral DNA and conformationally intact p72 capsid protein. Only GML, at 2%, reduced ($P<0.01$) the infectivity of ASFv at both 30 minutes and 24 hours, though there was a tendency demonstrated at 1% as well. There was no effect of any treatment on the presence of intact viral DNA, which is consistent with the double-membrane envelope structure of ASFv that protects inner genetic material and is markedly more robust than the typical single-membrane structure of other enveloped viruses. Lastly, there was a dose-dependent decline in conformationally intact p72 only in the GML-treated feed samples (Figure 4) [Jackman et al, under review]. This protein is the major capsid protein of ASFv and its conformational change is consistent with GML-induced virus particle disruption, especially since p72 is anchored to viral lipid membranes. As such, disrupting viral lipid envelopes can also impair membrane-associated proteins as well, which underscores the multifunctional impact of GML as a feed additive.

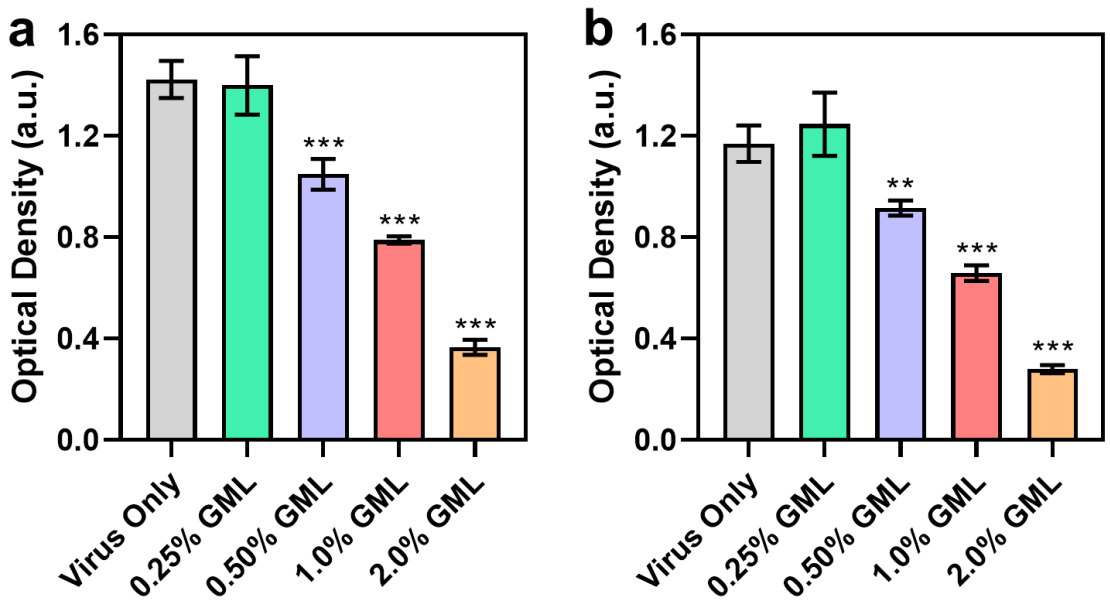



Figure 4. Effect of GML concentration in pig feed on the presence of structurally intact p72 capsid protein, as measured by ELISA, at 30 minutes (a) and 24 hours (b) post-inoculation (Jackman et al, under review).

Altogether, these findings demonstrate that MCFAs and MCMGs can serve as the basis for an effective and natural feed pathogen mitigation strategy. Importantly, their application should be investigated beyond monogastric animals as a potential mitigator of viral and bacterial challenges in pre-ruminants.

Delivery Strategies

Clearly the easiest means to deliver such treatments is via feed. However, when acute disease outbreaks occur, waiting for the next load of feed, or top-dressing treatments onto feed, are not always practical. In these instances, a drinking water or milk-deliverable treatment is the preferred method since it can be rapidly deployed in the initial stages of a disease outbreak. Even when animals go off feed, they typically continue to consume water so this approach would be highly advantageous.

## Conclusion

There is tremendous potential for MCFAs and MCMGs as feed additives in livestock production. Continued translation of molecular-level insights into engineered feed additive mixtures might enable the development of precision formulations with varying levels of membrane-damaging activity and pathogen targeting scope. At the same time, further investigation of the virus-killing mechanism of MCFAs and MCMGs in feed and in animals is warranted. Looking forward, this interdisciplinary approach to explore MCFAs and MCMGs as feed additives is highly relevant to a wide range of viral and

bacterial diseases in livestock production and could be extended across many animal species.

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