

Development and evaluation of emulsion-based adjuvants for vaccines against *Streptococcus suis* and classical swine fever virus in swine

by

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Abstract

Streptococcus suis and Classical Swine Fever pose significant threats to swine health worldwide, causing substantial economic losses in the pork industry. Effective vaccination is one of the most reliable strategies to control and prevent these infections. Adjuvants enhance immune responses, improve vaccine efficacy, and enable dose sparing, making them essential for large-scale immunization. Among various adjuvant types, emulsion-based systems are especially attractive due to their ability to promote strong humoral and cellular immune responses, extend antigen availability at the injection site, and support safe and scalable formulations.

This thesis focuses on developing and evaluating physically stable emulsion adjuvants suitable for swine vaccines. Multiple adjuvants based on different synthetic paraffin oils in conjunction with a surfactant were used to produce the emulsion at the nanoscale. Key physiological properties like droplet size, polydispersity, zeta potential, and emulsion stability were systematically analyzed. The surfactant ratio was optimized to produce nanoscale droplets and ensure stable emulsion for veterinary use.

OW-14 (previously established adjuvant) and OW-415 (experimental adjuvant); When co-administered with a subunit protein antigen and inactivated bacteria induced high levels of immune responses in pigs. Cytokine profiling at Days 7 and 28 post-vaccination revealed balanced systemic immune activation, with OW-415 showing modest elevations in IFN- α and IL-10, indicating potential immunomodulatory effects. These findings support the promise of OW-415 as safe, immunogenic, and physically stable adjuvants for swine vaccine development.

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Dedication

To my parents, Rabindra Thapa and Rita Thapa, and my brother, Reemon Thapa,
Everything I am today is because of your love. I hope I've made you proud.

To my husband, Ankit,
My heart, my home, and my greatest support, I couldn't have done this without you.

Chapter 1 - Introduction

Vaccination and adjuvant

Vaccination remains one of the most powerful tools in preventing and controlling infectious diseases in human and veterinary medicine. Vaccines elicit protective immunity by presenting immunogenic components to the host immune system without causing disease, mimicking natural infection while minimizing pathogenic risk (Meeusen et al., 2007). The term “Vaccine” was first coined by Edward Jenner in the eighteenth century, and later, in the late 1800s, Louis Pasteur made the first classical vaccine, which helped eradicate smallpox in humans and rinderpest in bovines (Aida et al., 2021; Nooraei et al., 2023). Under optimal conditions, a vaccine should trigger both innate and adaptive immune responses. However, different vaccine platforms present varying degrees of immunogenicity and safety, necessitating the incorporation of immunologic enhancers known as adjuvants (Clem, 2011).

Various vaccine strategies are currently available, with the most traditional being live attenuated vaccines, subunit vaccines, toxoid vaccines, and inactivated whole organism vaccines (Nooraei et al., 2023). While traditional vaccines, such as live attenuated, inactivated, toxoid, and subunit vaccines, have been widely used, newer formulations emphasizing purity and safety exhibit reduced inherent immunogenicity. Consequently, adjuvants play a critical role by enhancing antigen uptake, activation of antigen-presenting cells (APCs), and stimulation of cytokine production, thereby promoting robust humoral and cellular responses (Z.-B. Wang & Xu, 2020). One of the most widely used adjuvants, aluminum salts (Alum), effectively promotes Th2-biased antibody responses but offers limited capacity to induce strong cell-mediated immunity.

Therefore, there is a growing need for alternative adjuvant systems capable of eliciting more balanced or Th1/Th2 mixed immune responses, particularly for challenging pathogens.

In global swine production, *Streptococcus suis* (*S. Suis*) and Classical Swine Fever Virus (CSFV) remain among the most economically and clinically significant pathogens, contributing to severe morbidity, mortality, and trade restrictions (de Oliveira et al., 2020; Segura et al., 2020). Vaccination represents a cornerstone strategy for controlling these diseases, especially amid rising global restrictions on the prophylactic use of antimicrobials in livestock systems. However, existing vaccines have critical limitations.

Vaccine development for *S. Suis* presents significant challenges due to its Complex serotype diversity, antigenic variation, and polysaccharide nature (Kralova et al., 2022). Autogenous bacterins provide strain-specific protection but are often ineffective, costly, and challenging to scale across diverse production. Meanwhile, control of CSFV relies heavily on live attenuated vaccines, which, although efficacious, carry risks associated with viral persistence and cannot be easily distinguished serologically from wild-type infection. One promising approach is the development of emulsion-based adjuvants, which serve not only as antigen delivery systems but also as immune potentiators. Emulsions comprise an oil phase dispersed in an aqueous phase, enhancing antigen presentation, prolonging antigen release, and activating innate immune pathways through pattern recognition receptor (PRR) signaling.

Emulsions, which are typically composed of an oil phase and an aqueous phase, have shown significant potential as delivery systems for antigens and adjuvants (O'Hagan et al., 2021). Chapter 2 explores the formulation, stability, and physicochemical characterization of emulsion adjuvants. Chapter 3 provides a comprehensive overview of the pathogenesis and immunological

implications of *S. Suis* and CSFV. Chapter 4 details the in vivo evaluation of vaccine formulations in pigs, employing ELISA, virus neutralization assays, and cytokine profiling to assess both humoral and cellular responses. This chapter also explores the potential synergistic effects of the adjuvants and their impact on immune protection against both *S. Suis* and CSFV.

The central hypothesis suggests that emulsion-based adjuvants, OW-14 and OW-415, will significantly enhance the immunogenicity of vaccines against *S. Suis* and CSFV. This enhancement is expected to manifest through the induction of stronger and more balanced humoral and cellular immune responses compared to vaccines formulated with antigen alone or traditional adjuvants.

Background

Adjuvants used in veterinary medicine

In the 1920s, French veterinarian and immunologist Gaston Ramon discovered the concept of adjuvanticity while studying the vaccines for diphtheria and tetanus. He observed that certain compounds like calcium, starch, saponin, and vegetable oil induced heightened local inflammation at the injection site when delivered with a vaccine. These inflammatory responses were correlated with a rise in antibody production, leading to the idea of an "adjuvant" to enhance vaccine effectiveness (Burakova, Madera, McVey, et al., 2018). His findings laid the groundwork for developing modern adjuvants like aluminum salts (alum), which are commonly used in vaccines for humans and animals. Recently, a range of organic, inorganic, natural, and synthetic substances has been demonstrated to enhance the immune response and exhibit properties of adjuvanticity (Fox et al., 2011; Podda, 2001; Vogel & Powell, 1995; Wilkins et al., 2017; Yang et al., 2024).

Vaccine adjuvants are generally classified based on their mechanism of action and physiological characteristics into two main categories: delivery systems and immunostimulants (Facciola et al., 2022; Singh & O'Hagan, 2003). Delivery systems such as mineral salts, emulsions, and microparticles function primarily as carriers, ensuring efficient antigen presentation while concurrently inducing a localized inflammatory environment at the injection site. Local inflammatory response is initiated through activation of pattern recognition receptors (PRRs) expressed on innate immune cells, such as macrophages and dendritic cells, leading to the secretion of proinflammatory cytokines and chemokines. These mediators recruit other immune cells to the injection site, including antigen-presenting cells (APCs). APCs mediate antigen uptake and break down the antigen into peptide fragments for presentation on major histocompatibility complex (MHC) molecules at the cell surface. This enables recognizing and activating naïve T cells to

initiate the adaptive immune response. This interaction activates both CD4⁺ helper T cells and CD8⁺ cytotoxic T cells. Importantly, some activated T and B cells differentiate into long-lived memory cells, which allow the immune system to mount a faster and more effective response upon subsequent exposures (Pashine et al., 2005). Similar immune-enhancing mechanisms have been observed with immunostimulants such as Toll-like receptor (TLR) agonists, including TLR1, TLR4 ligand and saponins, which directly stimulate innate immune pathways and amplify overall immune activation (Shah et al., 2017).

An example, L-pampo, a combination adjuvant composed of Pam3CSK4 (a TLR1/2 ligand) and polyI: C (a synthetic TLR3 ligand), induced a stronger humoral immune response against hepatitis B virus (HBV) than aluminum hydroxide (Alum), enhancing cell-mediated immunity by promoting the presence of multifunctional CD4⁺ T cells (Lee et al., 2016). Another class of adjuvants, mucosal adjuvants, has a similar function to the previously described adjuvants; it eases antigen uptake by antigen-presenting cells (APCs). Unlike many systemic adjuvants, mucosal adjuvants specifically enhance immune responses at mucosal surfaces, promoting IgA production and mucosal-associated lymphoid tissue (MALT) activation (Lamichhane et al., 2014).

Adjuvants help increase seroconversion rates in individuals with weakened immune responses due to factors such as age, illness, or medical treatments. For instance, MF59, an oil-in-water adjuvant, has been used in influenza vaccines to enhance immune responses in elderly individuals (Podda, 2001). Furthermore, another key role of adjuvants is their ability to reduce the required vaccine antigen dose, and the number of booster doses needed to achieve protective immunity (Boyle et al., 2007; Facciola et al., 2022). However, the safety of the adjuvants varies from one species to another. Incomplete Freund's adjuvant (IFA) has been shown to induce tumors

in male Swiss mice, but long-term human studies have shown no increased risk of cancer in vaccinated individuals (Murray et al., 1972; Stuart-Harris, 1969). Despite its effectiveness, safety concerns based on animal data have limited its use in human vaccines (Stuart-Harris, 1969). Compared to human vaccines, animal vaccines receive fewer resources due to financial and regulatory constraints. Human vaccines require extensive safety testing and long-term studies, while animal vaccines prioritize cost-effectiveness and practicality (Carpenter et al., 2022; Knight-Jones et al., 2014; Monath, 2013).

Table 1.1: Adjuvant types used in commercial vaccines

Adjuvant Type	Active ingredient(s)	Vaccine Application
Alum (Aluminum Salts)	Hydroxide, Phosphate, or Hydroxy-phosphate Sulfate	Influenza (H5N1); DTaP, Hepatitis A; Hepatitis B
AS04	MPL (TLR4 Agonist) adsorbed onto Alum	Hepatitis B; HPV-16/18
Virosomes	Dispersed lipid vesicles with viral membrane proteins	Hepatitis A; Influenza (Seasonal)
Montanide® ISA51	Oil, non-ionic surfactants, and stabilizers	Cancer
Synthetic MPL	Naturally derived TLR4 ligand	Hepatitis B
O/W emulsions	Squalene, non-ionic surfactants, Gum Arabic, and other potential oils and stabilizers	Influenza (H1N1); Influenza (H5N1)
AS03	α -Tocopherol (Vitamin E), Squalene	Influenza (H5N1)
Freund's adjuvant	Paraffin Oil; Lanolin	<i>Mycobacterium tuberculosis</i>

Emulsions

Emulsions are combinations of two or more immiscible liquids stabilized by a surfactant. The two main classes of emulsions are oil-in-water (O/W) and water-in-oil (W/O) emulsions. In oil-in-water emulsions, oil serves as a dispersed phase within the continuous water phase, whereas in water-in-oil emulsions, the water phase is dispersed within the continuous oil phase (O'Hagan et al., 2021).

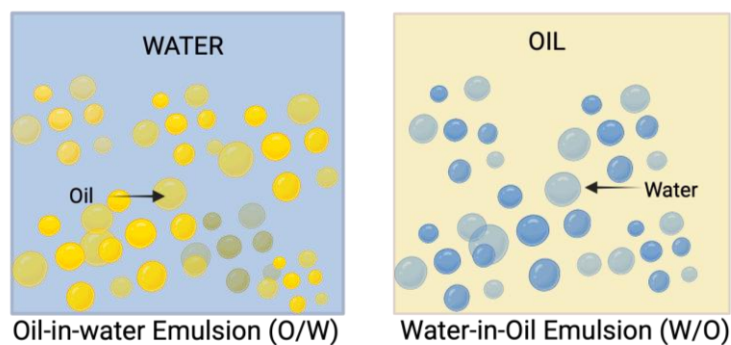


Figure 1.1: Two types of emulsion, oil-in-water, and water-in-oil emulsion (Created with BioRender.com)

From a thermodynamic standpoint, emulsions are inherently unstable and are prone to degradation over time through several physicochemical processes: Gravitational separation, flocculation, coalescence, particle aggregation, Ostwald ripening, and phase separation. During the encapsulation process, single emulsions typically show instability and are limited in their ability to encapsulate water-soluble substances. In contrast, double emulsions, such as water-in-oil-in-water (w/o/w) or oil-in-water-in-oil (o/w/o), provide a more versatile approach, as they can encapsulate both hydrophilic and hydrophobic compounds. These emulsions are inherently more

complex systems wherein the droplets of the dispersed phase contain one or more smaller dispersed phases within them (A. Kumar et al., 2022; Tyowua et al., 2017).

Emulsion stability problems

Conventional emulsions systems typically have droplet sizes ranging from 0.1 to 100 μm (McClements, 2004; Shi et al., 2024). These systems are prone to degrade over time and are thermodynamically unstable (Fig.1.2). Understanding these mechanisms is therefore essential in making a stable emulsion. Creaming and sedimentation occur when external forces, like gravity or centrifugal force, exceed thermal motion (Brownian motion), causing larger droplets to move toward the top or bottom of the container, creating a concentration gradient (Angardi et al., 2022). Coalescence is the merging of droplets as the liquid film between them thins, potentially leading to phase separation (McClements & Jafari, 2018). Flocculation (similar to coalescence) is the result of van der Waals attraction, a universal force that acts on all dispersed systems; it causes the aggregation of emulsion droplets that form a large cluster without mixing (Ravera et al., 2021). During this process, an interfacial film of the continuous phase remains between the adjacent droplets. Over time, the liquid film between two droplets becomes thinner, reaching its lowest stable size, typically 50-100nm, which determines whether the two droplets will fuse (Coalescence) or loosely cluster together (Flocculation). Therefore, the stability of these ultra-thin liquid films is critical in emulsion aging and linked to the stability characteristics of the emulsion (Ravera et al., 2021).

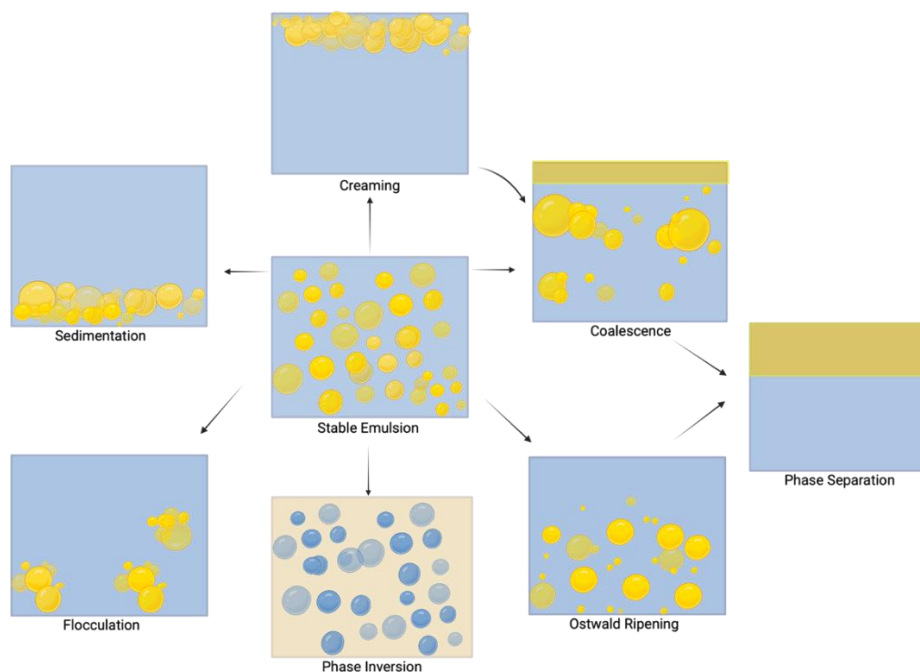


Figure 1.2: Schematic illustration of emulsion breakdown processes (Created with BioRender.com)

Ostwald Ripening, the system naturally moves toward a stable state by reducing the energy at the droplet surface. In this phenomenon, droplets with a small diameter tend to dissolve in the continuous phase and then redeposit onto the larger droplets (Kim et al., 2024). The various destabilization mechanisms are closely interlinked with one another throughout the aging process. Lastly, Phase inversion occurs when the two phases (dispersed and continuous) exchange, such as an oil-in-water emulsion, turns into a water-in-oil emulsion (Tadros, 2013).

Emulsion formulation

The emulsion can be prepared using either low-energy or high-energy emulsification techniques (Gonçalves et al., 2018; Jafari et al., 2005; Solans & Solé, 2012).

The nano emulsions (droplet sizes usually less than 100 nm) are often prepared using high-energy methods including high-speed homogenization, high-pressure homogenization and ultrasonication (Gonçalves et al., 2018; McClements & Jafari, 2018; Mehrnia et al., 2016). To formulate nano emulsions, mechanical energy is used to apply intense forces that break up larger droplets into nanometer-sized droplets (M. Kumar et al., 2019). High-pressure and high-speed homogenization are two techniques often used for emulsion preparation, especially in food and pharmaceutical applications. High-pressure homogenizers are designed to process liquid samples under intense pressure through a narrow valve and interaction chamber containing microchannels. The combined effects of high shear forces, sudden pressure drops, and cavitation within these microstructures lead to the effective disruption of large droplets into fine and uniform nano-sized droplets, often below 1.0 μm . This results in the formation of stable emulsions with improved physical properties (McClements & Jafari, 2018; Rai et al., 2018; Santana et al., 2013).

In high-speed homogenizers, rotor-stator mixers are commonly used in both industrial and laboratory settings to prepare emulsions. These devices operate at high rotation speeds, typically ranging from 1,000 to 25,000 rpm. As the rotor spins, it generates lower pressure, causing the liquid to circulate and interact within the narrow gap between the rotor and stator. This shear force leads to the formation of coarse emulsions, with droplet sizes that can be reduced to as low as 1.0 μm (Santana et al., 2013). Ultrasonic emulsification relies on the phenomenon of cavitation, where acoustic waves cause the formation and collapse of microbubbles, generating intense turbulence that breaks larger droplets into nano-sized ones (Canselier et al., 2002; Jayasooriya et al., 2004).

Low-energy emulsification processes, which include phase inversion and spontaneous emulsification (SE), rely on the chemical energy of the system and require minimal mechanical

agitation. These methods typically depend on specific changes in temperature or composition to facilitate the formation of microemulsions with droplet diameters ranging from 5 to 50 nm (McClements & Jafari, 2018; Solans & Solé, 2012). In spontaneous emulsification, the dispersed phase is mixed with a surfactant of high affinity to the continuous phase. The surfactant molecules rapidly diffuse from the dispersed to the continuous phase to reduce interfacial tension between the two phases. This results in the formation of nano-sized emulsion droplets, without changing the spontaneous curvature of the surfactant (M. Kumar et al., 2019; Mehrnia et al., 2016).

In contrast, phase inversion involves a change in the spontaneous curvature of the surfactant, where surfactant molecules migrate from the oil (dispersed) phase into the water (continuous) phase. Transitional phase inversion occurs when changes in parameters such as temperature or composition lead to a shift in the surfactant's affinity for the phases or a change in its spontaneous curvature, triggering the inversion process (Mehrnia et al., 2016; Solé et al., 2010).

Oils used in emulsions.

Non-polar lipids play an important role in the formulation of pharmaceutical emulsions, primarily functioning as the oil phase responsible for solubilizing and delivering lipophilic active ingredients. Commonly used non-polar lipids include triacylglycerols (TAGs), diacylglycerols (DAGs), monoacylglycerols (MAGs), free fatty acids, mineral oils, essential oils, and various lipophilic nutraceuticals (McClements & Rao, 2011). Due to their highly hydrophobic nature, these lipids effectively encapsulate poorly water-soluble drugs, which can enhance both the chemical stability and bioavailability of the active compounds (Khanum & Thevanayagam, 2017).

Nevertheless, incorporating non-polar lipids into nano and microemulsion systems presents several formulation challenges. The high viscosity, low polarity, and the presence of long-chain

fatty acids can significantly increase interfacial tension, making it more difficult to produce uniform, sub-100 nm droplets necessary for forming stable nanoemulsions (Driscoll, 2017; McClements & Rao, 2011; Wooster et al., 2008). To address these challenges, high-energy methods like high-pressure homogenization and microfluidization are used to reduce droplet size, improving emulsion stability and drug delivery efficiency (Bhangde et al., 2024; Fox et al., 2011; McClements & Rao, 2011).

Adjuvant oils are classified into two primary types: petroleum-based and plant-based oils. Petroleum-based oils, such as mineral oils, are commonly used in pharmaceutical emulsions due to their longevity and chemical stability. These oils are particularly effective in forming water-in-oil (W/O) emulsions, which serve as antigen depots at the injection site, enabling a slow release of the antigen and facilitating a prolonged immune response (Rachel, 2024). One example of a widely used mineral oil is Penreco Drakeol 5, a pharmaceutical-grade oil often used to formulate oil-in-water (O/W) emulsions (Galliher-Beckley et al., 2015). Several commercially successful W/O emulsions (e.g., Montanide™ adjuvants, Incomplete Seppic Adjuvants (ISA)) are formulated with either squalene (a plant-based oil) or mineral oils. These emulsions have been effectively used in vaccines for diseases like Newcastle disease in chickens and subunit vaccines targeting *Mycoplasma hyopneumoniae* in pigs (Arous et al., 2013; Galliher-Beckley et al., 2015; Jorge et al., 2014).

In recent years, GTL (gas-to-liquid) synthetic paraffin oil has emerged as a promising alternative to traditional mineral oils. GTL oil has many potential advantages, particularly its superior retention profile. Unlike conventional mineral oils, GTL synthetic paraffin oil

demonstrates lower absorption and retention in the liver and other tissues, which reduces the risk of long-term toxicity (Carrillo, Kamelia, et al., 2022; Carrillo, Shen, et al., 2022).

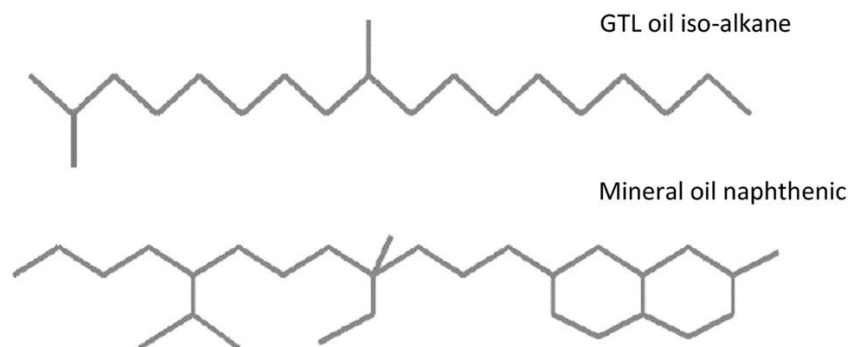


Figure 1.3: Chemical Structure of GTL oil vs Mineral oil naphthenic from (Carrillo, Shen, et al., 2022)

Hydrophilic-lipophilic balance and surfactants for emulsion preparation

To fully understand the function of emulsions and surfactants in vaccine adjuvant systems, it is important to first explore the concept of the hydrophilic-lipophilic balance (HLB). HLB serves as a predictive tool to evaluate the behavior of surfactants within emulsion systems. The HLB scale numerically expresses the relative tendency of a surfactant molecule to associate with either aqueous or oily phases. Surfactants are amphiphilic, meaning they possess both hydrophilic and lipophilic regions within their molecular structure (Tadros, 2013). Even at low concentrations, surfactants tend to accumulate at the interface between two immiscible phases and significantly reduce the surface tension. The greater the chemical difference between the two phases, the higher the interfacial tension (Rosen & Kunjappu, 2012).

Surfactants are categorized into several types, each playing a specific role in stabilizing emulsions. Non-ionic surfactants do not carry an electrical charge and are commonly used to prevent coalescence and flocculation. They stabilize emulsions through steric hindrance, forming a protective molecular layer around dispersed droplets and reducing aggregation. Adjuvant MF59

is formulated with Tween 80 and Span 85, both nonionic surfactants that contribute to its stability (Ko & Kang, 2018a). Another example is saponin, a plant-derived nonionic surfactant extensively used in cosmetics, the food industry, and the vaccine industry. Ou et al. developed a modular saponin-based nanoparticle (SNP) platform incorporating Toll-like receptor (TLR) agonists, including TLR1/2a, TLR4a, and TLR7/8a to evaluate their adjuvant potential. This platform induced cytokine production and immune signaling pathways, promoting T helper cell responses (Ou et al., 2024).

Polymeric surfactants, often referred to as non-ionic polymers, are larger and require more mechanical energy (stirring or homogenization) to form a stable emulsion. On the other hand, ionic surfactants carry either a positive or negative charge, which affects emulsion stability through electrostatic interactions. For example, sodium dodecyl sulfate (SDS), a common anionic surfactant, releases negatively charged molecules in water, and the electrostatic repulsion between droplets reduces the ability of droplets to coalesce, therefore improving long-term stability (Tadros, 2013). Furthermore, natural emulsifiers like Gum Arabic (a hydrocolloid) are widely used in both beverages and animal vaccine adjuvants. Gum Arabic's association with arabinogalactan protein (AGP) aids in droplet dispersion, significantly improving emulsion stability (Madera et al., 2022; Reiner et al., 2010).

Galliher-Beckley et al. demonstrated that the OW-14, an oil-in-water emulsion, formulated using food-grade emulsifiers, elicited a strong and long-lasting antibody response against swine influenza and *Mycoplasma hyopneumoniae* antigens (Galliher-Beckley et al., 2015). It even outperformed commercial vaccines such as FluSure® and RespiSure® in terms of immunogenicity. These findings highlight the importance of carefully selecting and optimizing the

type and concentration of surfactants used in emulsion formulations. Inadequate formulation compromises physical stability and increases the risk of undesirable biological effects, especially in sensitive applications such as vaccine development.

Mechanism of adjuvant action

Despite the extensive use of adjuvants for decades, the exact mechanisms through which they function remain unclear. However, recent studies have provided valuable insights into their underlying actions (Awate et al., 2013a; Billiau & Matthys, 2001). These studies suggest that adjuvants are crucial for initiating and amplifying immune responses to an appropriate level. Adjuvants primarily function by upregulating chemokines and cytokines through the sustained release of antigens at the injection site. Hence, triggering cellular recruitment and facilitating the uptake of the antigen by antigen-presenting cells (APCs). Following antigen capture, APCs undergo activation and maturation, which is characterized by an increased expression of major histocompatibility complex (MHC) II and co-stimulatory molecules (e.g., CD80, CD40, CD86). These changes enhance antigen processing and presentation. Following the maturation of the APCs, they migrate to draining lymph nodes to interact with antigen-specific B and T lymphocytes, leading to the activation of potent antibody production or cytokine secretion (e.g., IL-6, IL-12, IL-10, etc.). This cascade of events ultimately enhances the magnitude and specificity of both the cellular and humoral arms of immunity (Awate et al., 2013b; Garçon et al., 2011; Huang et al., 2024).

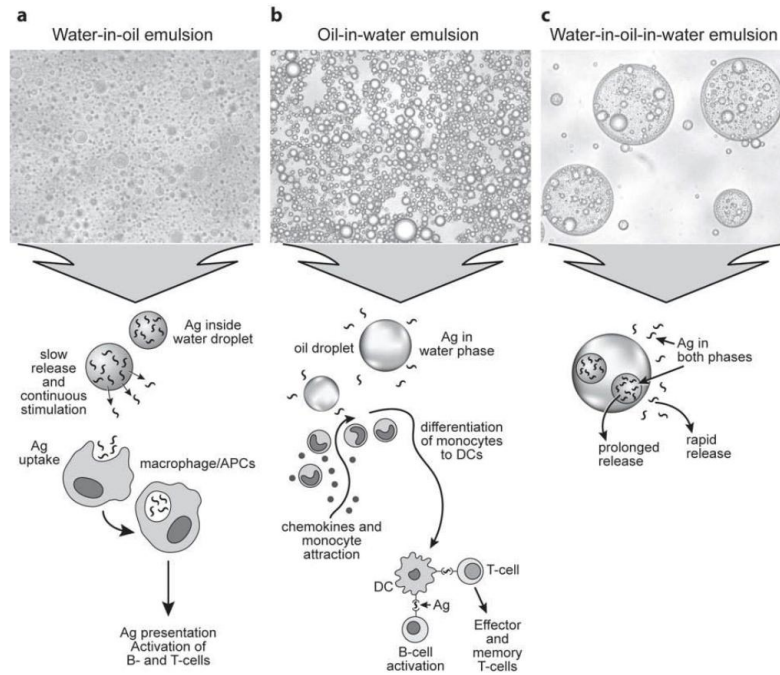


Figure 1.4: Emulsions and their mechanism of action as adjuvants from (Burakova, Madera, McVey, et al., 2018)

Mosca et al. used genome-wide microarray analysis to identify a set of genes commonly regulated by adjuvants, including alum. and MF59, at the injection site (Mosca et al., 2008). They found that this cluster includes genes involved in cytokine and chemokine production, innate immune receptors, interferon-induced genes, and adhesion molecules. Like PAMPs (pathogen-associated molecular patterns) and DAMPs (damage-associated molecular patterns), immunostimulants act as danger signal molecules. Immunostimulants bind to specific pattern recognition receptors (PRRs) on immune cells, triggering a cascade of signals that enhance the ability of antigen-presenting cells (APCs) to present antigens and provide the necessary co-stimulatory signals. This interaction leads to a stronger and more efficient adaptive immune response, helping the body recognize and respond effectively to potential threats (Zhao et al., 2023).

Emulsion as vaccine adjuvant

Emulsions are biphasic delivery system adjuvants and have been extensively utilized in veterinary science owing to their capacity to enhance adaptive immune responses, facilitate rapid immunity, and reduce the required antigen dose. Emulsion adjuvants have been part of licensed vaccines for over two decades, and more than 100 million doses have been delivered in over 30 countries, establishing their solid clinical effectiveness and safety (O'Hagan et al., 2021).

The two earliest emulsions, designed as adjuvants, are Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA), which is a mineral oil-based (W/O) emulsion adjuvant. CFA comprises mineral oil with an emulsifier and inactivated *Mycobacterium tuberculosis*, eliciting a strong and prolonged local inflammatory response. However, the use of CFA has been associated with several side effects, such as the formation of ulcers at the injection site and systemic effects such as weight loss and leukocytosis (Navarro-Alvarez et al., 2018). IFA, by contrast, consists of mineral oil and an emulsifier but lacks *Mycobacterium tuberculosis*. Its effectiveness is based on its ability to act as an oily antigen depot, allowing the gradual release of the antigen at the injection site (Billiau & Matthys, 2001). Despite these differences, studies have shown that CFA can stimulate both Th1 and Th2 responses, leading to the secretion of key cytokines such as IL-4, IFN- γ , and IL-13, which are critical for immune activation (Brunner et al., 2010).

Modified versions of IFA (e.g., Montanide ISA720) have demonstrated a more robust humoral response compared to the alum-derived adjuvant, Alhydrogel (B. S. Graham et al., 2010). Additionally, Montanide™ ISA 61 VG, when used in the formulation of a *Streptococcus suis* bacterin vaccine, provided significant protection against homologous challenge and reduced

morbidity in piglets, outperforming adjuvants such as aluminum hydroxide, Emulsan®-D, and Quill-A® (Obradovic, Corsaut, et al., 2021). In a separate study, researchers evaluated a modified oil-in-water emulsion adjuvant termed OWq, which incorporated a food-grade Quillaja saponin extract as both an emulsifier and an immunostimulant. This formulation elicited significantly higher levels of CSFV E2-specific IgG and virus-neutralizing antibodies in pigs when compared to oil-only adjuvanted vaccines. Furthermore, OWq induced a more balanced IgG1/ IgG2 antibody subclass response, indicating enhanced and broader immunogenicity without adverse health effects in vaccinated animals (Burakova, Madera, Wang, et al., 2018).

Oil-in-water (O/W) emulsions are a class of adjuvants with favorable safety and immunogenicity profiles, particularly in comparison to water-in-oil (W/O) systems. Unlike W/O emulsions, which form persistent antigen depots at the injection site, O/W emulsions typically do not induce prolonged local inflammation, contributing to their improved tolerability. These emulsions are characterized by their hydrophilic nature and relatively low viscosity, typically below 30 cP (MariamShakeel, 2023). The dispersed oil phase, constituting 5-30% of the formulation, enhances immune activation while minimizing reactogenicity (Tasheva et al., 2021). Clinically approved squalene-based O/W emulsions adjuvants, MF59 and AS03, have demonstrated potent adjuvanticity in human vaccines, particularly during influenza pandemics (Ko & Kang, 2018b; Wilkins et al., 2017). It has been suggested that MF59 acts primarily through chemokine-mediated recruitment of monocytes, macrophages, and granulocytes at the injection site, producing MCP-1, IL-8, CCL3, and CCL4 and reinforcing a positive feedback loop that amplifies immune cell infiltration (O'Hagan et al., 2012; Seubert et al., 2008).

The effectiveness and safety of emulsion adjuvants are intricately linked to their components, including the type of oil used and the concentration of surfactants in the formulation. Toxicological concerns often arise from the use of high concentrations of low-quality, non-biodegradable oils. The metabolic profile of the oil used, whether a poorly metabolized mineral oil or a vegetable-based oil, can substantially impact the strength and nature of the immune response (Stone, 1997). Notably, excessive surfactant levels can induce adverse reactions following vaccination (Ascarateil & Dupuis, 2006).

In addition to compositional factors, the physical characteristics of emulsions, particularly droplet size, play a critical role in immunogenicity. Studies have shown that droplet size influences immune cell recruitment and activation. Droplet size plays a pivotal role in the recruitment and activation of immune cells. Emulsion adjuvants with particle sizes ranging between 100-200 nm have been reported to facilitate uptake by dendritic cells, thereby enhancing antigen presentation and subsequent immune activation (Ott et al., 1995). In a comparative study, emulsions with droplet sizes near 160 nm (e.g., SEA160) demonstrated superior immunogenicity over smaller droplets (e.g., 20 nm), with SEA160 showing comparable efficacy to the licensed MF59 adjuvant. Smaller droplets did not enhance immune responses; instead, they showed reduced cellular infiltration and antigen transport (Iyer et al., 2015; Shah et al., 2019). The potency of emulsion adjuvants appears to depend not only on droplet size but also on surfactant interaction with host cells, potentially involving intracellular pathways and danger-associated molecular pattern (DAMP) release (Shah et al., 2019; Vono et al., 2013).

Understanding the interactions between oils, surfactants, and the immune system can lead to improved vaccine efficacy while minimizing the risk of adverse reactions. Continuous research

and rigorous clinical evaluations are essential to advance safe and effective vaccine adjuvants, particularly in the context of emerging infectious diseases.

Chapter 2 - Formulation and Characterization of Gum Arabic-Stabilized Emulsion Adjuvants Using GTL Oils for Vaccine Applications

Abstract

Subunit and inactivated vaccines often require adjuvants to enhance immune responses and ensure long-lasting protective immunity. Among the various adjuvant systems, oil-in-water emulsions have gained attention due to their ability to improve antigen delivery, enhance immunogenicity, and maintain stability over time. In this study, oil-in-water emulsions were formulated using gum Arabic as a natural emulsifier at concentrations of 5%, 7.5%, and 8% for Ondina X-415 GTL (gas to liquid) oil, while a constant 7.5% concentration of gum Arabic was used for the other GTL oils tested: SL-3008, SL-3007, and Ondina X-420. The formulation containing 7.5% gum Arabic and Ondina X-415 GTL oil, a medium-viscosity oil, exhibited the most favorable characteristics. This emulsion demonstrated a droplet size of 221.7 ± 1.2 nm, a low polydispersity index of 0.093 ± 0.029 , and a zeta potential below -30 mV, indicating electrostatic stability. All emulsions remained physically stable for 90 days at 20°C , without macroscopic phase separation. However, the transmission electron microscopy (TEM) analysis revealed differences in microstructural stability. Coalescence was observed in emulsions formulated with SL-3007 GTL oil, while SL-3008 GTL oil emulsions exhibited flocculation. Ondina X-420 GTL oil emulsions displayed stable microstructures, with minimal aggregation or coalescence. The study found that increasing the gum Arabic concentration from 5% to 7.5% improved droplet stability. It resulted in a lower polydispersity index (PDI), without reducing the droplet size, whereas further increasing to 8% resulted in higher polydispersity without additional size reduction. These results

highlight the importance of optimizing both oil type and emulsifier concentration to achieve stable and effective adjuvant formulations in vaccine development.

Introduction

Oil-in-water (O/W) emulsions have become a cornerstone in vaccine adjuvant technology due to their proven ability to enhance antigen delivery and stimulate robust immune responses (Fox et al., 2013; Galliher-Beckley et al., 2015; Iyer et al., 2015; O'Hagan et al., 2012; Y. Zhang et al., 2024). These emulsions consist of oil droplets dispersed in a continuous aqueous phase, and surfactants to stabilize the interface between the oil and water. Historical records show that the concept of using oil emulsions in vaccines dates back to 1916, when Le Moignic and Pinoy demonstrated enhanced immune responses in mice vaccinated with inactivated *Salmonella typhimurium* suspended in a Vaseline oil and water emulsion (Nevagi et al., 2019). Later, during the 1990s, oil-in-water emulsions emerged as promising adjuvants for influenza vaccines, further validating their effectiveness (Fox & Haensler, 2013; Podda, 2001).

MF59 has been licensed for use in seasonal influenza vaccines in Europe since 1997 and has been used in more than 20 countries (Podda, 2001). MF59 is composed of squalene, a natural oil synthesized in the human liver and a precursor to cholesterol, combined with surfactants such as polysorbate 80 and sorbitan trioleate (Span 85) (Wilkins et al., 2017). Similarly, OW-14, a low-cost O/W emulsion adjuvant made from light mineral oil and food-grade gum Arabic, has shown promising results. It has been reported to induce strong antigen-specific antibody responses in vaccines targeting swine influenza (SI), *Mycoplasma hyopneumoniae*, and classical swine fever (CSF) (Galliher-Beckley et al., 2015; Madera et al., 2016). Another innovative emulsion adjuvant, MIL@A-SW01-C, incorporates an aluminum-based metal-organic framework (MIL-53(Al)) with

carbomer, offering enhanced and balanced Th1/Th2 responses in both pigs and mice (Yang et al., 2024).

A critical determinant of emulsion adjuvants is droplet size, and the stability of droplet size has been linked to the overall effectiveness of the emulsion in enhancing immune responses and ensuring long-term stability (Haensler, 2017; Huang et al., 2024; Iyer et al., 2015). Ideally, particle sizes between 80 to 200 nm provided stable formulations and efficient antigen delivery to lymph nodes (Huang et al., 2024; O'Hagan et al., 2021; Stelzner et al., 2018). Commercial adjuvants used in licensed vaccines, such as MF59 and AS03, typically have particle sizes around 150–160 nm (Huang et al., 2024; Shah et al., 2019). Smaller particles, particularly less than 100 nm, are more stable and resistant to phase separation or breakdown, even under harsh conditions like elevated temperatures or fluctuating pH levels (Iyer et al., 2015). For example, emulsions with an average particle size of 80 nm showed structural stability for longer than 30 days at 40°C and were stable in variable pH and salt concentration (Iyer et al., 2015). Larger particles (above 150 nm, on the other hand, are more prone to instability, as they tend to separate or break down when exposed to stressors such as shaking or heat, which compromises their reliability for long-term storage or in challenging conditions (Shah et al., 2019).

Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA) are two common techniques used to characterize particle size distributions in nanoformulations. These methods are particularly useful for analyzing emulsion samples, as they can measure particles ranging from the nanometer to the micron scale, covering systems such as emulsions, liposomes, and macromolecules like proteins (Filipe et al., 2010; *Orthogonal and Complementary Nanoparticle Characterization Techniques* | Malvern Panalytical, n.d.). Both techniques rely on Brownian motion, which is the random movement of particles in suspension in a liquid medium.

As the size of the particle increases, the particle will diffuse much more slowly, and the degree of Brownian motion can be quantitatively determined to estimate particle size.

DLS works by detecting fluctuations in scattered light intensity over time, which are used to calculate the diffusion coefficient and subsequently the hydrodynamic diameter through the Stokes-Einstein equation. Key parameters, such as the refractive index and viscosity of the sample medium, allow for the determination of values like the Z-average particle size and Polydispersity Index (PDI), which reflects the distribution width of particle size (Friskin, 2001; Mailer et al., 2015; Pecora, 2013). On the other hand, Nanoparticle Tracking Analysis (NTA) is an advanced technique that tracks the movement of individual particles in solution using laser illumination and imaging by a high-speed camera and microscope. This method excels at assessing particle size distributions with reduced susceptibility to errors from polydispersity compared to DLS (Chan et al., 2017; Filipe et al., 2010). Both techniques complement each other, providing comprehensive insights into the particle size and distribution in emulsion systems, crucial for evaluating their stability and suitability as vaccine adjuvants.

Despite significant advancements in emulsion-based adjuvants, limited research has investigated the use of gas-to-liquid (GTL) synthetic oils in their formulation. This chapter addresses this gap by formulating and characterizing Gum Arabic-stabilized emulsions incorporating various GTL oils, with a focus on evaluating their physical properties, microstructure, and storage stability. The findings provide valuable insights for the rational design of thermally stable and immunologically effective emulsion adjuvants.

Materials and methods

Emulsion preparation

Oil-in-water emulsions were formulated using Ticamulsion A-2010, a gum Arabic-based emulsifier, at a final concentration of 7.5% (w/v) in deionized water (Galliher-Beckley et al., 2015). Synthetic paraffin oils (Ondina X-415, X-420, SL-3007, and SL-3008), produced via gas-to-liquid (GTL) technology supplied by Shell Catalysts & Technologies, were used as the oil phase at 15% (w/v). Nanopure deionized water was prepared using the Barnstead Nanopure Infinity water system (ThermoScientific, Marietta, OH, USA). Pre-emulsions were generated by high-shear mixing (10,000 rpm, 15 min) using a Silverson mixer (L5M-A, Silverson, East Longmeadow, MA), followed by homogenization via five passes through a high-pressure Microfluidizer (M-110P, Microfluidics, Newton, MA) at 10,000 psi. Emulsions were characterized by droplet size using dynamic light scattering and Nanoparticle Tracking Analysis to ensure accuracy and reliability.

Table 2.1: Physical characteristics of synthetic paraffin oils used in this study

Oils	Viscosity at 40 °c, cSt	Density (Kg/m ³)15°c
SL- 3007	3.3 mm ² /s	-
SL-3008	6.0 mm ² /s	800
Ondina X- 415	9.3 mm ² /s	806
Ondina X- 420	18 mm ² /s	816

The effect of varying emulsifier concentrations on particle size distribution was also evaluated using emulsions formulated with the Ondina X-415 oil phase. Specifically, emulsifier concentrations of 5%, 7.5%, and 8% (w/v) were compared to identify the optimal formulation parameters that promote enhanced droplet stability and uniformity.

Emulsion characterization

Droplet size

Droplet size was measured using a Zetasizer Nano ZS 90 (Malvern Instruments, Westborough, MA) via dynamic light scattering (DLS) technology. All emulsion samples were diluted 1:100 in deionized water before analysis to minimize multiple scattering. Measurements utilized a 633 nm helium-neon laser at a backscatter angle of 173°, enabling determination of the hydrodynamic diameter of the droplets. The polydispersity index (PDI) was also assessed, where values below 0.2 indicate a uniform droplet size distribution, while values equal to or above 0.7 reflect significant heterogeneity (Danaei et al., 2018). In parallel, NTA was performed (NanoSight LM10, Malvern Instruments) to visualize and quantify individual droplet motion based on Brownian movement, and the Stokes-Einstein equation to calculate the hydrodynamic diameter of the particles. This method provided complementary data on particle size and concentration within the suspension. All measurements were carried out in triplicate at 25 °C, and results were expressed as mean $\pm$ standard error of the mean (SEM).

Zeta potential measurements

The zeta potential of the emulsion samples was determined using a Zetasizer Nano ZS 90 (Malvern Instruments, Westborough, MA) equipped with a disposable folded capillary cell. Before analysis, samples were diluted 1:100 with deionized water. An external electric field was applied to evaluate the electrophoretic mobility of the droplets, from which the zeta potential was calculated using Henry's equation. To ensure reproducibility, each formulation was analyzed in triplicate, using two separately prepared batches. Results are expressed as the mean $\pm$ standard error of the mean (SEM).

pH measurement

The pH of the emulsions was determined using a VWR SympHony digital pH meter SB21 (VWR International, Radnor, PA, USA). Before measurement, the instrument was calibrated using a three-point method, following the manufacturer's instructions.

Transmission electron microscopy (TEM)

Different emulsions were examined using a Transmission Electron Microscope (TEM) located in the Department of Biology at Kansas State University. Before imaging, the emulsion samples were diluted 20-fold and applied onto 200-mesh formvar-carbon-coated grids (Electron Microscopy Sciences, Hatfield, PA). The samples were allowed to adsorb for 5 minutes at room temperature, followed by staining with 2% uranyl acetate for an additional 5 minutes under the same conditions. After air drying, the 3 mm diameter grids were imaged using a FEI CM100 TEM operated at an accelerating voltage of 100 kV and a magnification of 45,000x. Images were captured with an AMT digital imaging system.

Long-term stability

The physical stability of the emulsions was monitored under two storage temperatures: 4 °C and 20 °C. Emulsions were considered visually stable if they showed no evidence of creaming, sedimentation, or phase separation. For formulations that remained visually stable throughout the storage period, droplet size measurements were performed using dynamic light scattering (DLS) at the end of each storage interval. All measurements were conducted in triplicate, and results are expressed as the mean $\pm$ standard error of the mean (SEM). In addition to passive storage, a centrifugation method was employed to simulate stress conditions and accelerate

instability detection. Emulsions were subjected to high-speed centrifugation using a laboratory centrifuge to evaluate their resistance to phase separation.

Results

The effect of emulsion concentration is different on the droplet size of synthetic paraffin oil emulsion.

As shown in Figure 2.1, an emulsion stabilized with 5% (w/v) gum Arabic produced the smallest mean droplet diameter of 211.8 ± 1.2 nm. Increasing the emulsifier concentration to 7.5% increased droplet size, with a mean diameter of 221.7 ± 2.3 nm. At 8% gum Arabic, the droplet diameter decreased slightly to 214.1 ± 1.7 nm. All emulsions exhibited growth in droplet size over a 90-day storage period at both 4°C and 20°C, indicative of physical aging processes. Among the oil types tested, OW-415 showed the highest droplet stability, with final diameters of 258.03 ± 2.10 nm at 4°C and 252.97 ± 0.92 nm at 20°C. OW-3008 emulsions exhibited greater droplet growth, reaching approximately 280.0 ± 3.4 nm after 90 days. Emulsions formulated with OW-3007 were excluded from long-term analyses due to phase separation and instability during storage.

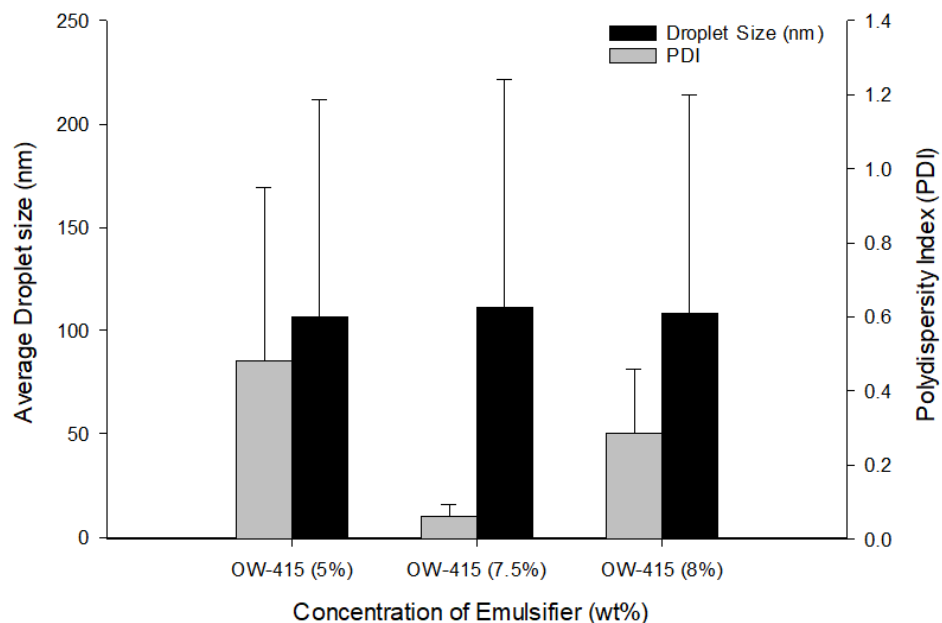


Figure 2.1: Effect of gum Arabic concentration on the droplet diameter and polydispersity index (PDI) of synthetic paraffin oil emulsions. Data expressed as mean $\pm$ SEM.

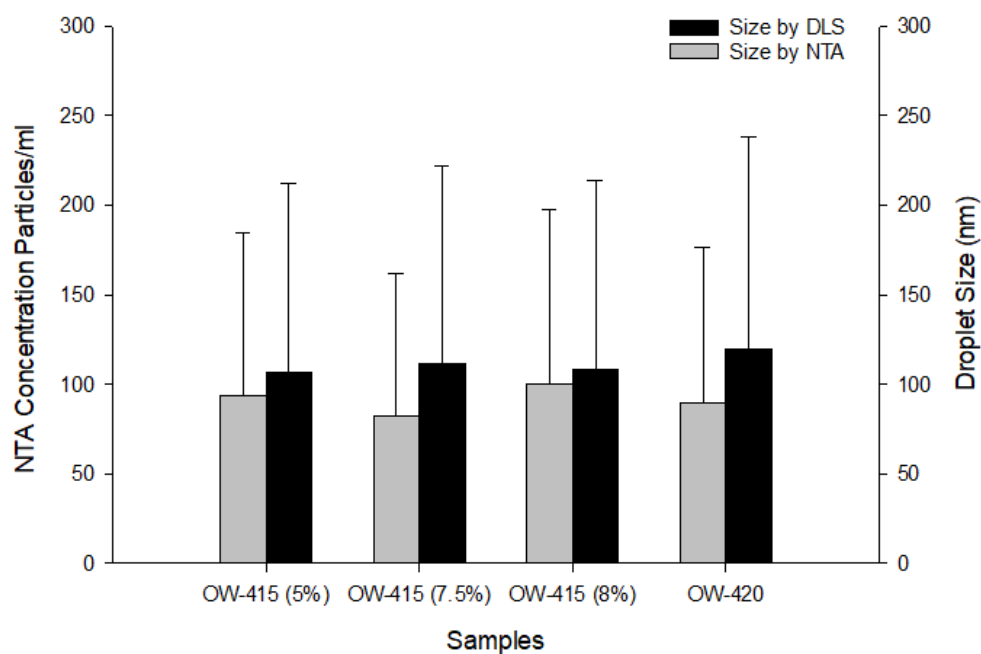


Figure 2.2: Comparison of droplet size distributions for OW-415 emulsions formulated with 5%, 7.5%, and 8% gum Arabic, measured by DLS and NTA. Data expressed as mean $\pm$ SEM.

Comparison of droplet size distribution by DLS and NTA

Complementary analyses using dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) were performed to evaluate the droplet size distribution across formulations (Figure 2.2). For the 5% gum Arabic formulation, the mean droplet size determined by DLS was 211.8 ± 1.2 nm, while NTA reported a closely comparable size of 184.5 ± 2.3 nm, corresponding to a difference (Δ) of 27.3 nm. In contrast, the 7.5% formulation exhibited a greater divergence between the two analytical techniques, with DLS measuring 221.7 ± 1.2 nm and NTA reporting 161.8 ± 3.0 nm ($\Delta = 59.9$ nm). The 8% formulation presented an intermediate discrepancy, with droplet sizes of 214.1 ± 3.0 nm by DLS and 197.8 ± 2.5 nm by NTA ($\Delta = 16.3$ nm). These observed differences highlight the inherent methodological distinctions between intensity-weighted (DLS) and number-weighted (NTA) particle sizing, reflecting the influence of larger particles on scattering-based measurements.

The emulsions demonstrated varying degrees of distribution uniformity, as indicated by PDI values. The 5% gum Arabic emulsion, while producing the smallest droplet size, showed a high PDI of 0.102, indicative of heterogeneous droplet populations. The 7.5% formulation presented the lowest PDI (0.093), suggesting more uniform particle sizes and improved droplet stabilization. At 8%, the PDI increased to 0.110, indicating a broader size distribution and a less stable emulsion system at higher emulsifier concentrations (Shown in Fig. 2.1).

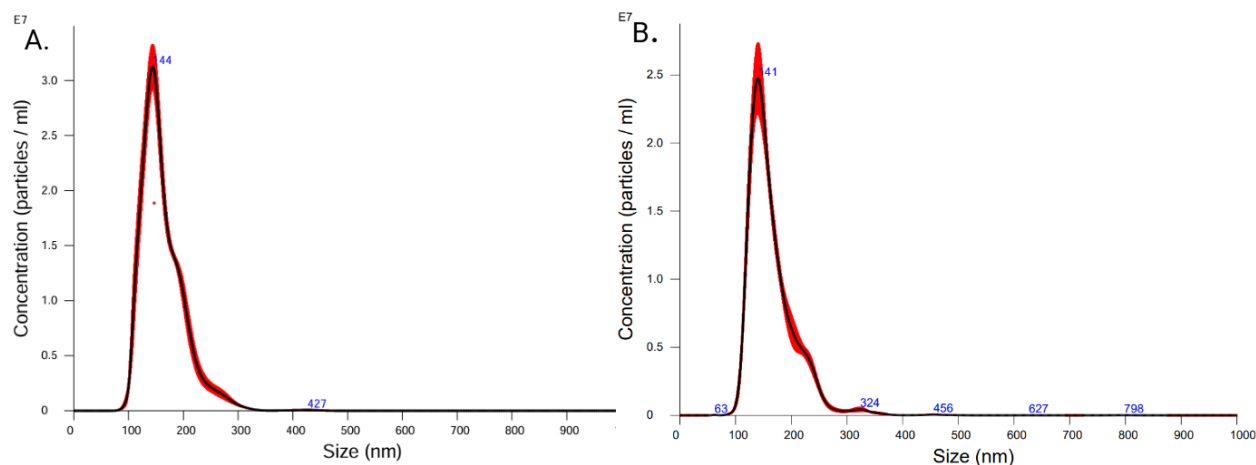


Figure 2.3: Droplet size distribution of OW-415 emulsions (A) Droplet size distribution of freshly prepared OW-415 emulsion analyzed by Nanoparticle Tracking Analysis (NTA); (B) droplet size distribution after 90 days of storage.

Zeta potential and droplet morphology

All emulsions showed strong electrostatic stabilization, with zeta potential values remaining consistently below -30 mV across the study period. OW-415 exhibited the most stable zeta potential values (≥ -35 mV), correlating with its robust colloidal stability (Table 2.3). OW-420 and OW-3008 showed slightly lower values, but still within the threshold for electrostatic repulsion and colloidal stability. Slight decreases in zeta potential over the 90-day storage period were observed across all formulations, consistent with minor physicochemical aging effects, but these reductions did not compromise overall stability. OW-3007, which had the least negative zeta potential and showed coalescence, was physically unstable at 20°C and therefore excluded from further consideration.

Transmission electron microscopy further validated droplet morphology (Fig.2.6A-D). OW-415 and OW-420 displayed well-dispersed, spherical droplets with minimal aggregation (Fig.2.6C-D). OW-3008 exhibited evidence of flocculation, while OW-3007 showed pronounced coalescence (Fig.2.6A-B and E-F), visually confirming the physical instability previously observed during thermal storage studies.

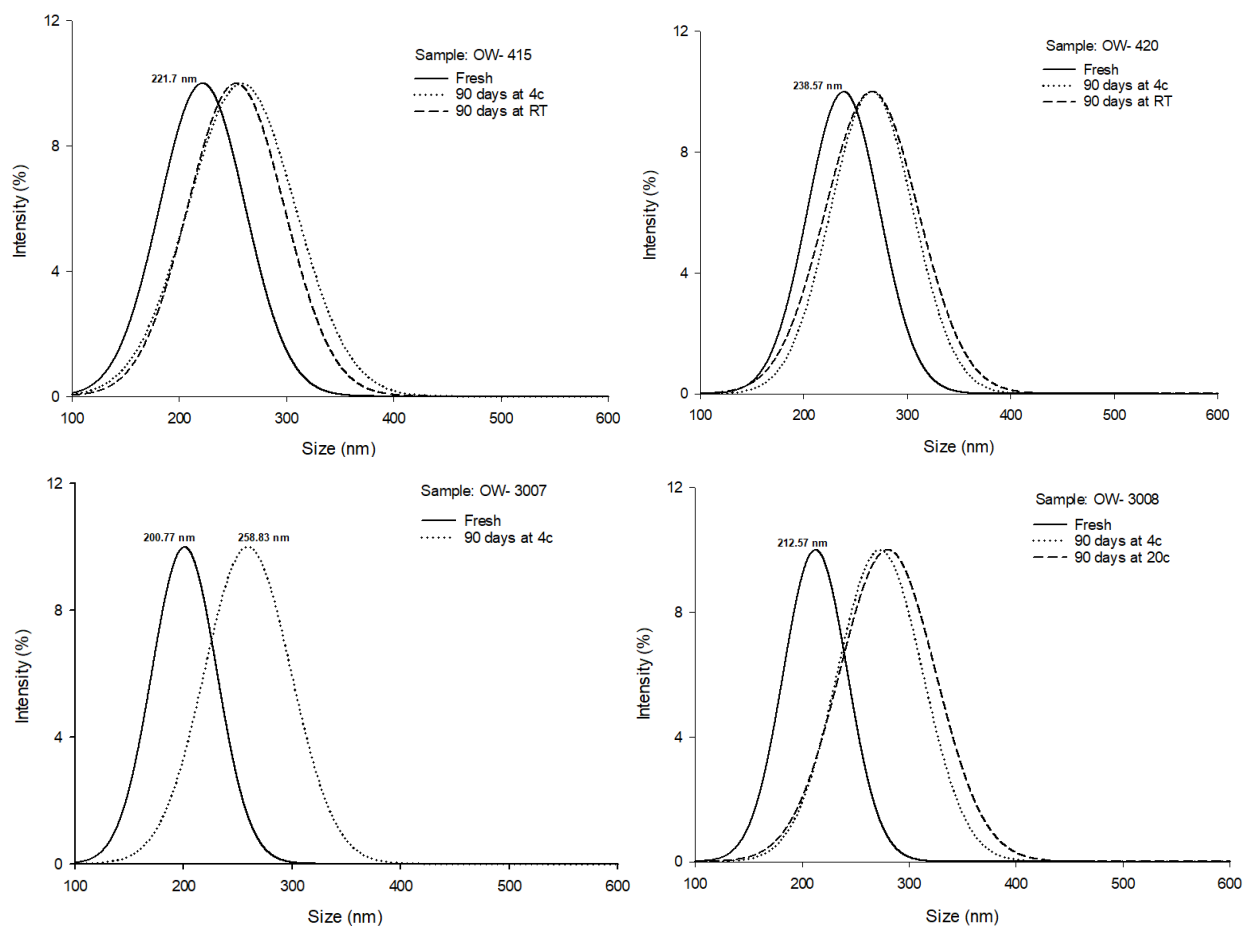


Figure 2.4: Variation in emulsion droplet size across different samples after three months of storage under varying conditions.

Table 2.2: Centrifugation test results for the different emulsions, (+ stable, - phase separation)

Formulations	(FRESH)	(3 MONTHS)
	5000 rpm for 15 minutes	5000 rpm for 15 minutes
OW-415 (with 7.5% emulsifier)	+	+
OW-420 (with 7.5% emulsifier)	+	+
OW-3007(with 7.5% emulsifier)	+	+
OW-3008(with 7.5% emulsifier)	+	+

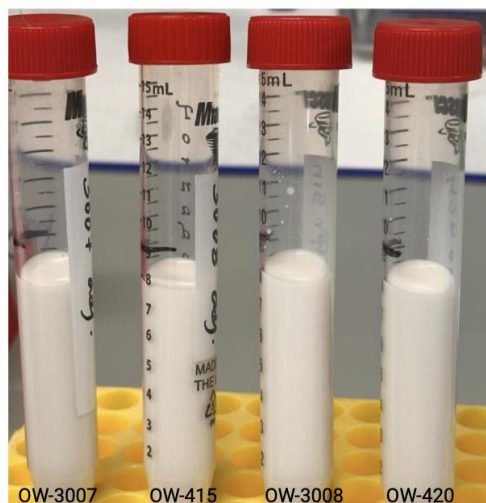


Figure 2.5: Photograph depicts emulsion appearance (stable) after centrifugation at 5000 rpm for 20 minutes

Long-term stability assessment

Following storage at 4°C and 20°C for 90 days, droplet size increases were observed across all formulations, aligning with aging mechanisms such as Ostwald ripening (Fig. 2.4). The droplet size distribution of OW-415 emulsions, assessed by Nanoparticle Tracking Analysis, showed that the freshly prepared emulsion had a sharp and narrow size distribution with a dominant peak around 144 nm, reflecting uniform particle size and effective emulsification. After 90 days of storage, the primary peak remained near 141 nm, indicating good size retention, although minor peaks between 324 nm and 798 nm suggested limited droplet aggregation or coalescence (Fig.2.3.). These findings demonstrate that OW-415 preserved its nanoscale characteristics and physical stability over time under storage conditions. OW-420 and OW-3008 remained within acceptable ranges, while OW-3007 was excluded due to early destabilization. Centrifugation at 5000 rpm for 20 minutes (Fig. 2.5) did not result in visible phase separation or coalescence across all formulations (Table 2.2).

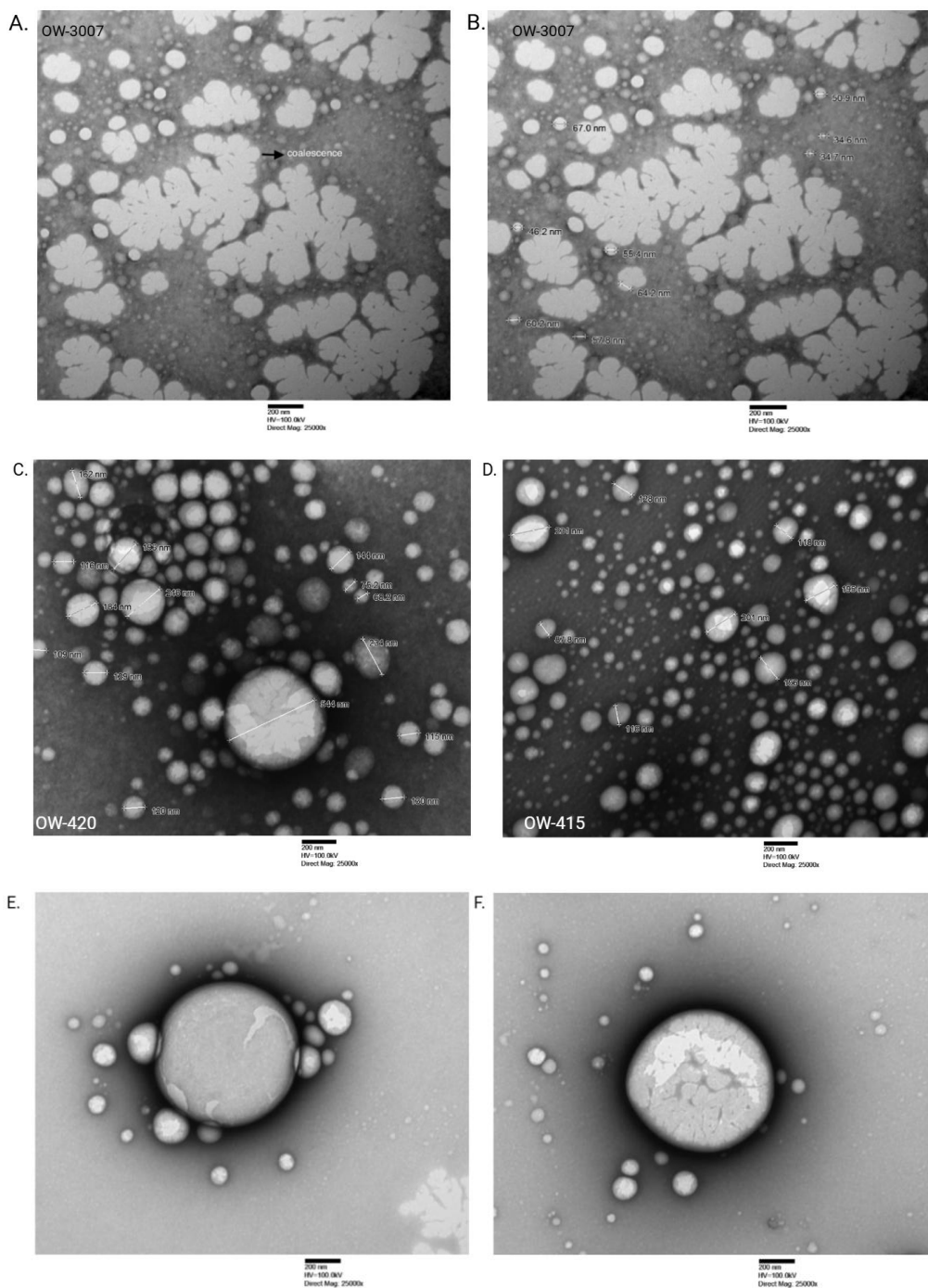


Figure 2.6: Transmission Electron Microscopy (TEM) images of emulsions (A and B) 3007 exhibiting coalescence of droplets; (C and D) OW-420 and OW-415 formulations showing stable oil-in-water droplet structures; (E and F) Sample 3008 displaying flocculation behavior

Table 2.3: Physical characteristics of fresh emulsion adjuvants and after 90-day storage at different temperatures

	Emulsion Sample	Mean droplet size $\pm$ SD, nm	Polydispersity $\pm$ SD	Zeta potential $\pm$ SD, mV	pH
OW- 415	Fresh	221.7 $\pm$ 1.2	0.093 $\pm$ 0.029	-53.53 $\pm$ 0.39	6.63
	90 days at 4°C	258.03 $\pm$ 2.10	0.128 $\pm$ 0.009	-37.87 $\pm$ 0.33	6.64
	at 20°C	252.97 $\pm$ 0.92	0.155 $\pm$ 0.011	-35.53 $\pm$ 0.45	
OW- 420	Fresh	238.57 $\pm$ 1.24	0.12767 $\pm$ 0.0118	-25.0 $\pm$ 3.7	6.81
	90 days at 4°C	265.47 $\pm$ 1.48	0.184 $\pm$ 0.013	-36.53 $\pm$ 0.71	6.68
	at 20°C	265.8 $\pm$ 2.33	0.126 $\pm$ 0.015	-38.03 $\pm$ 0.42	
OW- 3007	Fresh	200.77 $\pm$ 2.02	0.105 $\pm$ 0.006	-55.47 $\pm$ 2.5	6.5
	90 days at 4°C	258.83 $\pm$ 1.83	0.124 $\pm$ 0.009	-41.47 $\pm$ 1.98	6.5
	at 20°C	Discarded	Discarded	Discarded	
OW- 3008	Fresh	212.57 $\pm$ 1.06	0.128 $\pm$ 0.018	-53.6 $\pm$ 0	6.52
	90 days at 4°C	272.67 $\pm$ 3.38	0.168 $\pm$ 0.016	-37.33 $\pm$ 0.45	6.6
	at 20°C	280.13 $\pm$ 5.58	0.124 $\pm$ 0.028	-35.17 $\pm$ 0.26	

RT, room temperature; SD, standard deviation

Discussion

Various studies have highlighted the importance of emulsifiers, such as gum Arabic, in stabilizing emulsions (Benabbes et al., 2019; Liang et al., n.d.). Destabilization of the emulsions is typically associated with gravity separation (creaming and sedimentation), flocculation, coalescence of droplets, and osmosis, all of which are influenced by factors including droplet size distribution, emulsion concentration, the properties of the interfacial film, and the phase state of the film (Costa et al., 2019; Liang et al., n.d.).

Our findings suggest that emulsifier concentration plays a pivotal role in influencing the equilibrium between minimizing droplet size and achieving uniform particle distributions. Dynamic Light Scattering (DLS) analysis revealed that a lower Arabic concentration of 5% yielded the smallest average droplet size, approximately 211.8 nm, and an elevated polydispersity index (PDI) of 0.102. In colloidal systems, lower PDI values correspond to greater stability due to

reduced rates of Ostwald ripening; however, in this case, the elevated PDI suggests the presence of size heterogeneity despite the small average diameter (Solè et al., 2012). Reduced emulsifier levels may facilitate the formation of smaller droplets. However, they may not provide adequate steric or electrostatic stabilization to prevent droplet size variation or aggregation, thereby compromising physical stability (Ebrahimi et al., 2020). This is a critical consideration, as achieving a small droplet size alone does not guarantee formulation success if uniformity and long-term stability are not maintained.

Among the formulations evaluated, the OW-415 emulsion, composed of 15% Ondina X-415 oil and 7.5% Ticamulsion A-2010, demonstrated optimal physicochemical characteristics for potential application as a vaccine adjuvant. This formulation exhibited the smallest and most monodisperse droplet size, measuring 221.7 nm, the lowest PDI of 0.093, and a highly negative zeta potential of -53.53 mV, all of which are indicative of excellent colloidal stability and a high potential for immune system uptake. Notably, these favorable characteristics were preserved after 90 days of storage at both 4°C and 20°C, confirming the formulation's extended stability. Across all emulsions tested, zeta potential values consistently remained below -30 mV, indicating strong inter-droplet electrostatic repulsion, a key factor in preventing aggregation (Sharma et al., 2014).

The droplet size distributions determined by DLS and Nanoparticle Tracking Analysis (NTA) were comparable for the OW-415 emulsion, with the mean sizes falling within the 100-400 nm range. The stability trends observed in the DLS data were further corroborated by the NTA results, where the number-based particle sizes were marginally smaller than the intensity-based sizes. This expected discrepancy arises from DLS's greater sensitivity to larger particles, which disproportionately contribute to light scattering intensity, whereas NTA provides a more accurate representation of the actual droplet population (Chan et al., 2017). Transmission Electron

Microscopy (TEM) imaging provided visual confirmation of the DLS and NTA findings and the zeta potential measurements. OW-415 and OW-420 displayed discrete, spherical droplets without any visible aggregation, a hallmark of kinetically stable emulsions. In contrast, OW-3008 exhibited partial flocculation, consistent with its larger droplet size and slightly reduced zeta potential (Fig.2.6E and F). OW-3007 showed evidence of advanced coalescence, confirming its instability and justifying its exclusion from subsequent analyses (Fig.2.6A and B). These morphological observations align with previous reports indicating a strong correlation between droplet growth and zeta potential reduction over time, ultimately leading to coalescence (P. et al., 2018).

Notably, all tested emulsions (OW-415, OW-420, OW-3007, and OW-3008) remained stable and showed no signs of phase separation after centrifugation at 5000 rpm for 15 minutes, both when freshly prepared and after three months of storage under the same conditions. The homogeneity of an emulsion is crucial for its stability, as phase separation of the dispersed phase from the continuous phase compromises the formulation's usability. Studies have established that high stability observed immediately following centrifugation, typically at around 5000 rpm for 15–20 minutes, exhibits a strong correlation with the long-term physical stability of emulsions (Khatchadourian, n.d.; Navarro-Pérez et al., 2021). Emulsions that maintain their integrity, without exhibiting phase separation, creaming, or coalescence, after undergoing such rigorous testing tend to remain stable for periods ranging from 6 months to up to 1 year under standard storage conditions (Lerche & Sobisch, 2011). Based on these comprehensive physicochemical analyses, OW-415 emerged as a promising candidate for further evaluation in immunogenicity studies.

Conclusion

The optimization of Synthetic paraffin oil emulsion formulations was achieved by adjusting the concentration of gum Arabic, which significantly affected both droplet size and emulsion stability. A 5% gum Arabic concentration yielded the smallest droplet size, but with a higher polydispersity index (PDI), reflecting a broader size distribution. Increasing the emulsifier concentration to 7.5% led to improved droplet uniformity, as indicated by a lower PDI, although the droplet size slightly increased. Emulsions containing OW-415 oil exhibited greater stability compared to other oils, showing less droplet growth and superior long-term stability during storage. Zeta potential values remained consistently below -30 mV for all formulations, indicating strong electrostatic stabilization. Further optimization of emulsifier concentration and the exploration of alternative emulsifiers for the GTL oil phase could improve the physical stability of these emulsions. Additional research is needed to investigate the potential of different oil types and emulsifier combinations for enhancing vaccine adjuvant formulations.

Chapter 3 - *Streptococcus suis* and CSFV E2 Protein

Abstract

This chapter provides a comprehensive overview of the pathogenesis, clinical manifestations, and immunological responses associated with *Streptococcus suis* and Classical Swine Fever Virus (CSFV), emphasizing their relevance to vaccine development. CSFV primarily spreads through oronasal exposure, targeting lymphoid tissues and evading immune defenses via proteins such as Npro and Erns, leading to systemic dissemination and immunosuppression. Its clinical outcomes range from acute hemorrhagic disease to persistent infections, shaped by viral virulence and host immune status. In contrast, *S. Suis* is an opportunistic bacterial pathogen responsible for severe systemic infections in swine, and it poses a growing therapeutic challenge due to rising antimicrobial resistance. While antimicrobial agents remain critical for short-term management, effective long-term control requires the development of broadly protective vaccines. Current efforts against *S. Suis* include autogenous bacterins and experimental multi-epitope vaccines, though challenges remain due to antigenic diversity. For Classical Swine Fever Virus (CSFV), live attenuated vaccines have been effective in controlling the disease. However, a lack of DIVA (Differentiating Infected from Vaccinated Animals) compatibility poses challenges for surveillance and eradication efforts. To address this issue, E2-based subunit vaccines have been developed as DIVA-compatible alternatives that can provide robust and long-lasting immune protection. This chapter also explores the impact of co-infections in intensive farming environments, highlighting the shared cytokine responses and immune pathways activated by both pathogens. These findings emphasize the importance of integrated vaccine strategies to address the complex immunological interactions between co-circulating viral and bacterial pathogens in swine populations.

Introduction

Streptococcus suis

General characteristics

Streptococcus suis (*S. Suis*) is regarded as a significant infectious disease in the swine industry, causing high economic losses in pig husbandry due to mortality, increased management, treatment, and prevention costs (Uruén et al., 2022). *S. Suis* is a gram-positive, facultative anaerobe and is a zoonotic pathogen that can cause several diseases in pigs and domestic animals (Staats et al., 1997). The bacterium is oval-shaped and exists in long chains in liquid media. The formation of longer chains in *S. Suis* is intricately linked to its pathogenic potential. Research indicates that the length of the bacterial chain correlates with an enhanced capacity for pathogenicity (Segura et al., 2017). When cultured on 5% sheep blood agar at 37°C, *S. Suis* forms round, moist, smooth colonies. *S. Suis* typically exhibits α -hemolysis on blood agar initially, transitioning to β -hemolysis with extended incubation (Kilpper-Bälz & Schleifer, 1987). In some cases, there may be no visible hemolysis around the colony, but scraping the colony can reveal either alpha or beta hemolysis. As shown in Figure 3.1, *Streptococcus suis* type 2 demonstrated alpha hemolysis on a sheep blood agar plate.



Figure 3.1: *S. Suis* grown on a 5% sheep blood agar plate

In swine, it causes meningitis, arthritis, endocarditis, pneumonia, and septicemia. Additionally, it poses a significant public health risk, causing meningitis, septicemia, and endocarditis in humans, particularly among individuals working in slaughterhouses, butchers handling infected pork, pig farmers, and veterinarians (Brizuela et al., 2023; Chattopadhyay, 1979). Notably, *S. Suis* outbreaks in China in 1998 and 2005 have changed the perspective of the threat posed by the pathogen to human health (Brizuela et al., 2023; P. Liu et al., 2023). In Swine, control of *S. Suis* typically relies on antimicrobial treatments. However, antimicrobial resistance (AMR) is a growing concern (Lunha et al., 2022). Moreover, the genetic diversity of *S. Suis* and the lack of cross-protection between different serotypes have made the development of a universal vaccine for this pathogen a persistent challenge (Lu et al., 2024).

Serotypes, distribution, and prevalence

S. Suis has been classified into 35 serotypes based on the capsular polysaccharide (CPS) antigen, with serotype 2 being primarily associated with virulence and commonly linked to disease-causing strains (Gottschalk et al., 1989; Staats et al., 1997). However, serotypes 20, 22, and 26 have been reclassified as *Streptococcus parasuis* following phylogenetic and sequence analyses, while serotypes 32 and 34 have been reclassified as *Streptococcus orisratti* (Hatrongjit et al., 2020; Hill et al., 2005). It is also important to note that not all type 2 isolates exhibit virulence, as virulence can vary among different isolates of the same serotype (Vecht et al., 1989).

Various methods, including the capillary precipitation test, coagglutination test, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), have been used for *S. Suis* serotyping (Athey et al., 2016; Goyette-Desjardins, Auger, et al., 2014). Traditional *S. Suis* serotyping relies on the use of antisera (Estrada et al., 2019), with reference

strains mainly isolated from diseased pigs, clinically healthy pigs, calves, and lambs (Segura et al., 2020). However, one limitation of the coagglutination test is that many field isolates do not express capsular polysaccharide (CPS) after culture (Lacouture et al., 2020). Conventional PCR-based serotyping targets the capsular polysaccharide (*cps*) locus and can serotype encapsulated isolates as well as those that do not express CPS targets. Nonetheless, this method cannot differentiate between serotypes 14 and 1, as well as serotypes 2 and 1/2, due to the highly similar CPS loci shared by these serotypes, differing only by a single nucleotide polymorphism (SNP) at position 483 of the *cpsK* gene (G→C/T) (Goyette-Desjardins, Auger, et al., 2014). Matiasovic et al. developed a PCR-restriction fragment length polymorphism (RFLP) method using the BstNI enzyme, which can detect the SNP and differentiate serotypes 2 and 1/2 and serotypes 1 and 14 (Matiasovic et al., 2020). Additionally, Lacouture et al. demonstrated that the mismatch amplification mutation assay (MAMA)-PCR could successfully differentiate 148 field isolates recovered from the internal organs of diseased pigs (Lacouture et al., 2020). A multiplex PCR method described by Thu et al. enables the identification and differentiation of *S. Suis* serotypes 1, 1/2, 2, and 14 in a single reaction (Thu et al., 2021).

The distribution and prevalence of *S. Suis* serotypes can vary geographically. In North America, serotypes 2 and 1/2 are most prevalent, followed by serotypes 7, 3, 5, and 14 (Lacouture et al., 2022). Recent studies indicated that predominant serotypes in Europe are serotypes 2, 9, and 3, with serotype 9 being the most common in Spain, the Netherlands, as well as in China and Canada (Aradanas et al., 2021; Petrocchi Rilo et al., 2024; Vilaró et al., 2025). Serotype 14, known for its high virulence, is the second most frequently recovered serotype from human cases of *S. Suis* worldwide and has been identified for the first time in a zoonotic case in the Netherlands (Goyette-Desjardins et al., 2014) (Brizuela et al., 2025). These findings underscore the global

spread and shifting patterns of *S. Suis* serotypes, emphasizing the importance of continued surveillance and research across different regions.

Pathogenesis and clinical signs

Although *S. Suis* is of concern to public health, its exact pathogenesis remains unclear. It is a complex commensal bacterium primarily found in the tonsils, nasal cavities, and alimentary tracts of healthy pigs, where it typically does not cause disease under normal conditions (Dekker et al., 2013). However, the shift from harmless colonization to active disease involves the bacteria traversing the mucus layer secreted by respiratory tract epithelial cells or by suppressing mucus production. The specific mechanisms driving this shift are yet to be understood (Segura et al., 2016).

The tonsillar lymphoid tissue, with its deep epithelial invaginations, plays a critical role in initiating immune responses by sampling antigens. However, these same structures can provide shelter to pathogens like *S. Suis*, aiding in their persistence and evasion of host defenses (Dee et al., 1993). Norton et al. found that virulent *S. Suis* strains were able to invade epithelial cells more effectively in vitro when analyzed with the Hep-2 epithelial cell line (Norton et al., 1999). While *S. Suis* is known to adhere to epithelial cells, studies indicate that this attachment does not always result in bacterial invasion (Lalonde et al., 2000). One of the key virulence factors in *S. Suis* is its polysaccharide capsule, which offers protection against complement-mediated opsonization, particularly at mucosal surfaces, thereby helping the bacterium evade immune clearance (Segura et al., 2016, 2017).

Beyond the respiratory route, the gastrointestinal (GI) tract has been suggested as a potential secondary site of infection for *S. Suis*, with possible routes for the bacteria to cross the

gut barrier and cause infections in various tissues and organs (Segura, 2015; Segura et al., 2016). However, many of these findings are based on experiments involving encapsulated strains that are resistant to gastric acid, which may not fully reflect the behavior of non-encapsulated strains in natural conditions. Warneboldt et al. emphasized the challenges of *S. Suis* survival in the gastric environment, raising questions about the viability of GI transmission under typical physiological conditions (Warneboldt et al., 2016). The exact mechanisms by which the bacteria may translocate across the intestinal barrier and contribute to systemic infection remain unclear (Fittipaldi et al., 2012). Understanding these pathways and their clinical impacts is crucial for developing effective management and prevention strategies in the swine industry. Further research is needed to clarify the conditions that facilitate bacterial translocation and its role in disease progression.

Although *Streptococcus suis* is associated with over a hundred potential virulence-related genes, none have been definitively proven to be solely responsible for its pathogenicity, suggesting that its virulence likely results from the interaction of multiple factors (Estrada et al., 2021). Among the most well-studied virulence genes are *sly*, *mrp*, and *epf*, which encode proteins such as suilysin (SLY), muramidase-released protein (MRP), and extracellular factor (EF) (Y. Feng et al., 2014; Segura, 2015). Suilysin, a key toxin found in virulent *S. Suis* serotypes 2 and 9, belongs to the cholesterol-dependent pore-forming toxin family (Y. Feng et al., 2014). While SLY is associated with bacterial adherence and host-cell entry, its mechanistic involvement is not yet fully understood (Tenenbaum et al., 2016). He et al. reported that upregulated SLY expression promotes the ability of *Streptococcus suis* to invade epithelial cells, thereby enhancing its pathogenicity during the initial stages of infection (He et al., 2014).

Once colonized, *S. Suis* employs specific mechanisms to breach the epithelial barriers of the tonsils and upper respiratory tract. By utilizing its CPS, the bacterium can evade immune

recognition and phagocytosis, facilitating its passage through mucosal linings (Segura et al., 2016; X. Wang et al., 2025). Vanier et al. highlighted the role of CPS in influencing the interaction between *S. Suis* serotype 2 and porcine brain microvascular endothelial cells (PBMEC), facilitating adherence and invasion of host cells (Vanier et al., 2004a). CPS also contributes to biofilm formation, which enhances the bacterium's ability to evade immune responses, exchange genetic material, and resist antibiotics (Donlan, 2002; Tohya et al., 2017).

Upon entering the bloodstream, *S. Suis* triggers the production of key pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, and IL-8, which contribute to the severe inflammation characteristic of septic shock syndrome (Fittipaldi et al., 2012; Segura et al., 2006; Q. Zhang et al., 2015). After surviving the initial inflammatory response, *S. Suis* may invade the central nervous system (CNS), leading to conditions such as meningitis (Fittipaldi et al., 2012). The bacterium crosses the blood-brain barrier (BBB), a vital component of the neurovascular unit, using surface proteins to interact with PBMECs. This interaction triggers cytokine production, disrupting the BBB and facilitating bacterial entry into the CNS (Vanier et al., 2004b; Xia et al., 2019). Furthermore, CPS partially inhibits *S. Suis* adhesion to PBMECs, with unencapsulated mutants adhering more strongly and earlier than encapsulated strains. This suggests that adhesins, critical for adhesion, may be located in the bacterial cell wall and are partially masked by the CPS (Vanier et al., 2004b).

Meningitis and Septicemia are the most common clinical manifestations of *S. Suis* infection in humans and pigs. Early signs of *S. Suis* infection in pigs include elevated rectal temperature, intermittent fever, reduced feed intake, signs of lethargy, and lameness that may shift between limbs (Besung et al., 2019).

Table 3.1: Clinical manifestations observed in animals infected with *Streptococcus suis*

Animal	Clinical signs
Human	Septicemia, Meningitis, toxic-shock syndrome, bronchopneumonia
Pigs	Meningitis, polyarthritis, septicemia, pneumonia, endocarditis
Cats	Pneumonia, moist dermatitis, and meningoencephalitis
Horses	Meningitis, guttural pouch, pneumonia, osteomyelitis
Ruminants	Meningitis, arthritis, pneumonia, peritonitis, septicemia
Birds	Septicemia with multiple sudden deaths

Several factors influence the development of clinical symptoms in *Streptococcus suis* infections. Environmental stressors such as poor ventilation, high humidity, and inadequate sanitation, along with management practices like overcrowding and frequent cross-fostering, contribute to increased disease incidence (Vötsch et al., 2018). Clinical signs are most observed in piglets between 5 and 10 weeks of age, corresponding with the decline of maternally derived antibodies following weaning. However, *Streptococcus suis* has been isolated in pigs from just hours after birth up to 32 weeks of age (Neila-Ibáñez, Casal, et al., 2021; Segura, 2015). Co-infections with immunosuppressive viruses, particularly porcine reproductive and respiratory syndrome virus (PRRSV), significantly increase host susceptibility. These viral infections impair immune defenses, facilitating *S. Suis* colonization and systemic spread (Obradovic, Segura, et al., 2021; Vötsch et al., 2018). W. Feng et al. reported that piglets exposed to PRRSV in utero showed heightened vulnerability to *S. Suis* type I, while Hoa et al. found that pigs from PRRSV-affected farms had higher systemic infection rates of serotype 2 (SS2) than non-outbreak herds (W. Feng et al., 2001) (Hoa et al., 2013). Recognizing the initial symptoms of infection in pigs is essential for timely intervention and effective disease control.

Role of maternal immunity in *S. Suis*

Newborn piglets acquire passive immunity against *S. Suis* primarily through colostrum ingestion shortly after birth. Colostrum provides high concentrations of IgG, along with IgM and IgA, with IgG absorption being critical for early systemic protection (Campbell et al., 2012). The concentration of these maternally derived antibodies reaches its highest shortly after ingestion but begins to wane by 18 to 19 days of age, creating a window of susceptibility before the piglet's adaptive immune system matures. High levels of maternal IgG during this phase may also interfere with the piglet's response to vaccination, reducing the effectiveness of early immunization strategies (Corsaut et al., 2020; Lapointe et al., 2002; Obradovic, Corsaut, et al., 2021; Rieckmann et al., 2020). Following the systemic phase, lactogenic immunity continues through the secretion of IgA in milk, which offers localized mucosal protection in the gastrointestinal tract via plasma cells originating from mucosa-associated lymphoid tissue (MALT) (Hurley & Theil, 2011).

CSFV and the E2-glycoprotein

Classical swine fever virus (CSFV) remains a re-emerging pathogen of significant concern to swine health and the global pork industry (Postel et al., 2019; Xing et al., 2019). Initially referred to as hog cholera, the disease was first described by farmworkers in Tennessee in 1810, with multiple outbreaks subsequently recorded across ten U.S. states during the 19th century (Edwards et al., 2000; Hanson, 1957). By the 1860s, CSF had disseminated extensively across Europe and North America, reflecting the virus's rapid transcontinental spread during the 19th century (Choori et al., 2015; Ganges et al., 2020; Rios et al., 2017a; Tu et al., 2001). Since the 1960s, aggressive vaccination programs and systematic depopulation have facilitated the successful eradication of CSF in regions such as North America and Australia (Coronado et al.,

2021). In contrast, the European Union adopted a non-vaccination strategy in the 1990s, relying on stamping-out policies, movement restrictions, and surveillance to progressively eliminate the virus (Postel et al., 2012). Despite these efforts, CSF continues to persist either endemically or sporadically in various regions of Asia, Europe, and the Americas, posing continued challenges for disease control and trade biosecurity (Blome et al., 2017; Moennig, 2015; Schettino et al., 2021).

CSFV is a small, enveloped virus exhibiting icosahedral symmetry, with a particle diameter of 40–60 nm. It is classified within the genus *Pestivirus* of the *Flaviviridae* family (Vuono et al., n.d.). The viral genome consists of a single-stranded, positive-sense RNA with a single large open reading frame (ORF), flanked by untranslated regions (UTRs) at both ends (Y. Liu et al., 2022; Rios et al., 2017b). The ORF encodes a ~3,900 amino acid polyprotein, subsequently processed into four structural (C, Erns, E1, E2) and eight non-structural proteins (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) by viral and host proteases (Lamp et al., 2011; Meyers & Thiel, 1996; Tang et al., 2010; Vuono et al., n.d.). These proteins play critical roles in the virus lifecycle, including virus assembly, replication, and modulation of host interactions.

The envelope glycoproteins Erns, E2, and E1 together form the outer membrane of the virion. Viral attachment and entry are mediated through interactions involving Erns and the E1-E2 complex, enabling infection in both host tissues and cultured cells. Among these, E2 is the main immunogenic protein, critical for inducing protective immune responses in pigs. Erns, which possesses ribonuclease (RNase) activity, also contributes to viral pathogenesis and immune evasion (Hulst et al., 1998; Kwang et al., 1992). Previous studies have shown that modifications in the glycosylation sites within the E2 gene can lead to the attenuation of a virulent CSFV strain (Leifer et al., 2013; Risatti et al., 2005; Velazquez-Salinas et al., 2016). For instance, a single

amino acid substitution (N116A) in the Brescia strain's E2 protein significantly reduced pathogenicity (van Gennip et al., 2004), while mutation of the N805 glycosylation site similarly impaired virulence (Sainz et al., 2008).

The E2 glycoprotein, composed of 373 amino acids with a molecular mass of 51–55 kDa, is the primary surface antigen recognized by the host immune system and is involved in key stages of the viral life cycle, including receptor binding, membrane fusion, and immune evasion (N. Chen et al., 2008). Its N-terminal ectodomain facilitates interactions with the host environment, while its C-terminal region contains a hydrophobic segment of approximately 40 amino acids that embeds the protein within the viral envelope (Thiel et al., 1991). This segment adopts a helical hairpin structure stabilized by a conserved arginine residue, a feature shared with other flaviviruses (Vuono et al., 2021; J. Wang et al., 2014). E2 is recognized as the most immunogenic glycoprotein of CSFV (Gavrilov et al., 2011; König et al., 1995; Weiland et al., 1990). E2 harbors multiple linear neutralizing epitopes and is the most potent antigen for inducing virus-neutralizing antibodies and protective immunity (Bouma et al., 1999; S. Liu et al., 2006). Due to these properties, E2 serves as the principal antigenic target for DNA-based and subunit vaccine development against CSFV (Bouma et al., 1999; Ganges et al., 2005; S. Liu et al., 2006; Qi et al., 2009). While traditional live attenuated vaccines remain effective, their limitations in terms of safety and DIVA (differentiating infected from vaccinated animals) compatibility have prompted a shift toward subunit vaccine strategies. Studies have confirmed that immunization with purified E2 glycoprotein elicits strong protective responses and represents a promising alternative to conventional vaccines (Blome et al., 2017; Madera et al., 2016; Tong et al., 2020).

Pathogenesis and clinical signs

Classical swine fever virus (CSFV) is primarily transmitted through the oronasal route via direct contact with infected pigs, ingestion of contaminated feed, or through vertical transmission. Mucosal shedding facilitates additional routes of infection, including sexual transmission (Blome et al., 2017; Floegel et al., 2000). Upon entry through the oronasal cavity, CSFV initially targets the epithelial cells of the tonsillar crypts, before disseminating to adjacent lymphoid tissues (Summerfield & Ruggli, 2015). The virus subsequently infects cells of the reticuloendothelial system, including macrophages, dendritic cells, and endothelial cells, promoting immune cell activation and systemic dissemination (Ruggli et al., 2003). Through lymphatic and blood circulation, CSFV causes viremia and is distributed to secondary lymphoid organs and parenchymal tissues (Blome et al., 2017; Ganges et al., 2020).

A pivotal aspect of CSFV pathogenesis lies in its capacity to evade innate immune responses. Two viral components, the Npro protein and Erns glycoprotein, play central roles in subverting host immunity. Npro interferes with type I interferon (IFN) signaling by degrading interferon regulatory factors, while Erns, through its ribonuclease (RNase) activity, degrades extracellular viral RNA, further dampening the IFN response (L.-J. Chen et al., 2012; M. Wang et al., n.d.). Although plasmacytoid dendritic cells (pDCs) are potent producers of IFN- α following viral detection, CSFV-mediated suppression mechanisms, particularly in infections caused by highly virulent strains, limit this critical antiviral response (Auray et al., 2020; Franzoni et al., 2014).

Beyond innate immunity, adaptive immune responses play a crucial role in controlling CSFV infection. The E2 glycoprotein harbors multiple cytotoxic T lymphocyte (CTL) epitopes capable of inducing strong CTL responses essential for the early control of viral replication. The

NS3 protein also contributes to T-cell activation, but is less effective in conferring protective immunity compared to E2 (Ceppi et al., 2005; Rau et al., 2006). CTL responses are marked by the production of cytokines such as IFN- γ , IL-2, and TNF- α , in addition to perforin, reflecting potent cytolytic activity. With disease progression, the immune profile gradually shifts toward a Th2-dominant response characterized by increased IL-4 and IL-10, supporting B cell differentiation and neutralizing antibody production (S. P. Graham et al., 2010).

The clinical outcome of CSFV infection is strongly influenced by the virulence of the strain. Infections caused by highly virulent CSFV strains can result in rapid mortality, often preceding seroconversion. In contrast, infections with less virulent strains tend to persist, often presenting as chronic disease characterized by transient lymphopenia, prolonged viral shedding, and incomplete immune clearance (Summerfield & Ruggli, 2015). Notably, early-phase serum IFN- α levels correlate with disease severity; elevated levels have been linked to pronounced lymphoid depletion and leukopenia, particularly in young pigs (Summerfield & Ruggli, 2015). The clinical spectrum of CSFV infection ranges from acute hemorrhagic fever with severe lymphoid depletion, leukopenia, thymic atrophy, and thrombocytopenia to subclinical or chronic disease (Blome et al., 2017; Summerfield & Ruggli, 2015).

Treatment and vaccines for *Streptococcus suis* and CSFV

Historically, *Streptococcus suis* infections have been managed with antimicrobial therapy for treatment and disease prevention in swine populations. However, the increasing prevalence of antimicrobial resistance (AMR) among *S. Suis* isolates, particularly in regions such as Vietnam, where resistance to commonly used agents like tetracycline and enrofloxacin is widespread, underscores the growing limitations of this approach (Hoa et al., 2013). Nevertheless, some

antibacterial agents, namely ampicillin, ceftiofur, enrofloxacin, florfenicol, penicillin, and trimethoprim/sulfamethoxazole, continue to show clinical efficacy, with studies showing low resistance rates (Dechêne-Tempier et al., n.d.; El Garch et al., 2016). In conjunction with antibiotic therapy, supportive care is crucial to improving the chances of recovery in affected animals. Ensuring adequate hydration is important, especially in severe *Streptococcus suis* infections involving septicemia or meningitis (Halbur et al., 2000).

Although antibiotic therapy retains clinical relevance, long-term disease control depends on effective vaccination. largely due to the pathogen's genetic heterogeneity and incomplete understanding of its virulence mechanisms (Neila-Ibáñez, Brogaard, et al., 2021). In the absence of effective commercial vaccines, autogenous (farm-specific) bacterins are widely used across countries, despite variable efficacy and limited cross-serotype protection.

Several studies have evaluated autogenous vaccines under field conditions. In one study, piglets vaccinated with a sonicated *S. Suis* serotype 1/2 vaccine formulated with Rehydragel® and Emulsigen® developed elevated antibody levels. However, the presence of maternal antibodies likely interfered with vaccine responsiveness, and disease outbreaks in the study population prevented a thorough assessment of the vaccine's efficacy (Lapointe et al., 2002). Similarly, Hopkins et al. assessed an autogenous vaccine in a herd infected with a penicillin-resistant *S. Suis* strain, administering doses at weaning and three weeks later; however, vaccination did not significantly reduce clinical cases in piglets aged 6–9 weeks (Hopkins et al., 2019). In contrast, Corsaut et al. demonstrated that vaccinating replacement gilts with three doses of a serotype 7 bacterin substantially enhanced maternal antibody transfer and recall responses (Corsaut et al., 2021). The findings suggest that immunization strategies targeting breeding stock may be more effective, though further comparative studies are needed to optimize protocols. Significantly, the

immunogenicity and efficacy of autogenous vaccines vary widely with formulation specifics such as bacterial concentration, adjuvant composition, and production processes.

Recent advances in reverse vaccinology offer a potential breakthrough in *S. Suis* vaccine development. J. Liu et al. designed three multi-epitope antigens (MEASs) incorporating conserved epitopes from multiple *S. Suis* serotypes, fused to dendritic cell-targeting peptides (J. Liu et al., 2024). These constructs elicited broad humoral and cellular immune responses in mice and conferred cross-serotype protection, with MEAS 1 and MEAS 3 demonstrating strong efficacy against virulent strains. While still in the experimental stage, these findings highlight the potential of epitope-based vaccine designs to overcome the challenges posed by serotype diversity. While promising advances such as multi-epitope antigen designs show cross-serotype protection in experimental models, further research and standardized evaluation are essential to translate these strategies into effective, broadly protective vaccines for use in the field.

Similarly, for classical swine fever (CSF), vaccination remains a key component of disease control, particularly in endemic regions or in areas working towards eradication. Live attenuated vaccines, such as the C-strain (hog cholera lapinized virus, HCLV), have achieved widespread success in preventing outbreaks and eradicating CSF in several countries (Coronado et al., 2021; Risatti et al., 2005). However, these vaccines cannot differentiate between infected and vaccinated animals (the DIVA concept), posing challenges for disease surveillance and international trade (Greiser-Wilke & Moennig, 2004). To address this limitation, research has focused on developing safer and more diagnostically compatible vaccines, particularly targeting the immunodominant E2 glycoprotein. One promising example is the KNB-E2 vaccine, which utilizes a baculovirus-expressed E2 protein formulated with an oil-in-water adjuvant. A single 75 µg dose conferred strong protection against clinical CSF, while even 25 µg was sufficient to prevent disease. The

vaccine elicited protective immunity within two weeks, with immunity persisting for at least four months (Gallagher-Beckley et al., 2015; Madera et al., 2016).

Co-infections involving multiple pathogens are common under intensive swine production conditions, with viruses like PRRSV, PCV2, swine influenza A virus, and bacteria such as *Streptococcus suis* and *Mycoplasma hyopneumoniae* frequently detected together in pigs exhibiting respiratory and other symptoms. Such co-infections often result in more severe or chronic disease manifestations, highlighting a synergistic pathogenic effect between pathogens (Obradovic, Segura, et al., 2021). The immune responses to CSFV and *S. Suis* demonstrate overlapping immunological pathways, including upregulation of SLA Class II molecules and cytokines such as IL-1, IL-6, IL-10, and TNF- α , suggesting potential cross-activation or synergism in immune modulation (Letendre et al., 2018; M. Liu et al., 2011). However, in the context of vaccination, these same viral infections may exert antagonistic or suppressive effects, undermining vaccine efficacy rather than enhancing it.

In veterinary applications, an important yet often overlooked consideration is whether the effectiveness of bacterin-based vaccines is affected by concurrent or preceding viral infections. Despite its practical significance, this area remains underexplored. Halbur et al. investigated the efficacy of various antibiotic treatments and autogenous bacterin vaccination in piglets experimentally co-infected with the virulent VR-2385 strain of PRRSV and *Streptococcus suis* (Halbur et al., 2000). In this model, serotype 2 *S. Suis* bacterin vaccination did not confer notable protection against homologous challenge, whereas ceftiofur treatment significantly reduced mortality. However, the absence of a non-PRRSV-infected control group limits conclusions about the direct impact of PRRSV on vaccine efficacy. Nonetheless, supporting evidence comes from other studies where the same bacterin formulation (Emulsigen-adjuvanted serotype 2) conferred

significant protection in PRRSV-free settings (Baums et al., 2009). Thus, while co-infections may exacerbate disease through synergistic pathogenic mechanisms, they may also diminish vaccine performance through immunological interference, underlining the complex and context-dependent nature of pathogen interactions.

Chapter 4 - Animal Study

Abstract

Effective vaccines are crucial for controlling swine diseases like *Streptococcus suis* and Classical Swine Fever Virus (CSFV), helping to safeguard both animal health and the economic stability of the swine industry. This study investigates the effectiveness of two emulsion-based adjuvants, OW-14 and OW-415, in combination with the CSFV E2 subunit protein and inactivated *S. Suis* antigen for vaccine development. The CSFV E2 protein was selected for this study due to its well-established capacity to enhance immune responses and its proven ability to increase antibody protection when formulated with the OW-14 emulsion adjuvant. Emulsions were prepared using Ticamulsion A-2010 as a surfactant, with mineral oil (OW-14) and synthetic white oil (OW-415) serving as the oil phases. Physical properties, including viscosity and density, were assessed, and both adjuvants demonstrated stability and safety in a pig study. Immunological analysis revealed that OW-14 significantly enhanced E2-specific IgG and neutralizing antibody titers compared to OW-415. When the *S. Suis* antigen was co-administered with the CSFV E2 protein, the OW-14 group exhibited a diminished immune response, suggesting a potential antigenic interference. In contrast, OW-415 induced a robust immune response to both antigens, highlighting the potential for synergistic effects. These findings emphasize the importance of adjuvant selection in optimizing vaccine formulations for swine immunization, offering valuable insights for improving vaccine efficacy in swine populations.

Materials and methods

A well-characterized virulent *Streptococcus suis* serotype 2 strain was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

at the Veterinary Diagnostic Laboratory, Kansas State University. Briefly, the bacteria were grown overnight on TSA supplemented with 5% sheep blood at 37 °C. Isolated colonies were grown in 5 mL of Todd–Hewitt Broth (THB; RPI, USA) and incubated for 8 hours at 37 °C. Optical density at 600 nm (OD₆₀₀) was recorded at 2-hour intervals to monitor bacterial growth (Fig. 4.1B). A 1:100 dilution (250 µL) of the 8-h culture was added to tubes containing 25 mL fresh THB each and incubated for 16 h at 37 °C. Bacteria were centrifuged at 10,000 × g for 10 min at 4 °C, and pellets were re-suspended in 1 mL of sterile phosphate-buffered saline (PBS), centrifuged at 10,000 × g for 10 min at 4 °C. Two additional washing steps of the bacterial pellet with PBS were performed using the same centrifugation settings. After the final wash, the pellet was re-suspended in 13 mL of sterile PBS, and the bacterial count was performed. To inactivate the bacteria, formaldehyde was added to a final concentration of 0.5% and incubated for 48 h at 4 °C. The mixture was screened for sterility and washed three times with sterile PBS, using the same centrifugation settings. The bacterin pellet was re-suspended in 13 mL of sterile PBS, and thiomersal was added to a final concentration of 0.01% v/v. Final vaccine preparations contained the equivalent of 10⁹ CFU/mL per dose.

Sterility testing of the final bacterin preparation was conducted by plating samples on Thioglycolate Agar (TGA) for anaerobic bacteria, Tryptic Soy Agar (TSA) for aerobic bacteria, TSA with 5% defibrinated calf blood for fastidious organisms, and Sabouraud Dextrose Agar (SDA) for fungal contaminants. Plates were incubated at 37 °C (TSA, TSA with blood, and TGA) or 25 °C (SDA) for 14 days. No microbial growth was observed, confirming effective inactivation and aseptic preparation of the bacterin (Fig. 4.2).

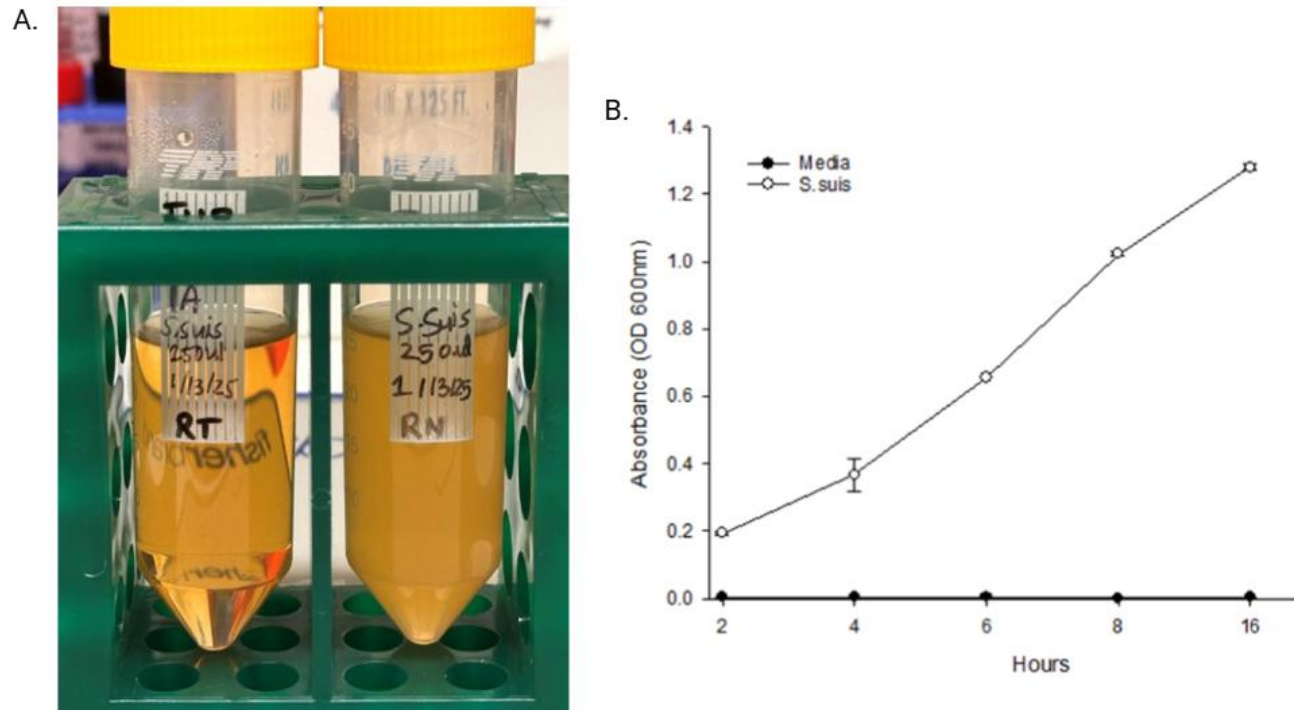


Figure 4.1: (A) Turbidity of *Streptococcus suis* after 16 hours of growth (T16). (B) Optical density (OD₆₀₀) of *S. Suis* measured at different time points

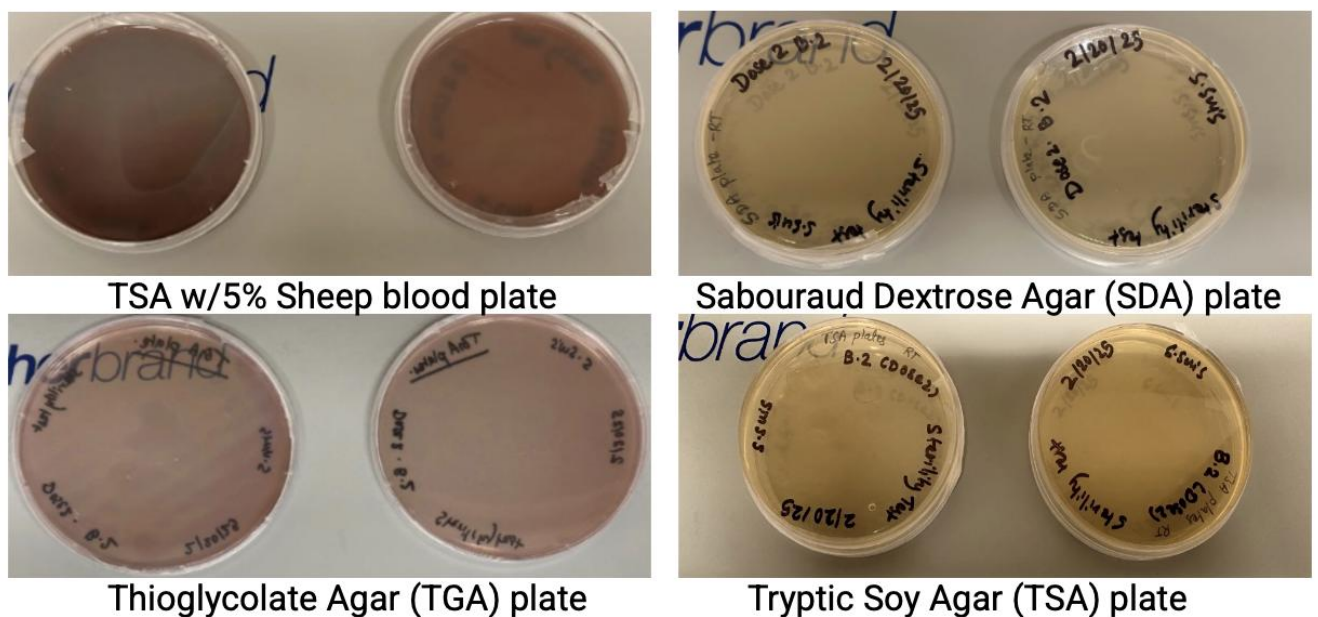


Figure 4.2: Sterility test of formalin-inactivated *Streptococcus suis* preparation. No growth was observed after 14 days of incubation.

Multiplex PCR for *Streptococcus suis* serotyping

Multiplex PCR (mPCR) assays were performed as described previously (Z. Liu et al., 2013). Bacterial colonies were streaked from glycerol stocks onto TSA with 5% defibrinated calf blood and incubated overnight at 37 °C.

Genomic DNA was extracted using the thermal lysis protocol (Espinosa et al., 2013). For the dry block method, colonies were suspended in 50 µL nuclease-free water, heated at 100 °C for 5 min, cooled at –20 °C for 10 min, and centrifuged at 3,000 × g for 10 min. For the thermal cycler method, samples were heated at 95 °C for 15 min. Supernatants were used as DNA templates.

Multiplex PCR reactions were performed in a final volume of 20 µL, consisting of 10 µL of DreamTaq Green PCR Master Mix 2x (ThermoScientific, USA), 0.2 µM of primer targeting serotypes $\frac{1}{2}+2$, 9, 11, and 1+14, 5 µL of the extracted DNA template, and nuclease-free water. PCR amplification was carried out using a Bio-Rad thermal cycler (Bio-Rad, USA) under the following conditions: an initial denaturation at 95°C for 3 minutes; 30 cycles of denaturation at 95°C for 20 seconds, annealing at 62°C for 90 seconds, and extension at 72°C for 1 minute; followed by a final extension at 72°C for 5 minutes. mPCR products were separated by electrophoresis on a 2% agarose gel stained with SYBR Safe DNA stain (Invitrogen, USA) in 1X TAE buffer. Electrophoresis was performed at 100V for 40–45 minutes. A 1 kb and 100 bp DNA ladder was used as a molecular weight marker. Gels were visualized using an iBright500 imaging system (ThermoScientific, USA). The expected sizes of the mPCR products were 450bp for serotype $\frac{1}{2}+2$, 330bp for serotype 9, 896bp for serotype 11, and 550bp for serotype 1+14, as previously described (Z. Liu et al., 2013). Samples were considered positive when a specific amplification product corresponding to the expected size for each serotype was observed. Negative controls remained amplification-free throughout all reactions.

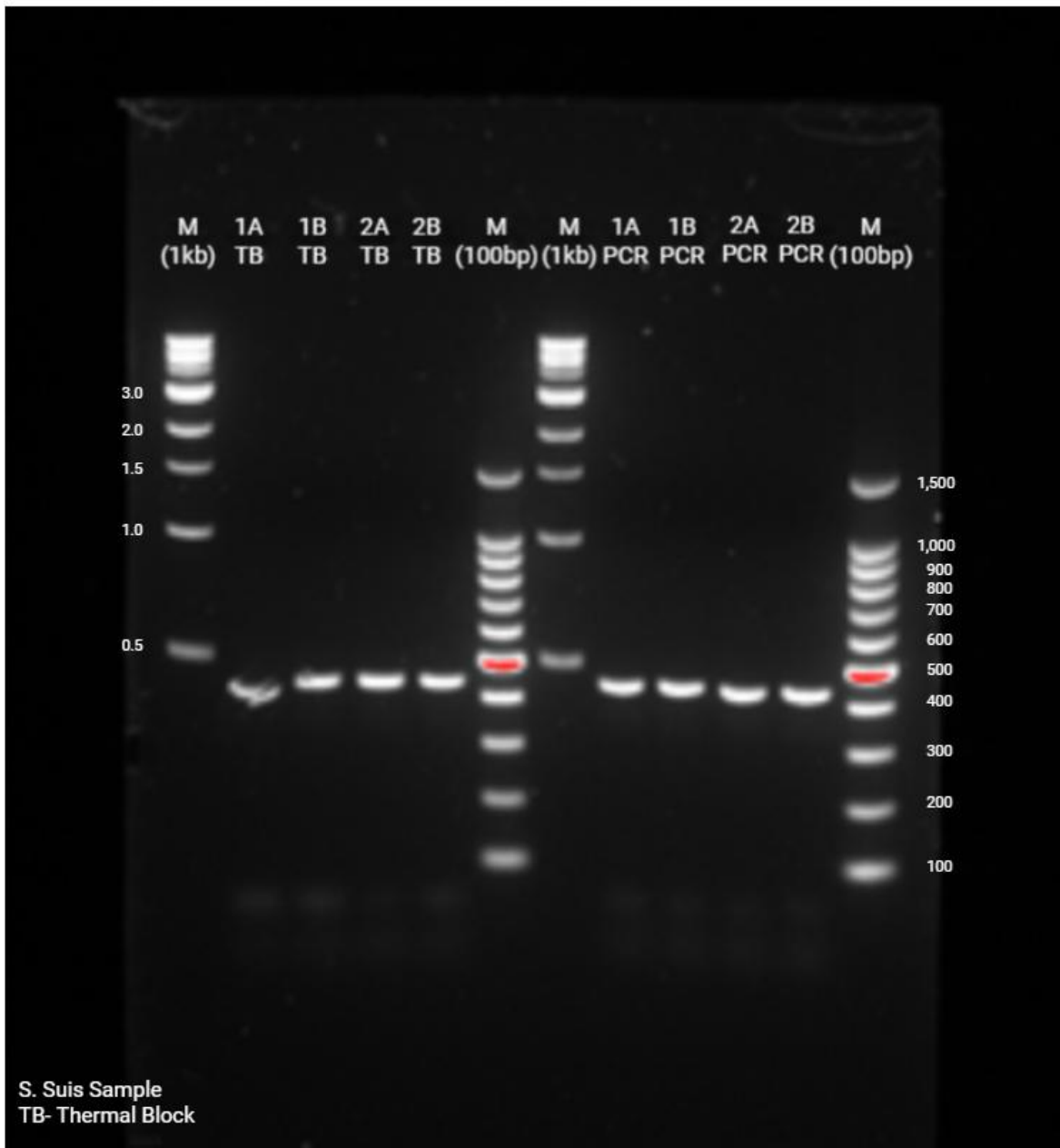


Figure 4.3: Agarose gel electrophoresis of mPCR products of *Streptococcus suis* serotype 2.
Samples 1 and 2 were obtained from the same *S. Suis* bacterial source in two separate tubes. DNA extraction was performed by two methods (PCR and thermal block), with two technical replicates (A and B) per method. M: molecular weight marker.

Formulation of *S. Suis* bacterin and CSFV E2 subunit vaccine with different adjuvants

Two emulsion-based adjuvants, OW-14 and OW-415, were formulated and used to prepare bivalent vaccines containing formalin-inactivated *S. Suis* serotype 2 at a final concentration of 10^9 CFU/mL and CSFV E2 subunit vaccine at a concentration of 50 µg/dose.

Adjuvant emulsions were prepared by first dissolving Ticamulsion A-2010, a gum Arabic-based emulsifier, in deionized water at 7.5% (w/v). For OW-14, mineral oil (Penreco Drakeol 5) was incorporated into the aqueous phase at 15% (w/v), while for OW-415, the same procedure was followed using Shell Ondina X 415 as the oil phase. The mixtures were homogenized at 10,000 rpm for 15 minutes using a Silverson L5M-A mixer, followed by five passes through a Microfluidizer M-110P operating at approximately 10,000 psi to achieve stable and uniform emulsions. Prepared emulsions were stored at 4 °C until use. On the day of vaccination, the inactivated *S. Suis* antigen and CSFV E2 subunit protein were manually mixed with each emulsion adjuvant at a 2:1 (v/v) antigen-to-adjuvant ratio. This resulted in final adjuvant concentrations of 5% (w/v) oil (Drakeol 5 or Ondina X 415) and 2.5% (w/v) Ticamulsion A-2010 per vaccine dose. The final formulation had a pH of 7.58. The physical properties of the emulsions were evaluated to assess formulation stability. The dynamic viscosity of OW-14 and OW-415 was measured using a Gilmont® Falling-ball Viscometer at 25 °C. For each measurement, 7 mL of the emulsion was loaded into the viscometer, and the time required for the ball to descend through the fluid was recorded. The viscosity of OW-415 was 17.70 cP. The density of OW-14 and OW-415 was measured and recorded as 1.01 g/mL and 0.99 g/mL, respectively.

EMULSION PREPARATION

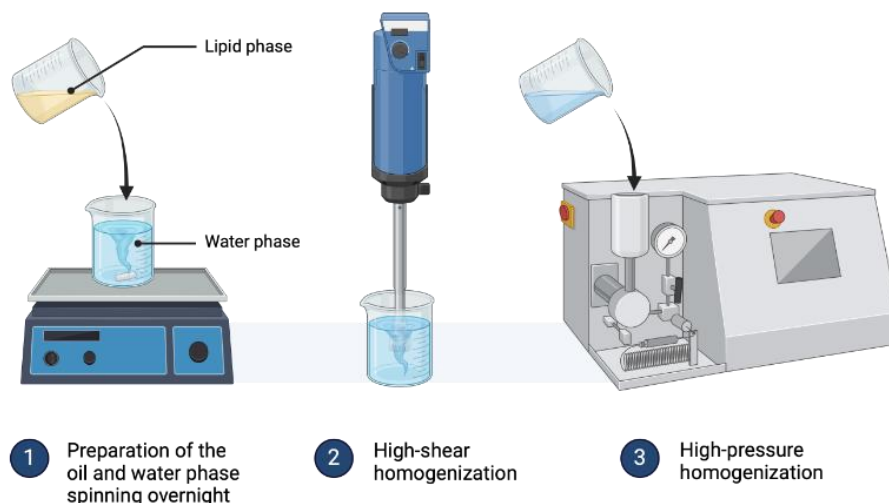


Figure 4.4: Schematic illustration of emulsion preparation (Created with BioRender.com)

Swine vaccination experiments

The animal study was conducted in compliance with guidelines approved by the Institutional Animal Care and Use Committee (IACUC). All animals were housed at the Large Animal Research Center (LARC) at Kansas State University. A total of 30 conventional Large White –Duroc crossbred pigs, specific-pathogen-free (SPF) and weaned at three weeks of age, were randomly assigned to six experimental groups ($n = 5$ per group). Pigs were immunized with 2 mL subunit and inactivated vaccine formulations in a 2:1 (v/v) antigen-to-adjuvant ratio. Group 1 received the CSFV E2 subunit protein co-administered with OW-14 adjuvant (OW-14 + E2). Group 2 was immunized with CSFV E2, *Streptococcus suis* antigen, and OW-14 adjuvant (OW-14 + E2 + *S. Suis*). Group 3 received E2 combined with OW-415 adjuvant (OW-415 + E2), while Group 4 was administered E2, *S. Suis*, and OW-415 adjuvant (OW-415 + E2 + *S. Suis*). Group 5

received E2 protein alone, and Group 6 was administered *S. Suis* antigen only. Vaccinations were administered intramuscularly with a 21-day interval between the two doses.

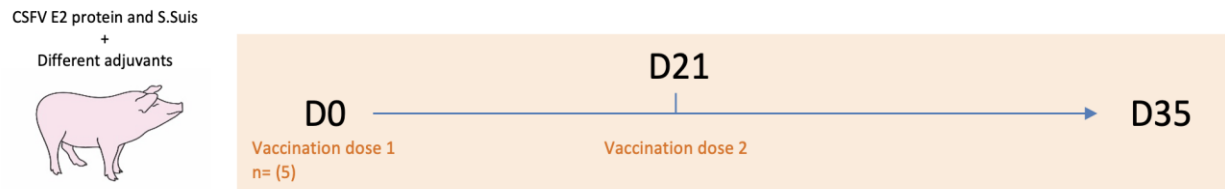


Figure 4.5: Animal Study Plan: CSFV E2 Protein and *S. Suis* Vaccination with Different Adjuvants

Clinical evaluation of pigs

During the study, the clinical evaluation of pigs was carried out by our research team. Daily monitoring included subcutaneous temperature measurement and routine clinical assessments. The weight of the pigs was recorded weekly, while pig health and vaccine injection sites were monitored daily. Sera were collected on days 0, 7, 14, 28, and 35 for subsequent immunological analyses. Following vaccination, all pigs grew consistently, and no difference in weight gain was observed between the animals in separate groups (Fig. 4.6B). Small (1–2 cm) subcutaneous bumps were observed at the injection sites in OW-14 + E2 and OW-14 + E2 + *S. Suis* vaccinated pigs. By day 28 of the study, they decreased in size and mostly disappeared (data not shown). No issues were observed in pig health, and all animals survived by the end of the study.

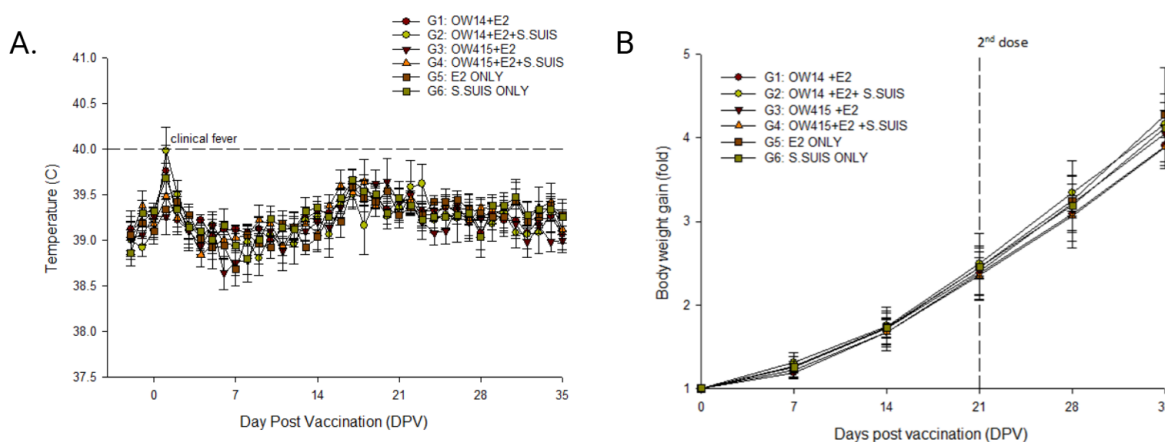


Figure 4.6: Safety effect of OW-14 and OW-415 emulsions administered with CSFV-E2 antigen and Inactivated *S. Suis*. (A) shows subcutaneous temperatures in all pig groups from Day 0 to Day 35. (B) shows body weight gain in pigs throughout the study period. Data are presented as mean $\pm$ SD for pigs in each group.

Quantification of anti-E2 and anti-*Streptococcus suis* antibodies

E2-specific IgG antibody titers in pig sera were determined using an enzyme-linked immunosorbent assay (ELISA), as described previously (Madera et al., 2016). Briefly, 96-well flat-bottom microtiter plates (Corning®) were coated overnight with 100 ng/ml of purified E2 antigen. Plates were washed three times with ELISA Wash Buffer (0.05% Tween 20 in PBS) and blocked with 2% skim milk in PBS for 2h at room temperature (RT). Diluted sera (each sample in duplicate) were added to the plates and incubated for 1 hour at RT, followed by washing 3 times with ELISA Wash Buffer. Goat anti-swine IgG conjugated with horseradish peroxidase (HRP; A5607, Sigma, USA; dilution 1/5,000) in ELISA Blocking Buffer was added to wells at 100 μ l/well and incubated for 1 hour at RT, followed by washing 5 times with ELISA Wash Buffer. 3,3',5,5'-Tetramethylbenzidine- (TMB-) stabilized chromogen (Invitrogen, USA) was used to develop the ELISA plates for 10 minutes at RT, followed by the addition of 2N sulfuric acid (RICCA Chemical Co., USA). Optical density (OD) was measured at 450 nm with SoftMax® Pro 6.4 Software (Molecular Devices, USA).

Anti-*Streptococcus suis*-specific IgG responses were evaluated by indirect ELISA, as previously reported (Corsaut et al., 2020), with minor modifications. Briefly, 96-well high-binding microtiter plates were coated with *S. Suis* antigen suspension (1×10^8 CFU/mL in PBS), prepared from the same inactivated strain used for vaccine formulation, and incubated overnight at 4 °C. Plates were washed three times with PBS containing 0.05% Tween-20 (PBS-T) and blocked with 2% skim milk in PBS for 2h at RT. Serum samples were diluted starting at 1:50 in blocking buffer and added to the plates, incubated for 1h at RT. After washing 3 times with PBS-T, plates were incubated with horseradish peroxidase (HRP; A5607, Sigma, USA; dilution 1/5,000) in blocking buffer for 1 h at RT. Following five washes with PBS-T, plates were developed using TMB substrate for 10 min at RT. The reaction was stopped with 2 N H₂SO₄, and absorbance was measured at 450 nm using a microplate reader.

A neutralizing monoclonal antibody-based competitive ELISA for E2

To detect E2-specific neutralizing antibodies, a competitive ELISA was performed, as previously reported (L. Wang et al., 2020). Briefly, 96-well high-binding clear flat-bottom microtiter plates (Corning®) were coated overnight at 4°C with 0.625 µg/ml of purified C-strain E2 antigen and subsequently washed 3 times with ELISA Wash Buffer (PBS containing 0.05% Tween-20). Plates were blocked with 200 µl/well of Blocking Buffer (2% fetal bovine serum in PBST) for 1 hour at 37°C, followed by a wash with 300 µl/well of Wash Buffer. Pig serum samples (each sample in duplicate), diluted 1:5 in Blocking Buffer, were added at 50 µl/well and mixed with an equal volume (50 µl/well) of HRP-conjugated monoclonal antibody (HRP-6B211; 2.5 µg/ml in Blocking Buffer). Plates were incubated at 37°C for 1 hour and washed 5 times with Wash Buffer. TMB substrate was added for 10 min at RT, and the reaction was stopped by adding

100 µl/well of 2N sulfuric acid. Absorbance was measured at 450 nm with SoftMax® Pro 6.4 Software (Molecular Devices, USA).

Cytokine assays

Serum cytokine levels were quantified using the ProcartaPlex Porcine Cytokine 9-Plex Assay (Invitrogen, Waltham, MA), following the protocol provided by the manufacturer. Each sample was diluted 1:2 and analyzed in duplicate. Cytokines were measured using a Luminex MAGPIX platform, with Bio-Plex Manager MP software (Bio-Rad Laboratories, Hercules, CA). A substantial number of samples yielded cytokine values below the assay's detection threshold, limiting the dataset for statistical evaluation. The median fluorescence intensity for each cytokine was subjected to $\log_{10}$ transformation before analysis (Breen et al., 2016) .

Statistical analysis

Experimental data were expressed as the mean $\pm$ standard error (SE), calculated from groups of five animals. Group comparisons were analyzed using one-way ANOVA with SigmaPlot 11.0 software (Systat Software Inc., USA). Differences were considered statistically significant when * $p < 0.05$.

Results

E2-specific IgG and neutralizing antibody responses in vaccinated pigs

The E2-specific ELISA results are presented in (Fig. 4.7A and B). By day 28 post-immunization, pigs vaccinated with OW-14 + E2 exhibited significantly higher serum IgG titers than those receiving OW-415 + E2 ($p = 0.009$) (Fig. 4.7B). A marked increase in IgG titers was observed across all groups following the booster dose administered on day 21. However, when E2 was co-administered with inactivated *Streptococcus suis*, no significant difference in E2-

specific IgG titers was found between the OW-14 and OW-415 groups on day 28 ($p = 0.729$). These results suggest that while OW-14 enhances E2-specific IgG responses, the addition of *S. Suis* may be detrimental to the OW-14 adjuvant.

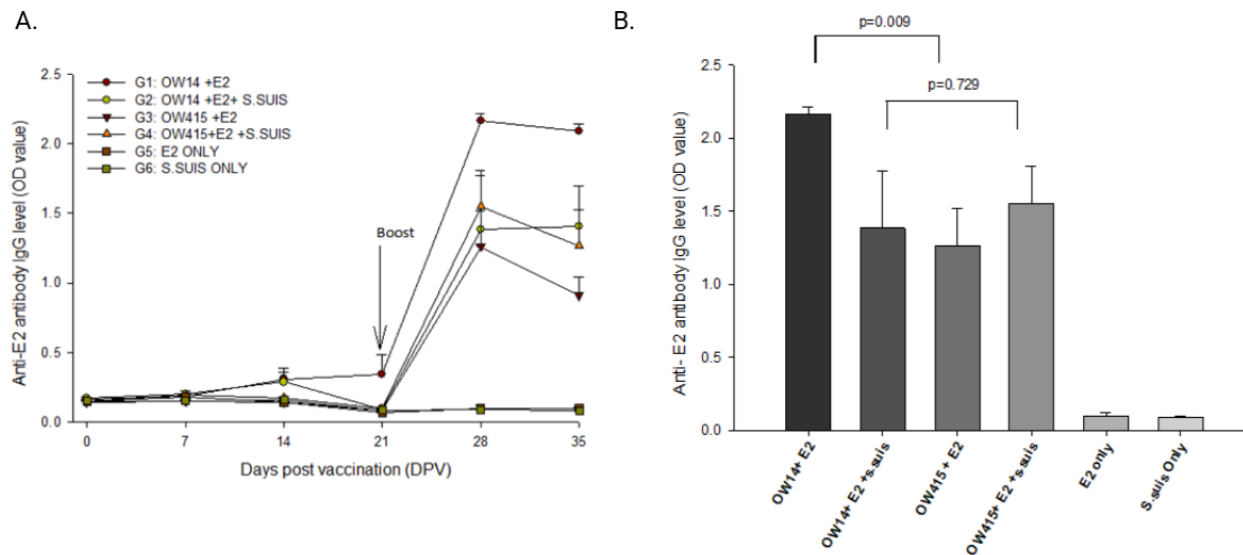


Figure 4.7: Immunological effect of OW-14 and OW-415 injected with E2 antigenic protein from days 0 to 28 [dilution 1/1000] (A) on day 28 [dilution 1/1000] (B). Error bars: $\pm$ SEM.

(Fig.4.8A and B) represents Neutralizing antibody titers, assessed by a monoclonal antibody-based competitive ELISA for classical swine fever virus (CSFV) C-strain. The highest neutralizing antibody titers were detected in pigs immunized with OW-14 + E2, consistent with the ELISA IgG results. Although neutralizing titers in the OW-14 + E2 vaccine group exceeded those in OW-415 + E2 on day 28, the difference was insignificant ($p = 0.138$; Figure 4.8B). Notably, the OW-14 + E2 + *S. Suis* vaccinated group showed significantly higher neutralizing titers than the OW-415 + E2 + *S. Suis* vaccine group on day 28 ($p = 0.049$). Higher neutralizing antibody levels were observed for OW-14 + E2 and OW-14 + E2 + *S. Suis* on both day 28 and day 35 compared to other OW-415 vaccinated groups.

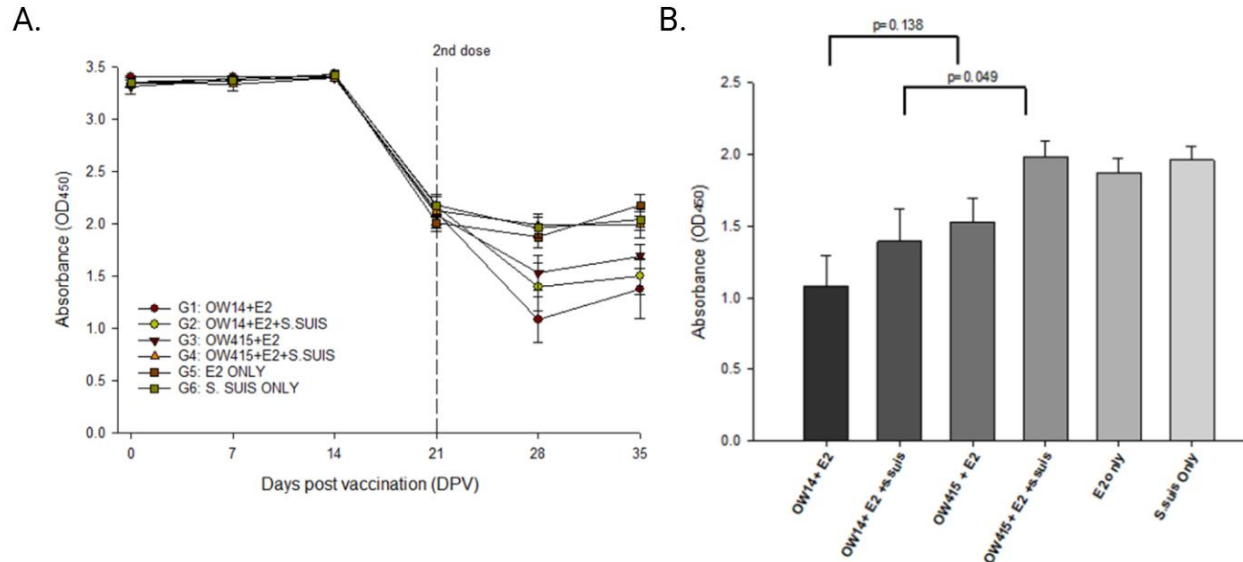


Figure 4.8: Neutralizing antibody responses against E2 measured by monoclonal antibody-based competitive ELISA from days 0 to 28 (A) on day 28 (B). Error bars: $\pm$ SEM.

***S. Suis*-specific IgG antibody responses in vaccinated pigs**

The inactivated vaccine containing *Streptococcus suis* serotype 2 antigen successfully induced measurable IgG antibody responses in all vaccinated groups. (Fig. 4.9A) demonstrates the results of the ELISA of serum samples from the pig study. At day 7 post-vaccination, antibody titers peaked across all groups, with a subsequent decline observed over time. No significant antibody titers were produced by the CSFV E2-only group (Fig. 4.9). On day 28, pigs immunized with OW-415 + E2 + *S. Suis* and *S. Suis* antigen-only vaccines had very similar IgG levels in the serum ($P = 0.358$), while the groups vaccinated with OW-14 + E2 + *S. Suis* had significantly lower titers of IgG antibodies ($p < 0.001$) (Fig. 4.10). On day 7, the IgG antibody titers were higher in the OW-415 + E2 + *S. Suis* vaccine group compared to the OW-14 + E2 + *S. Suis* and *S. Suis*-only vaccine group, although it should be noted that there was no statistically significant difference between the three groups ($p = 0.391$ and $p = 0.061$ respectively) (Fig. 4.9C and D). OW-415 demonstrated a boosted antibody response compared to subsequent time points; however, antibody

titers did not reach the initial peak observed on day 7. Interestingly, the *S. Suis* only group also had high antibody levels, but no significant difference was observed compared to the OW-415 adjuvanted group ($P = 0.359$). This finding suggests that the antigen alone could produce a robust immune response comparable to the response generated by the OW-415 adjuvanted vaccine.

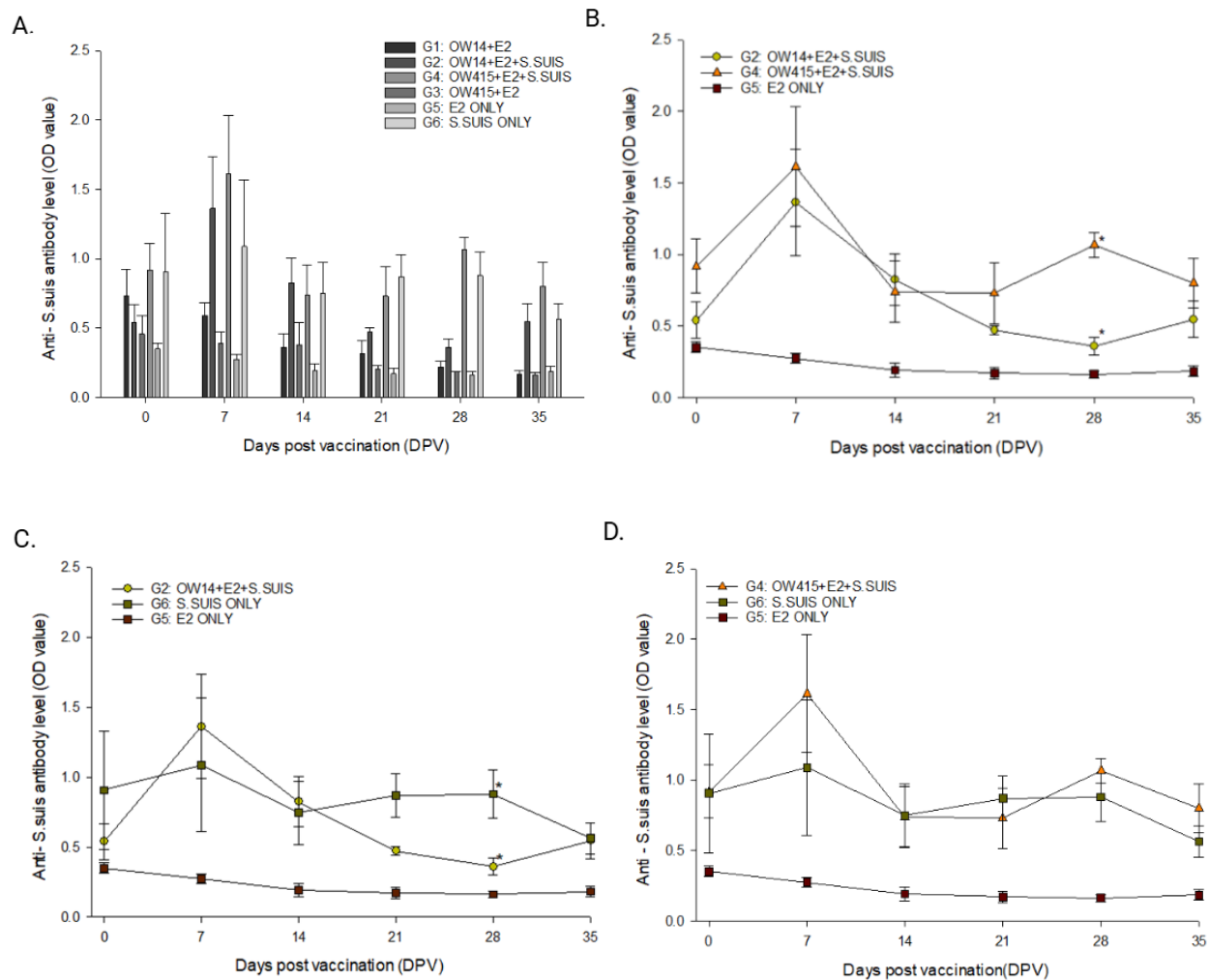


Figure 4.9: *S. Suis*-specific IgG responses measured by indirect ELISA.

(A) Anti-*S. Suis* IgG antibody levels detected in pig samples from day 0 post vaccination to day 35 [dilution 1/1000] **(B)** Comparison of IgG responses among Group 2, 4 and 5 **(C)** Comparative IgG responses between OW-14 + E2 + *S. Suis*, OW-415 + E2 + *S. Suis*, and *S. Suis* only groups **(D)** IgG antibody comparison among Group 4, 6, and 5.

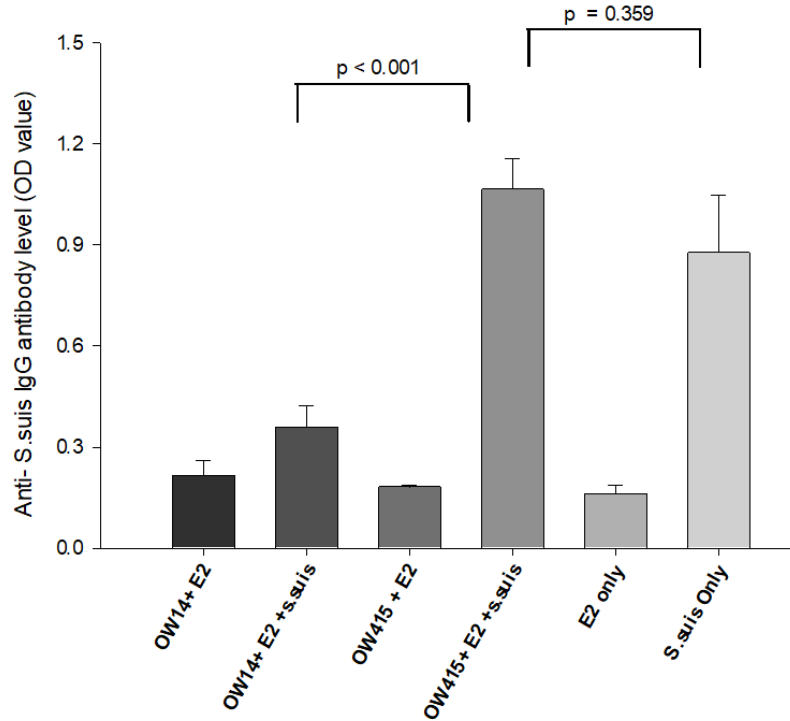


Figure 4.10: *S. Suis*-specific IgG responses measured by indirect ELISA on day 28 of the study (dilution 1/1000). Error bars: $\pm$ SEM.

Serum cytokine responses following vaccination

The systemic cytokine profiles of pigs immunized with *Streptococcus suis* (*S. Suis*) antigens were evaluated at 7 and 28 days post-vaccination (DPV) by quantifying nine cytokines in serum: IFN- α , IFN- γ , IL-1 β , IL-4, IL-6, IL-8, IL-10, IL-12, and TNF- α . As shown in Figure 4.11, comparisons were made across four experimental groups: OW-14+E2+*S. Suis*, OW-415+E2+*S. Suis*, *S. Suis*-only, and a negative control group receiving E2-only. To facilitate interpretation, the cytokines were categorized into Th1-associated markers (IFN- α , IFN- γ , IL-1 β , IL-8, IL-12, TNF- α) and Th2-associated markers (IL-4, IL-6, IL-10). All fluorescence intensity (FL) values were log₁₀-transformed before statistical analysis. One-way ANOVA was employed to identify significant differences in cytokine expression among treatment groups at each time point.

Th1-associated cytokine IFN- α levels increased from Day 7 to Day 28 across all groups, with the OW-415+E2+S. *Suis* group showed the highest expression on Day 28. However, this difference was not statistically significant compared to the OW-14+E2+S. *Suis* group ($P = 0.439$). In contrast, IFN- γ responses remained relatively unchanged across all groups between Day 7 and Day 28. A modest elevation in IL-12 was observed in the OW-14+E2+S. *Suis* group relative to OW-415+E2+S. *Suis*, but this difference also did not reach statistical significance ($P = 0.259$).

Assessment of the Th2 cytokines IL-4, IL-6, and IL-10 revealed no statistically significant differences among groups on Day 7. By Day 28, the OW-415+E2+S. *Suis* group showed increased IL-10 levels compared to the OW-14+E2+S. *Suis* group; however, this difference was not statistically significant ($P = 0.354$). IL-4 and IL-6 levels remained comparable across all groups in both day 7 and day 28 (Fig. 4.12).

Overall, no statistically significant differences between the Th1- or Th2-associated cytokine responses were detected between treatment groups at either Day 7 or Day 28. These findings suggest that the adjuvant formulations OW-14 and OW-415, when combined with E2 and inactivated *S. Suis* antigens, did not differentially skew the immune response toward either a Th1 or Th2 profile. Instead, the cytokine patterns observed indicate a generally balanced systemic immune response across all vaccinated groups, without evidence of polarization.

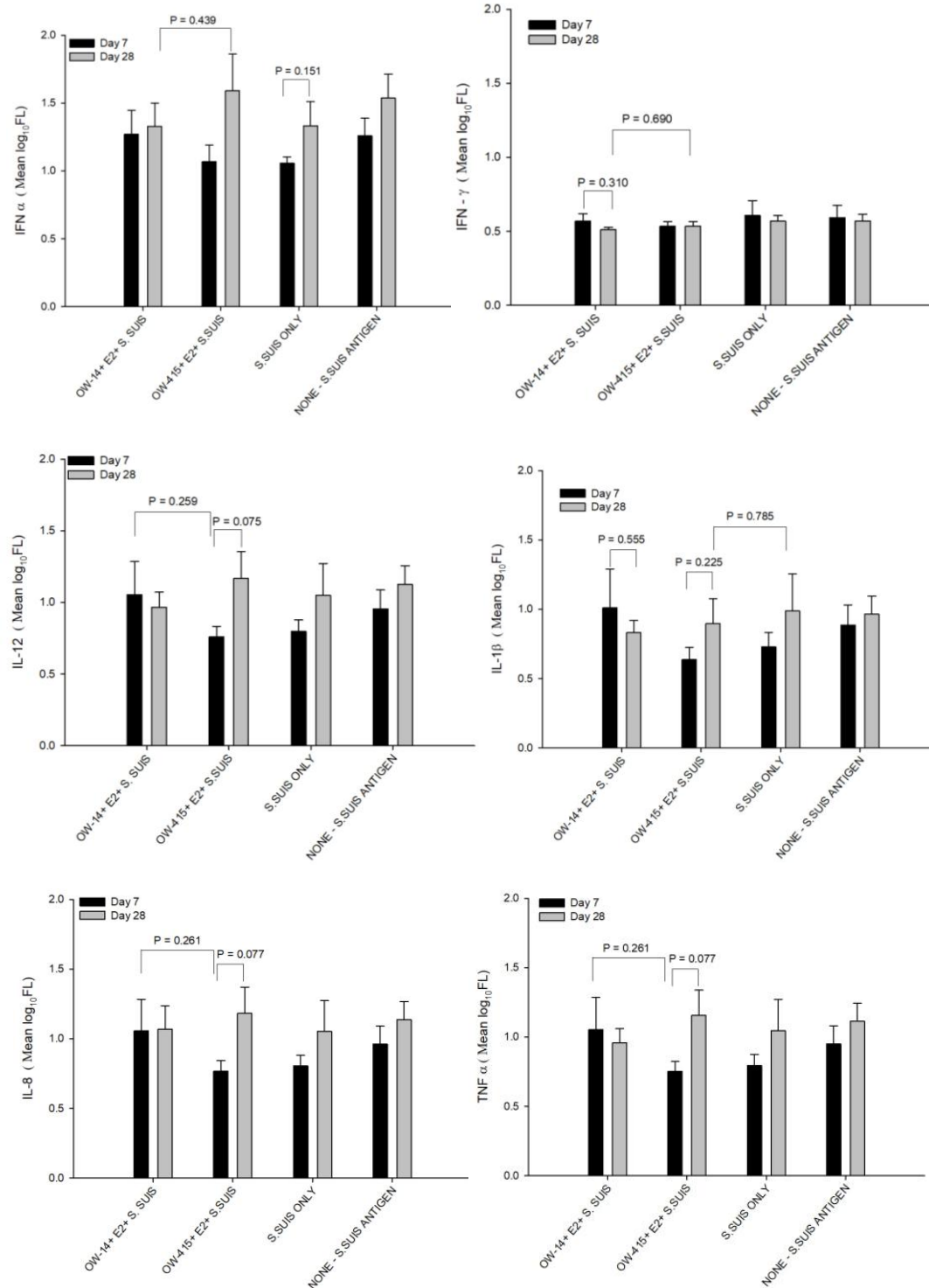


Figure 4.11: Th1-associated cytokine responses in serum of vaccinated pigs at 7 and 28 days post-vaccination. Log₁₀-transformed median fluorescence intensity (FL) values of IFN- α , IFN- γ , IL-12, IL-1 β , IL-8 and TNF α measured by cytokine assay in serum from pigs immunized with OW-14+E2+S. *Suis*, OW-415+E2+S. *Suis*, *S. Suis* only, or NONE - *S. Suis* antigen (E2-only, negative control). Bars represent means $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA.

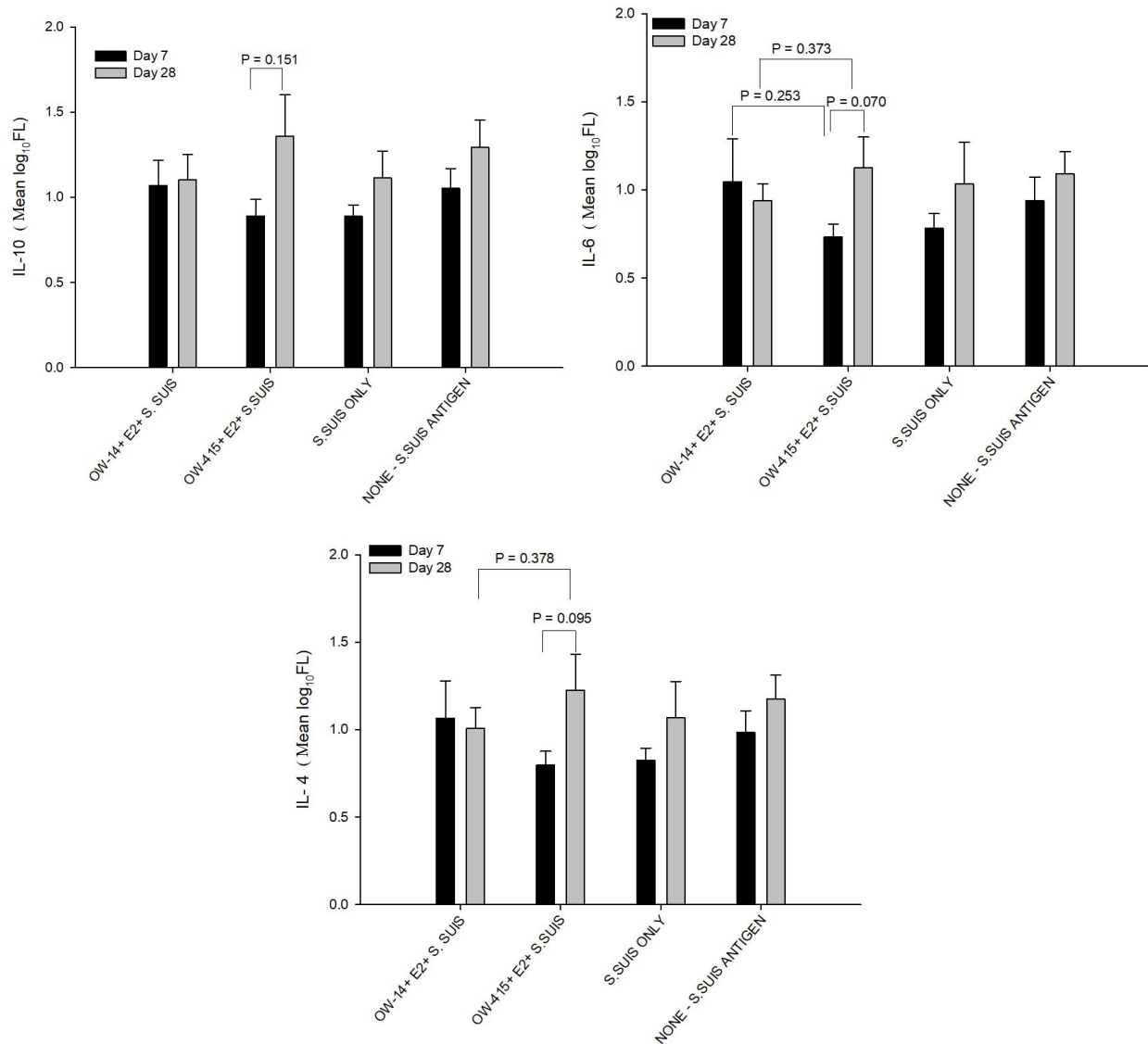


Figure 4.12: Th2-associated cytokine responses in serum at 7 and 28 days post-vaccination. Log₁₀-transformed mean fluorescence intensity (FL) values for IL-10, IL-6, and IL-4 were measured in serum from pigs immunized with OW-14+E2+S. *Suis*, OW-415+E2+S. *Suis*, *S. Suis* only, or NONE – *S. Suis* antigen (E2-only control). Bars represent means $\pm$ SEM (n = X per group). Statistical comparisons between selected groups were performed using one-way ANOVA. No statistically significant differences were detected among groups for any cytokine at either time point.

Discussion

Adjuvanted vaccines have played a pivotal role in veterinary immunization for decades, underscoring the importance of adjuvants in enhancing immune responses, particularly against antigens with inherently low immunogenicity (Di Pasquale et al., 2015; Madera et al., 2022). Among these, emulsions are widely recognized as cost-effective and versatile adjuvants. The combination of Ticamulsion A-2010 (modified starch-based plant emulsifier) as a surfactant with mineral oil has been studied to optimize droplet size and distribution, enhancing antigen bioavailability and prolonging the immune-stimulating depot effect (Galliher-Beckley et al., 2015; Madera et al., 2022). Despite their widespread use, mineral oil-based adjuvants, especially water-in-oil (w/o) emulsions, are associated with local and systemic adverse reactions (Stils, 2005).

In this study, we evaluated two novel emulsion adjuvants, OW-415 and OW-14, formulated with the E2 subunit antigen and inactivated *Streptococcus suis* antigens. OW-14 has previously been shown to induce robust IgG responses against the CSFV E2 subunit vaccine (Madera et al., 2016). OW-415 emulsion, formulated with synthetic-white oil, is free from impurities common in conventional mineral oils, such as aromatics and sulfur, resulting in greater chemical stability and lower reactivity (*Shell-Gtl-Performance-Fluids-Brochure-2024-Digital.Pdf*, n.d.). Based on the emulsion stability studies in Chapter 2, OW-415 remained physically stable for over 90 days under various temperature conditions, with no evidence of phase separation or creaming. This stability is essential for vaccines intended for livestock populations where cold chain infrastructure may be inconsistent or unavailable.

Both adjuvants exhibited favorable safety profiles; vaccinated pigs showed no clinical abnormalities and maintained consistent weight gain throughout the study (Fig.4.6). Notably, pigs immunized with the OW-415 adjuvant showed no subcutaneous bumps at the injection site, while

those receiving OW-14 developed a transient bump that cleared by day 28. This highlights the difference in tissue retention, with OW-14 potentially staying longer at the injection site, which may influence the duration and magnitude of the immune response. OW-14 adjuvanted E2 induced stronger E2-specific IgG, and higher anti-CSFV neutralizing antibody titers compared to OW-415 or E2 alone. When co-formulated with E2 and inactivated *S. Suis*, the OW-415 adjuvant group induced higher antibody titers than the OW-415 + E2 vaccine group. This suggests a synergistic effect in the multivalent context, possibly driven by enhanced activation of antigen-presenting cells (APCs) and broader humoral responses (J. Liu et al., 2024).

Although OW-415 + E2 induced a relatively weaker antibody response, this may be partially attributed to its mean droplet size of 221.7 nm, which exceeds the <200 nm range typically considered optimal for uptake by antigen-presenting cells (APCs) (Kanchan & Panda, 2007; Oyewumi et al., 2010; Shah et al., 2015). However, this size-based explanation alone does not fully account for the observed immunogenicity differences. Notably, OW-14 demonstrated superior immunological performance despite having an even larger average droplet size of 240 nm, suggesting that the droplet size, while relevant, is not the sole determinant of adjuvant efficacy. This discrepancy aligns with previous findings that report inconsistent correlations between droplet size and immune activation (Oyewumi et al., 2010). The surfactant composition may contribute to this discrepancy, as it plays a critical role in stabilizing the emulsion and enhancing both humoral and cellular immune responses by promoting antigen uptake and immune cell stimulation (Ko & Kang, 2018b).

Given the increasing regulatory restrictions on prophylactic and metaphylactic antibiotic use and the lack of effective commercial vaccines for *S. Suis*, autogenous bacterins remain a primary strategy for managing *S. Suis* outbreaks in swine herds (Donlan, 2002; Rieckmann et al.,

2020). Few studies have systematically examined how different adjuvants influence immune responses to *S. Suis* bacterin vaccines (Obradovic, Corsaut, et al., 2021; Pallarés et al., 2004; Wisselink et al., 2001). Understanding these differences is critical for guiding vaccine formulation strategies. In ELISA analysis of anti-*S. Suis* IgG responses, distinct differences in immunogenicity were observed across vaccine formulations. On Day 0, pigs exhibited elevated antibody titers, likely due to maternally derived antibodies (Corsaut et al., 2021). These titers declined overtime in all *S. Suis*-free groups, reaching the lowest point on Day 35. Following the first immunization, IgG titers were notably elevated in the OW-14 group by Day 7. Although the OW-415 + E2 + inactivated *S. Suis* group showed enhanced antibody responses, no statistically significant differences were detected at Day 7 compared to the OW-14 + E2 + *S. Suis* and *S. Suis* only groups. By Days 14 and 21, antibody titers declined significantly across all groups. After the booster on Day 28, a marked increase in anti- *S. Suis* antibodies was observed in the OW-415 + E2 + *S. Suis* group, although these levels remained lower than the Day 7 peak. Meanwhile, the OW-14 group maintained low antibody titers, with only a modest increase by Day 35.

While suggestive of differences in vaccine efficacy, it is essential to note that these trends may not necessarily imply a causative relationship between the adjuvant formulation and immune response. The observed lack of statistical significance between OW-415 + E2 + *S. Suis* and OW-14 + E2 + *S. Suis* at Day 7 indicates that further investigation with larger sample sizes and more timepoints is necessary to confirm these findings. The preparation method of inactivated bacterins may also influence immunogenicity. Formalin inactivation can damage key epitopes through protein cross-linking or denaturation, potentially impairing protective efficacy (Halbur et al., 2000; Holt et al., 1990; Pallarés et al., 2004). A previous study reported only 40% protection using a formalin-inactivated *S. Suis* bacterin with aluminum hydroxide adjuvant (Pallarés et al., 2004).

Consistent with these findings, we observed variability in *S. Suis*-specific IgG ELISA results, suggesting that formalin inactivation may reduce antigenic integrity and limit immunoassay sensitivity.

Interestingly, the *S. Suis*-only antigen group maintained relatively higher antibody titers than the OW-14-adjuvanted group. The immunogenicity of the inactivated *S. Suis* antigen may be more influential than initially anticipated. This finding implies that further optimization of the inactivation process and antigen formulation could enhance vaccine performance, even in the absence of adjuvants. The relatively high antibody titers observed in the *S. Suis*-only group also raise questions about the potential role of pre-existing maternal antibodies (Corsaut et al., 2021; Lapointe et al., 2002). Similar observations were reported by (Arndt et al., 2018; Obradovic, Corsaut, et al., 2021). The source of these baseline antibodies remains unclear, particularly since the pigs originated from a herd without reported clinical *S. Suis* disease at weaning. It is plausible that the detected antibodies reflect maternal transfer from sows previously exposed to non-pathogenic or commensal *S. Suis* strains, or even to antigenically similar bacteria residing in the upper respiratory tract (Corsaut et al., 2020). Furthermore, because a whole-cell bacterial antigen was used in the ELISA assay, it is possible that cross-reactive antibodies targeting other *S. Suis*-like organisms contributed to the measured titers (Obradovic, Corsaut, et al., 2021).

Cytokine profiling of serum samples at 7 and 28 days post-vaccination showed no significant differences in either Th1- (IFN- α , IFN- γ , IL-12, IL-1 β , IL-8, TNF- α) or Th2-associated (IL-4, IL-6, IL-10) cytokines across *S. Suis* groups. Although the OW-415 + E2 + *S. Suis* group exhibited a slight increase in IL-10 and IFN- α at Day 28, these differences were not statistically significant. While OW-415 showed modest elevations in specific cytokines, OW-14 consistently induced stronger humoral responses. This disconnect between cytokine profiles and antibody titers

suggests that adjuvants may differentially stimulate distinct arms of the immune system, and systemic cytokine levels alone may not fully reflect adjuvant-mediated immunomodulation. Monitoring cytokine kinetics within the first 72 hours post-immunization would be critical for capturing the acute innate immune response and understanding adjuvant mechanisms (Talaat et al., 2018).

Adjuvants play a crucial role in shaping the antibody response elicited by vaccines, influencing the quantity, quality, and subclass distribution of immunoglobulins produced. Multiple swine vaccination studies, including those targeting *Streptococcus suis*, have employed O/W adjuvant (Gallagher-Beckley et al., 2015; Obradovic, Corsaut, et al., 2021; J. Zhang et al., 2018) . However, studies have shown that while formulations containing Quil A® (saponin) or oil-in-water adjuvants like Emulsigen®-D can stimulate humoral responses in pigs, they often fail to confer adequate clinical protection following challenge with *S. Suis* serotype 2. Notably, these vaccines elicit a Th2-skewed IgG1-dominant antibody profile, which may contribute to suboptimal protective efficacy (Obradovic, Corsaut, et al., 2021). Thus, evaluating functional immunity, including T-cell responses and protection after pathogen challenge, remains essential.

Conclusion and future directions

Our findings reinforce the conclusion that adjuvant formulation is pivotal in the immunogenicity and protective potential of *S. Suis* bacterin vaccines. The enhanced responses observed with OW-415 in the multivalent formulation highlight the potential for synergistic effects when combining adjuvants and antigens. However, the comparatively high antibody levels in the *S. Suis*-only antigen group, alongside possible maternal antibody interference and antigen-related limitations, underscore the importance of optimizing antigen integrity and adjuvant selection to

achieve effective vaccination outcomes. Future studies should focus on evaluating alternative antigen inactivation methods, characterizing cellular immune responses, and assessing protective efficacy in challenge models to further inform the development of effective, safe, and practical vaccines for the swine population. Our data also highlight the importance of incorporating cellular immune readouts beyond antibody and serum cytokine profiles. Including T-cell assays, early timepoint cytokine data, and local immune responses (e.g., lymph node or injection site) would provide a more holistic assessment of adjuvant function and vaccine efficacy.

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