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Yield improvement of a plant-made antibody against Enterohemorrhagic E. coli

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree
in Biology

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ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) is a foodborne pathogen that harbors asymptotically in a bovine intestinal reservoir and has been a consistent global health risk for the last thirty years. In this thesis, I have functionally characterized an anti-EHEC antibody and shown it to bind and neutralize four of the seven most prevalent EHEC strains. However, yield is arguably the greatest technical hurdle in advancing such an antibody from proof of principle to market. Although there have been many advances in the last thirty years in improving plant-based recombinant yields, little is still known about both the mutational potential in engineering the structure for better yield as well as the limitations of the antibody's oxidative folding environment. This thesis explores two main strategies toward yield improvement: first, rational design of the antibody and second, targeting it to the thylakoid lumen. I have identified and characterised six rationally designed mutations that can be pyramided to improve the yield of the antibody by twenty-fold. I show that despite engineering its structure, the antibody retains its ability to assemble into a polymeric complex as well as its binding and neutralization efficacy against EHEC in *in vitro* assays. Because endoplasmic reticulum (ER)-associated degradation and misfolding may potentially be limiting factors in the oxidative folding of antibodies in the ER, I sought to explore oxidative folding in an alternative subcompartment, the chloroplast thylakoid lumen, and determine its viability in a molecular farming context. I developed a set of in-house expression vectors targeting the antibody to the thylakoid lumen via either Sec or Tat import pathways. Compared to stromal, cytoplasm and Tat-imported pathways, the Sec-targeted antibody showed superior accumulation, but about one third less than its ER-targeted counterpart. Sec-targeted antibodies also retain binding and neutralization

efficacy in *in vitro* assays. Additionally, the introduction of a rationally designed *de novo* disulfide enhances *in vivo* accumulation when introduced into the Sec-targeted antibody. These results collectively provide a proof of concept on the viability of rational design and thylakoid targeting as novel, broadly applicable strategies for yield improvement and potentially advancing an anti-EHEC antibody closer toward market adoption.

Keywords: Plantibodies, Enterohemorrhagic *E. coli*, O157, Antibodies, Rational design, Thylakoid, EHEC, Stability engineering, Molecular farming, Plant production platform, VHH, Fc, IgA, sIgA, Nanobodies.

SUMMARY FOR LAY AUDIENCE

Enterohemorrhagic *Escherichia coli* (EHEC) is a pathogen that harbors in the intestines of cattle and can be transmitted through the food supply chain to cause foodborne illness in a population. An appealing intervention strategy is the use of antibodies that can be fed to the animal to clear EHEC from its intestines prior to harvest. In this thesis, I describe the design of such an antibody and demonstrate it to bind and neutralize four of the seven most prevalent EHEC strains that collectively account for 72% of related foodborne illnesses. However, because yield is a major hurdle preventing transitioning of this antibody from proof of principle to market, there is a need to explore yield optimization strategies. Therefore, this thesis explores two novel strategies for improving yield: engineering of the structure of the antibody and targeting it to a cellular compartment known as the thylakoid lumen.

I designed a screen of mutational candidates and assessed their yield when expressed in tobacco. Out of 24 candidates, I identified six mutations that individually improve the yield of the antibody. Furthermore, these mutations could be pooled to incrementally increase yield to give a twenty-fold improvement of the antibody. To ensure that the engineered antibody was still functional, I tested its binding and neutralization across the same seven strains and found that it retained efficacy.

The thylakoid lumen is a cellular subcompartment inside the chloroplast of plants that may potentially allow for higher antibody yields because of a different intracellular environment. However, when targeted to the thylakoid lumen, the antibody reached a yield about 60% of the conventional endoplasmic reticulum compartment. Still, functional assays indicated that the thylakoid-targeted antibody retained efficacy.

Overall, this thesis is notable because it demonstrates the viability of rational design and thylakoid targeting as strategic research directions for yield improvement of an anti-EHEC antibody.

CO-AUTHORSHIP STATEMENT

The following thesis contains material from manuscripts that have been published or are in preparation which are coauthored by Adam Chin-Fatt (ACF), Reza Saberianfar (RS), Kevin Henry (KH) Andrew Scott (AS), Ed Topp (ET) and Rima Menassa (RM). My supervisor Rima Menassa provided guidance and strategic direction for the projects and, along with my co-supervisor, David Smith, edited the final manuscripts.

Chapter 1. Author's contributions

A portion of the introduction (1.1-14) concerning enterohemorrhagic *Escherichia coli* is an abridged version of a book chapter co-authored by Adam Chin-Fatt, Ed Topp and Rima Menassa and entitled, "The benefit of a plant-based cattle vaccine for reducing Enterohemorrhagic *Escherichia coli* shedding and improving food safety." MacDonald, J. (Ed.). (2018). *Prospects of Plant-Based Vaccines in Veterinary Medicine*. Springer.

ACF wrote the chapter. ET and RM edited the chapter.

Chapter 2. Author's contributions

Chapter 2 consists of an abridged version of a co-authored journal article published in *Frontiers Plant Science* entitled:

Saberianfar, R., Chin-Fatt, A., Henry, K. A., Scott, A., Topp, E., & Menassa, R. (2019). Plant-produced chimeric VHH-sIgA against enterohemorrhagic *E. coli* intimin shows cross-serotype inhibition of bacterial adhesion to epithelial cells. *Frontiers in Plant Science*, 10, 270.

I have included only the results that I was responsible for, namely the binding and neutralization assays as well as the phylogenetic analysis. The published abstract, introduction and relevant parts of the discussion are also my own work. Work done by other co-authors was incorporated in the introduction to provide a better idea of the study but their involvement has been explicitly indicated.

RM conceived the study. RS and RM designed the research. RS, KH, and ACF performed the experiments. RS, ACF, KH, and RM wrote the manuscript. AS assisted with the binding and adhesion experiments. ET provided feedback on experimental design and result interpretations. RS, KH, ACF, AS, ET, and RM edited the manuscript.

Chapter 3. Author's contributions

ACF conceived the study. ACF and RM designed the research. ET provided feedback on experimental design and result interpretations. ACF and RM wrote and edited the manuscript

Chapter 4. Author's contributions

ACF conceived the study. ACF and RM designed the research. ET provided feedback on experimental design and result interpretations. ACF and RM wrote and edited the manuscript

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LIST OF ABBREVIATIONS

A/E	Attaching and effacing
ANOVA	Analysis of Variance
AvNAPSA	Average number of neighboring atoms per side chain atom
CDC	Centre for disease control
CFIA	Canadian Food Inspection Agency
CH	Heavy chain
DAEC	Diffusely adherent <i>E. coli</i>
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
dpi	Days post infiltration
DTT	1,4-Dithiotheitol
EAEC	Enteroggregative <i>E. coli</i>
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic <i>Escherichia coli</i>
EIEC	Enteroinvasive <i>E. coli</i>
EPEC	Enteropathogenic <i>E. coli</i>
ER	Endoplasmic reticulum
Esp	<i>E. coli</i> secreted protein
ETEC	Enterotoxigenic <i>E. coli</i>
Fab	Antigen-binding fragment
Fc	Fragment crystallizable
Fc α R	Fragment crystallizable α receptor
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
Fv	Variable fragment
FW	Fresh weight
Gb3	Globotriaosylceramide
GFP	Green fluorescent protein
GI	Gastrointestinal
HC	Hemorrhagic colitis
HEp-2	Human epithelial cells type 2
HUS	Hemolytic uremic syndrome
Ig	Immunoglobulin
JC	Joining chain
LB	Luria-Bertoni medium
LEE	Locus of enterocyte effacement

LTO1	Lumen thiol oxidoreductase 1
mAb	Monoclonal antibody
NleA	Non-Lee encoded effector A
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PVDF	Polyvinylidene difluoride
PVPP	Polyvinylpyrrolidone
scFv	Single chain variable fragment
sdAb	Single domain antibody
SDS	Sodium dodecyl sulfate
Sec	Secretory
sIgA	Secretory Immunoglobulin A
SLIC	Sequence and ligation independent
SRP	Siderophore receptor and porin
STEC	Shiga toxin producing <i>E. coli</i>
Stx	Shiga toxin
T3SS	Type 3 secretion system
Tat	Twin-arginine translocation
TIR	Translocated intimin receptor
USDA	United States Department of Agriculture
VHH	Variable Heavy chain only
VIDO	Vaccine and Infectious Diseases Organization
VTEC	Verotoxigenic <i>Escherichia coli</i>

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1.0 INTRODUCTION

1.1 Research motivation and overview

Upon ingestion, enterohemorrhagic *Escherichia coli* (EHEC) can colonize intestinal mucosa and cause hemorrhaging of nearby tissue (Cleary 2004). The failure to adequately control its contamination of food and water can consequently compromise the health of a population and incur economic losses to all stages of the food supply chain. EHEC is currently one of the foremost foodborne pathogenic threats worldwide because of its virulence across all age groups and demographics, a low infective dose, a relatively high resilience in diverse environments and its widespread prevalence across cattle herds (Rangel et al. 2005). EHEC primarily colonizes the bovine digestive tract from which it can be transmitted via fecal shedding or during slaughter (Nart et al. 2008). Considering its threat to food security and in accord with the ‘One Health’ framework, the development of an antibody for enteromucosal passive immunization as a pre-harvest intervention strategy to curtail the transmission of EHEC is of great interest. However, its transition to market has been hampered primarily by a higher yield requirement. Therefore, strategies to improve its yield are also important considerations.

Overall, this thesis focuses on the plant-based production of an antibody against EHEC and explores two main strategies that are conceptually novel research avenues for yield improvement in plants. Chapter 1 summarizes the scope of the threat posed by EHEC and discusses current intervention strategies as well as the prospects of developing a commercial plant-based antibody for EHEC within the framework of the North American beef industry. Chapter 2 describes the original design and plant-based production of a

synthetic antibody and focuses on testing its binding and neutralization competency. Chapter 3 explores the use of protein engineering principles to improve yield of the antibody by stabilizing its structure. Chapter 4 explores the thylakoid lumen as an alternative subcompartment for folding the antibody to investigate if a different folding environment could improve yield. Finally, chapter 5 critically discusses the significance of the antibody and the described yield optimization strategies in terms of applicability and relevance.

1.2 Problem and context: Occurrence and disease symptoms of Enterohemorrhagic *E. coli*

Diarrhea is the second leading cause of death among toddlers under the age of five globally, with an estimated occurrence of 2.5 billion cases overall, and an estimated mortality of 1.5 million annually (Unicef 2010). While diarrhea may be a common symptom of a broad spectrum of gastrointestinal upsets, a relatively small handful of micro-organisms are the primary causes for most acute diarrheal cases, including *E. coli*. The pathogenic *E. coli* strains that cause diarrheal disease in humans, collectively known as diarrheagenic *E. coli*, are broadly categorized based on clinical symptoms and virulence attributes into: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and Vero toxin-producing/Shiga toxin-producing *E. coli* (VTEC/STEC). The latter category is further divided into enterohemorrhagic *E. coli* (EHEC) and non-enterohemorrhagic subgroups though in practice, the terms EHEC, STEC and VTEC are often used interchangeably. As the name suggests, the EHEC subgroup is typified by

hemorrhaging of the intestines but constitutes more than 100 different serotypes that are identifiable based on variations of their O (somatic lipopolysaccharide), H (flagellar) and K (capsular) antigens. While lipopolysaccharides are found in all Enterobacteriaceae, flagellar and capsular antigens are not always present in some strains. Therefore, routine epidemiological surveillance has conventionally screened primarily for O serogroups as the primary biomarker, with subsequent H-subtyping if presumptive pathogenic O strains are detected. Subtyping for the K antigen is not part of routine surveillance since few labs are equipped for the requisite assay. The most prevalent and virulent EHEC serotype in North America is O157:H7 and has been classified as a major food adulterant by the United States Department of Agriculture (USDA) and Canadian Food Inspection Agency (CFIA) for almost 20 years. Although non-O157 strains are individually less prevalent, the collective contribution of non-O157 strains to gastrointestinal illness has as of late been of growing concern, particularly since recent surveillance indicates a 41% increase in the average annual incidence of infection of non-O157 strains over the last five years across the US (Gill and Gill 2010). Six additional EHEC serogroups O26, O45, O103, O111, O121 and O145, known as the “Big Six”, generally comprise >90% of non-O157 infections of any given year and have been traced to at least 22 human disease outbreaks in the US since 1990. In the US, national surveillance was only recently enabled in 2012 by the USDA to individually track non-O157 serotypes in human illness (Mathusa et al. 2010). In 2011, Canadian national surveillance by the Public Health Agency of Canada expanded their monitoring of O157 to include all VTEC strains in agricultural, water, retail and human health components (Public Health Agency of Canada 2015).

Epidemiology of human infections

The recognition of EHEC as a discrete and important class of diarrheagenic *E. coli* originally stems from two reports in 1983. The first was a clinical report detailing two separate outbreak events in the United States of a distinctive gastrointestinal illness, subsequently called hemorrhagic colitis (HC), characterized by severe abdominal pain and acute watery diarrhea that later developed into bloody diarrhea (Riley et al. 1983). In both cases, the illness was associated with consuming undercooked hamburger meat from two fast food chains and dubbed by news media as the “hamburger disease”. Also, stool cultures sampled from the patients both yielded a previously unidentified *E. coli* strain. The second report provided strong association between fecal cytotoxin producing *E. coli* and the occurrence of hemolytic uremic syndrome (HUS) (Karmali et al. 1983). HUS is characterized by the triad combination of acute renal failure, thrombocytopenia and microangiopathic hemolytic anemia, and was already known to be preceded by a bloody diarrhea that was symptomatically similar to that observed in the two fast food chain outbreak events. The discovered link between EHEC, its enteric disease causing ability and its route of transmission via undercooked beef products has subsequently prompted a series of surveillance efforts in the food industry to curtail the outbreak potential of EHEC (Doyle et al. 2006).

Since then, EHEC, particularly the O157:H7 strain has been detected worldwide. Based on a data mining approach of incidence studies covering 21 countries, a review has conservatively estimated that each year VTEC causes 2,801,000 acute illnesses, 3,890 cases of HUS and 230 deaths (Majowicz et al. 2014b). Based on these estimates, on a

global ranking, VTEC places behind typhoid fever, foodborne trematodes and nontyphoidal salmonellosis in importance. EHEC is estimated to affect approximately 230,000 people in the United States each year, with ~73,000 of these being caused by O157:H7 (Hale et al. 2012). In terms of most frequently isolated overall food-borne pathogen ranking in North America, it places fourth after *Campylobacter*, *Salmonella* spp and *Shigella* spp based on stool samples collected from patients (Griffin 1995). However, if restricted to only stool samples with visible blood then EHEC, particularly O157:H7, is the most frequently isolated (Slutsker et al. 1997).

In the US, the national surveillance program for foodborne pathogens, FoodNet, reported that in 2015 (most recent available report) the average incidence rate for that year for O157 was 0.95 per 100,000 persons and for non-O157 strains was 1.65 per 100,000 persons (CDC 2017). Among the approximately 1200 EHEC infections (out of a total sample of ~49 million), the most common serogroups were O157 (39.8%), O26 (17.6%) and O103 (14.3%) (CDC 2017). Although surveillance for non-O157 strains is still fairly recent, the growth in incidence over the past five years is stark. Compared with the average annual incidence rate 2012-2014, non-O157 incidence has increased by 41% (CDC 2017). For that same period, there has been no significant change for O157 incidence (CDC 2017). This is possibly because most EHEC diagnostic and control measures have historically been specific for O157, despite the clinical relevance of non-O157 strains. Since discovering O157 in the 1980's, the trend of infection has progressively shown a decreasing incidence in North America, mirrored by a decrease in HUS (CDC 2011). Between 1996 to 2010, the incidence of infection for O157 has decreased by 44% and the

number of HUS cases has decreased by 90% (CDC 2017). There are many likely contributing factors such as improved regulatory and biosecurity control, cleaner slaughter methods, better microbial testing and improved food awareness by consumers. In Canada, the national surveillance system for foodborne pathogens, FoodNet Canada, reported an average incidence rate for VTEC to be 3.00 per 100,000 persons (Public Health Agency of Canada 2015). Targeted surveillance on retail ground beef products across Ontario for 2015 indicated VTEC in 2.3% of samples, with a similar prevalence to Salmonella (1.5%), and placing second behind the consistent frontrunner, *Listeria monocytogenes* (25%) (Public Health Agency of Canada 2015). The 10-year trend for VTEC in contamination in retail ground beef reveals that VTEC consistently hovers around 2% positive with the exception of 2010-2011 in Ontario when incidence spiked to ~8% during an outbreak (Public Health Agency of Canada 2015).

Both incidence rates and occurrence of HUS are consistently highest in toddlers <5 years compared with all other age groups. In FoodNet's latest report (2015), toddlers <5 had an incidence rates of 3.72 and 6.76 per 100,000 for O157 and non-O157 strains respectively (Gill and Gill 2010). In comparison, all other age groups ranged between 0.33-2.39 and 0.62-2.04 per 100,000 for O157 and non-O157 strains respectively (Gill and Gill 2010). Approximately 1 in 5 toddlers <5 years with an O157 infection will develop HUS. Out of all HUS patients, more than 90% are due to O157, followed by O121 (4.8%) and then O111 (2.4%) (Gill and Gill 2010). Compared with 2006-2008, the incidence of pediatric HUS has decreased by 32%, which likely corresponds to the 30% decrease in O157 infections².

Large-scale outbreaks are rare but can affect large numbers of people and may be transmitted from a variety of sources, though most commonly from raw foodstuff or untreated water. For example, the five largest EHEC outbreaks worldwide were from: radish sprouts in Japan (12,680 cases) (Fukushima et al. 1999), drinking water in Canada (2,300 cases) (Hrudey et al. 2003), well water in the US (>1000 cases) (Charatan 1999), raw beef in the US (788 cases)(Wendel et al. 2009) and undercooked hamburger meat in the US (>700 cases) (Bell et al. 1994). In comparison, sporadic EHEC infections are more frequent and comprise the major disease burden in a population. The average frequency of sporadic cases has slightly risen over the past five years of surveillance (CDC 2017; Public Health Agency of Canada 2015). Of these sporadic cases, the incidence is distributed unevenly across North America, being more common in Canada and the northern US states than the southern US states and more common in western Canada than eastern Canada (Griffin 1995).

EHEC primarily occupies a bovine intestinal reservoir and correspondingly, its main route of transmission is via cattle's excretion of fecal matter carrying the bacterium, a process known as 'shedding'. Sporadic EHEC incidence can be affected by seasonality with the most common reports of EHEC shedding occurring during the summer through fall seasons. An investigation by the USDA on the seasonal occurrence of O157 suggests that the increased shedding of *E. coli* O157 during the summer season is strongly associated with an increased likelihood of product contamination and a corresponding increase of enterohemorrhagic cases in humans (Williams et al. 2010). Both O157 and non-O157 serogroups exhibit this trend.

Disease symptoms in humans

Milder forms of EHEC infection are typically associated with watery diarrhea while more aggressive forms may develop into HC or HUS, and in uncommon cases, accompanied by cardiovascular or nervous system abnormalities (Griffin and Tauxe 1991). In humans, the incubation period for EHEC O157:H7 ranges from 1-16 days. Symptoms usually become apparent after 3-4 days, typically manifesting as moderate to severe diarrhea. Most resolve without treatment whereas others can progress to HC after a few days, characterized by severe, bloody diarrhea with abdominal tenderness and cramping. Mild fevers, nausea, vomiting and dehydration are also possible accompanying symptoms (Cleary 2004). Although this will typically resolve in approximately 1 week, 16% will develop into HUS, characterized by the triad combination of kidney failure, hemolytic anemia and thrombocytopenia. In more severe cases, paresis, stroke, cerebral edema or coma are accompanying symptoms. Although 65-85% of patients recover from HUS without permanent injury, long term complications including hypertension, renal insufficiency and end-stage renal failure are possible. Certain demographics of patients seem to be more susceptible to the development of the infection into more serious symptoms. Patients who are younger than five, older than 60 or who are immunocompromised are significantly more likely to develop HC or HUS (Gould et al. 2009; Karmali 2004; Tuttle et al. 1999). In the elderly, a form of HUS, known as thrombocytopenia purpura, is more common, characterized by less kidney damage but more severe occurrence of neurologic symptoms such as stroke, seizure and central nervous system deterioration.

Histopathology

The typical histopathology characteristic of EHEC infection includes hemorrhaging and edema of the lamina propria (Griffin et al. 1990). Biopsy samples taken from the colon of infected patients also show focal necrosis and neutrophil infiltration. One of the hallmarks of EHEC infection is the attaching-and-effacing (A/E) lesion. This histopathology is apparent by microscopy in a variety of animal models and can also be reproduced in *in vitro* cell cultures (Donnenberg et al. 1993; Ismaili et al. 1995; Pai et al. 1986). *In vitro* organ culture of human endoscopic biopsy samples suggests EHEC adhere and form lesions on the terminal ileum (Chong et al. 2007). This distinct phenotype is caused during the EHEC colonisation phase when microvilli become effaced and various secreted proteins enable the intimate adherence between the EHEC pathogen and the outer membrane of the intestinal epithelium. Following attachment, the accumulation and rearrangement of polymerized actin leads to an altered cytoskeleton in which a pedestal-like structure protruding from the epithelium emerges. These structures can extend up to 10µm in a pod-like formation upon which the bacterium is ensconced (Moon et al. 1983).

1.3 Transmission

Route of transmission

The intestines of ruminants, especially cattle, are considered the primary reservoirs of EHEC and can transmit EHEC via excreted fecal matter or after slaughter during processing (Beutin et al. 1993b; Montenegro et al. 1990b). High levels of EHEC colonization have been reported in cattle herds from various countries, ranging typically between 10-25%, but can be as high as 60%. Healthy cattle transiently host EHEC in their

gastrointestinal tract and can directly or indirectly transmit this pathogen to humans (Rangel et al. 2005b). EHEC can persist in various environments that range extensively from soil, to water to the ruminant GI tract. In North America, most cases are caused by ingestion of contaminated food or water (Rangel et al. 2005a). When shed in bovine feces, the pathogen can remain viable in the farm environment and may contaminate nearby agricultural crops, other holding pens and ground water (Sanderson et al. 2006). Aside from undercooked or unpasteurized animal products and contaminated fruits and vegetables, exposure may come from contaminated soil, such as at campgrounds or other sites grazed by cattle, or from open water sources, such as swimming lakes or private wells that are drainage sinks from agricultural run-off. O157:H7 has been reported to persist for up to a year in manure-treated agricultural soil and for 21 months in non-composted raw manure (Jiang et al. 2002). Its resilience in water especially is a major factor for its dissemination and persistence across various transmission routes. Culturable O157 has been demonstrated to be able to survive for at least 8 months in contaminated water troughs (LeJeune et al. 2001). Furthermore, O157 strains that survived longer than 6 months still retained the capacity to colonize cattle (LeJeune et al. 2001). EHEC's robustness has implications for crop contamination considering that bovine manure often is used as fertilizer as well as after irrigation when surface water containing EHEC collects in sumps. Even if the use of bovine fertilizer were to be avoided, a recent report indicated that airborne transport of O157:H7 could contaminate leafy greens that were up to 180m away from a cattle feedlot, particularly when pen surfaces were under arid conditions (Berry et al. 2015). A safe set-back distance between feedlots and crops has not yet been determined.

Additionally, EHEC requires a much lower infectious dose than other foodborne pathogens when ingested, with fewer than 40 bacterial cells being sufficient to cause illness (Strachan et al. 2005). To a lesser degree than contaminated food and water, EHEC can also be transmitted from direct contact between humans as well as from animal to human contact, likely via fecal residues (Heuvelink et al. 2002).

Although infected cattle remain asymptomatic, cattle that have been exposed to EHEC develop a local immune response, an associated inflammatory response and attaching-effacing (A/E) lesions suggesting not only that EHEC is an active bovine pathogen but also that there is a limit to which the bovine host will tolerate pathogen load and after which host resistance mechanisms may actively function to reduce pathogen burden (Baines et al. 2008; Nart et al. 2008).

Super shedders

Generally, there are three distinct patterns observed for EHEC carriage in cattle that are characterized in terms of increasing severity of intestinal colonization, duration of shedding and magnitude of shedding. First, some cattle, known as passive shedders, lack colonization, transiently shed for only a few days and in small numbers. Second, cattle that are colonized, shed for approximately 1-2 months (Besser et al. 1997). Third, a small subset of cattle populations, known as “super shedders”, are colonized for extended periods, shed EHEC for longer periods at 3 to 12 months and at significantly higher levels (between 10^4 and 10^4 colony forming units/g of faeces) (Omisakin et al. 2003; Stephens et al. 2009). These super shedders are suggested to be important hubs in a cattle population for maintaining the penetrance of EHEC infection that perhaps would otherwise be transient

and short-lived. While there is as of yet no definitive explanation of the causes of the super shedding phenomenon, it is thought to collectively be mediated by factors from the EHEC pathogen, the bovine host and the environment. Hide contamination associated with super shedders rapidly resulted in the transmission of *E. coli* O157:H7 among cattle housed in a common pen (Stanford et al. 2011). An assessment of the link between shedding density and human risk suggested that even though super shedding events were relatively rare, they dominated as the environmental contamination source as well as the relative human risk of acquiring illness (Matthews et al. 2013). Almost half of all EHEC shed from cattle in an Alberta feedlot was due to super-shedders, even though these animals represent less than a tenth of the cattle population (Stephens et al. 2009). While super-shedders are increasingly considered to have a significant role in population-level persistence of EHEC, this small proportion of super-shedding cattle is not a stable, consistent subset of the population but rather varies transiently and dynamically making quarantining of the super-shedding animal an unviable option. Consequently, targeting them for interventions such as vaccination is difficult, unless applied to the entire herd. However, the exception to this is if immediately prior to slaughter, there were tools such as antibodies available to quickly diagnose and identify these super-shedders, these could be targeted for intervention to reduce the likelihood of product contamination.

Mechanism of infection

The ability of EHEC to successfully colonize the gastrointestinal tracts of both humans and cattle despite peristaltic movements and resource competition with neighboring microflora is one of the most defining features across all strains. In particular,

although all *E. coli* strains have some form of fimbrial structure to enable surface adherence, EHEC strains express specific fimbrial antigens that seem to specialize in adherence to the gut mucosa, enhancement of colonization of the intestinal epithelium, and defining of host specificity (Vial et al. 1988). In cattle, EHEC principally adheres to and colonizes the lymphoid follicle dense mucosa at the terminal rectum known as the rectoanal junction, whereas in humans, it adheres to and colonizes the follicle-associated epithelium of ileal Peyer's patches (Lim et al. 2007; Naylor et al. 2003; Phillips et al. 2000). Successful colonisation in both humans and cattle will typically be marked by a canonical A/E lesion.

The mechanism of colonization by EHEC of a mucosal site in either cattle or humans is a conserved process requiring the expression of at least 59 genes (Büttner 2012; Dziva et al. 2004). The main virulence genes cluster together on a chromosomal 43-kb pathogenicity island known as the locus of enterocyte effacement (LEE), the presence of which is both necessary and sufficient for showing the A/E phenotype (Perna et al. 1998). The LEE contains 41 open reading frames including genes encoding various subunit proteins that assemble to form a type III secretion system (T3SS), the major adhesin protein known as intimin (Eae) and its cognate Translocated intimin receptor (Tir), a lytic transglycosylase EtgA to remove glycans near to the site of colonisation (Burkinshaw et al. 2015), various effector proteins that are secreted through this system and various chaperones to stabilize the folding and assembly of these proteins (Wong et al. 2011). The T3SS consists of a syringe-like structure that permits the secretion of multiple effector proteins stored within the bacterial cell and into the host cytosol (Jarvis and Kaper 1996).

The first step of colonization is likely through contact to an intestinal epithelial membrane by an extended hollow, filamentous structure consisting of multiple polymerized subunits of *E. coli* secreted protein A (EspA) (Delahay et al. 1999; Knutton et al. 1989). Upon initial contact, two other LEE-encoded proteins, EspB and EspD, are translocated via the EspA filament into the host cell where they will assemble along with EspA to form a translocon pore stabilizing the entry point (Fivaz and van der Goot 1999; Kenny and Finlay 1995; Lai et al. 1997; Warawa et al. 1999). At least 39 other effector proteins are then secreted into the host cell, altering a variety of host cell processes that ultimately improve the likelihood of the bacterium's survival and replication (Tobe et al. 2006; Wong et al. 2011). Several of these effectors along with components of the T3SS are potential vaccine candidates because of their efficacy in engaging the host's active immune response. One of these effector proteins known as the non-Lee encoded effector A (NleA) protein is also secreted into the host cell where it may have a role in disruption of intestinal tight junctions and inhibition of intercellular protein trafficking (Gruenheid et al. 2004; Kim et al. 2007). Another effector known as Tir integrates into the host cell membrane where it allows docking of the adhesin protein, intimin (Kenny et al. 1997). Docking enables intimate attachment of the bacterium to the host cell and signals the recruitment and polymerization of actin at the pore resulting in a protrusion of the membrane toward the bacterium forming the canonical A/E lesion (Garmendia et al. 2004).

Subsequent to colonization, EHEC will produce a variety of virulence factors including verocytotoxins, also called Shiga-like toxins (Stx) because of their similarity to toxins produced by *Shigella dysenteriae*. In humans, the production of Stx is the primary

cause of the microvascular endothelial damage associated with HUS and HC. There are two major immunologically distinct types of Shiga-like toxins, Stx1 and Stx2, that are encoded by separate phage-derived *stx* genes on the bacterial chromosome (Wagner and Waldor 2002). Although Stx1 tends to be highly conserved across serotypes, there are many variants for Stx2. Nonetheless, all Shiga toxins form a basic A-B₅ subunit structure. Typically, the 32-kDa A subunit is cleaved to yield an enzymatically active 28-kDa A1 peptide that is bridged via a 4-kDa A2 peptide to a pentamer consisting of five 7.7-kDa B subunits. The B subunit pentamer is able to bind to a specific glycolipid receptor, globotriaosylceramide (Gb3) that is found on the cell membrane surface of intestinal epithelial cells. A Gb4 receptor may also be targeted by some Stx2 variants. Upon successful binding to a receptor, the toxin is endocytosed via clathrin coated pits. The internalized toxin is then delivered to endosomes where they are primarily targeted to lysosomes for degradation. However, a fraction can be delivered to the trans-Golgi network, followed by retrograde transport via Golgi cisterns into the ER. Similar to the effects of ricin, the A1 peptide of the cytotoxin is an N-glycosidase that catalytically removes a single adenine residue from the 28S RNA of 60S ribosomal subunits to effectively suppress protein synthesis by preventing binding of tRNAs to the ribosome and consequently triggering apoptosis in affected cells (Endo et al. 1988). The presence of the Gb3 receptor on the cell surface is required for Stx toxicity (Jacewicz et al. 1995). Although Stx production occurs in both humans and cattle, the former exhibit Stx-related pathophysiology primarily because of vascular expression of the Gb3 receptor in intestinal epithelial cells while the latter lack vascular Gb3 receptor expression in their GI tracts

(Pruimboom-Brees et al. 2000). Although the Gb3 receptor is expressed in the bovine brain and kidney, cells in the recto-anal junction do not permit Stx to be endocytosed and transported across the GI tract vasculature and consequently, the toxin is isolated from susceptible cells (Pruimboom-Brees et al. 2000). In contrast, EHEC's colonisation of human ileal tissue is proximal to the intestinal epithelial cells that express Gb3. The selective apoptosis of absorptive villus tip intestinal epithelial cells, carrying the Gb3 receptor, and the preservation of Gb3-absent secretory crypt cells may then lead to the osmotic dysregulation that manifests as diarrhea (Kandel et al. 1989). The development to HUS is assumed to be based on the translocation of Stx across the epithelial cell layer and into the bloodstream. The Gb3 receptor is abundant in human renal tissue (Boyd and Lingwood 1989). Upon contact, Stx is cytotoxic to the glomerular endothelial cells leading to blocking of the glomerular microvasculature with platelets and fibrin (Louise et al. 1997). This disrupted ability to filter fluid through the glomerulus may lead to the acute renal failure characteristic of HUS.

The significance of Stx in intestinal pathology can vary depending on the animal model used. In cattle, which lack the Gb3 receptor, the occurrence of the diarrhea is independent of the presence or absence of Stx but is rather determined by the extent and distribution of the A/E lesions. This pattern is similar across cattle, sheep, goats, chickens and rabbits that do not display clinical symptoms despite the formation of A/E lesions in their GI tracts, presumably due to a lack of Gb3 receptors (Best et al. 2005; La Ragione et al. 2005; La Ragione et al. 2006; Tzipori et al. 1989; Tzipori et al. 1995; Woodward et al. 2003). Overall, reports from various animal models suggest that the occurrence of the A/E

lesions is sufficient to cause non-bloody diarrhea but the cellular entry of the Stx is essential for inducing clinically relevant symptoms such as bloody diarrhea, HUS and HC.

1.4 Interventions

Pre-harvest and post-harvest interventions against EHEC

EHEC can be transmitted to humans via multiple routes such as crops, water and meat products. Towards the implementation of strategies to prevent EHEC infection of humans, the prevailing train of thought is to both curtail its colonization of cattle and minimize its spread from fecal shedding or at harvest. These strategies are broadly grouped into pre-harvest and post-harvest interventions with the former typically being adopted by beef producers and the latter by meat processors. Intervention strategies that are most commonly used or are most promising have been summarized in Table 1.

Post-harvest interventions involve removing contamination from the hide and/or carcass with various antimicrobial agents such as organic acids, oxidizing agents, heat exposure, irradiation or high pressure systems. Hide contamination can occur during skinning of the animal and to a lesser degree rupturing of the intestines. As an initial step, the carcass is often rinsed or steamed and visibly contaminated parts removed by knife trimming. Subsequently, a combination of treatments is typically used to reduce the contamination. Acid treatment is the most commonly employed method in North America likely due to its cost effectiveness. Promising newer methods such as high pressure and electron beam irradiation are twice as effective as acid treatment and have the highest efficacy amongst known interventions, though they require specialized equipment for implementation (Wheeler et al. 2014).

Table 1. A summary of commonly used or most promising strategies intervention strategies that have been investigated in mitigating EHEC carriage in cattle.

Strategy	Description
<u>Pre-harvest interventions</u>	
1) Exposure reduction	Modulates rearing conditions to minimize transmission to cattle
Treatment of drinking water	Destroys bacteria residing in drinking water, typically by chlorination, electrolysis or ozonation
Feed strategies	Reduces ingested bacteria by change of standard grain-based feed a few days before slaughter, usually by fasting or replacement with forage or hay
Maintaining closed herds	Prevents cross-contamination across herds by quarantining of cattle herds and facilities
Pest and wildlife management	Prevents transmission from various pests and wildlife which can act as EHEC transmission vectors
Sanitation practices	Ensures clean pens, bedding and transport to prevent EHEC growth in immediate environment
2) Exclusion strategies	Alters the mucosal site of colonisation within the GI tract to either interrupt or displace attachment and colonisation
Vaccination	Engages host active mucosal immunity by immunization with an EHEC specific antigen
Probiotics	Alters the gut microbiota by a viable preparation of microorganisms that outcompete EHEC at the ecological niche needed for colonization
Prebiotics	Enriches native competitive microbiota species by providing selectively digestible organic compounds
Competitive exclusion	Competes for EHEC binding to sterically block EHEC access
Antibodies	Binds and agglutinates EHEC in the lumen sterically preventing attachment
3) Direct anti-pathogen strategies	Live animal treatments that specifically target and kill EHEC
Sodium chlorate	metabolized by an EHEC-specific nitrate reductase to chlorite, a bactericidal metabolite
Antibiotics (Neomycin sulfate)	A broad spectrum compound that binds 30S ribosomal subunit and inhibits protein translation.
Bacteriophages	Viruses specific for a narrow bacterial host range that infect and lyse the EHEC bacteria
Colicins	Antimicrobial proteins that bind EHEC outer membrane receptors and subsequently translocate to the cytoplasm where they exert various cytotoxic effects

Post-harvest interventions

Physical removal	removes visibly contaminated parts and rinses excess unattached EHEC off carcass, usually by knife trimming, steam-vacuuming and ambient temperature water washing
Acid antimicrobials	disrupts proton motive force and substrate transport mechanisms leading to bacteriostasis, usually acetic, citric and lactic acids
Oxidizer antimicrobials	generates oxidative damage to a broad array of cellular structures leading to cell death, usually by peracetic acid, acidified sodium chlorite, ozone or hypobromous acid
Heat exposure	uses heat treatment to denature bacterial enzymes and nucleic acid degradation, usually by hot water sprays or steam pasteurization
Irradiation	uses a stream of high energy electrons or UV light to damage bacterial genetic material leading to cell death
High pressure	uses hydrostatic pressure to damage bacterial cell membranes causing lysis

Pre-harvest interventions are further sub-grouped into 3 categories: 1) exposure reduction, 2) exclusion, and 3) direct anti-pathogen strategies. Exposure reduction strategies involve management of the rearing conditions of the herd to minimize EHEC exposure such as by water and feed hygiene, by limiting exposure to pests, wildlife, and other cattle herds and by sanitation of living and transport conditions. Exclusion strategies seek to interrupt or displace attachment and colonisation of EHEC to the GI tract by altering the site of colonisation such as by engaging active immunity with vaccination, outcompeting niches with prebiotics and/or probiotics or sterically hindering access with competitive exclusion. Direct anti-pathogen strategies are live animal treatments that directly kill EHEC such as by sodium chlorate, antibiotics, bacteriophages and colicins. Based on systematic reviews of published reports, only three methods of pre-harvest interventions for EHEC have been validated to be reliably efficacious in reducing colonisation in cattle– the feeding of the probiotic combination *Lactobacillus acidophilus*

NP51 (NPC 747) and *Propionibacterium freudenreichii*, feeding of sodium chlorate and vaccination with T3SS proteins or Siderophore Receptor and Porin proteins (SRPs) (Sargeant et al. 2007; Snedeker et al. 2012). Meta-analysis also indicated no consistent association of antimicrobials with degree of shedding, and indicated that there are still an insufficient number of studies to confirm efficacy of other promising interventions such as bacteriophages and colicins.

Immunotherapeutic products that have reached market

Only two immunotherapeutics have successfully transitioned from research to market: a T3SS formulation known as Econiche® (Bioniche Life Sciences Inc., Belleville, Ontario, Canada) and a SRP formulation known as Epitopix® (Epitopix LLC, Willmar Poultry Company (WPC), Minnesota, USA). EHEC secrete T3SS proteins during colonisation and when injected directly through a host cell wall, these secreted proteins enable a receptor-mediated bacterial adhesion event to firmly anchor the bacterium to the site of the A/E lesion. The plausibility of using T3SS proteins as a vaccine was first reported on by the Finlay lab which demonstrated the secretion of extracellular proteins via a putative T3SS in both EHEC and EPEC (Jarvis et al. 1995; Jarvis and Kaper 1996). After partnering with the Vaccine and Infectious Diseases Organization (VIDO) in Saskatchewan, they demonstrated in a pilot study using a bacterial production platform that these attachment proteins reduced shedding of O157:H7 in cattle. With the intent of moving this product to market, Bioniche Life Sciences Inc. was contacted for scale-up and commercial manufacture of the vaccine. The product, called Econiche™, obtained full licensure by the CFIA in 2008 after clearing safety and efficacy requirements but has since

been discontinued due to poor market penetration, likely because of the cost and the frequency of animal handling that fell outside of regular handling schedules. The vaccine required three doses and in Phase II and Phase III studies using about 30,000 cattle, the vaccine efficacy was demonstrated to reduce duration (by 64%) and magnitude of shedding (2.3 log₁₀ reduction), reduce mucosal colonization (by 98%) and reduce hide contamination (by 54%) (Smith et al. 2009a; Smith et al. 2009b).

A siderophore receptor protein vaccine developed by Pfizer and marketed by Zoetis, known as EpiTopix™, was granted a conditional marketing license by the USDA in 2009 and is currently the only licensed vaccine available on the market. Siderophore receptor proteins are highly conserved outer membrane proteins that use high affinity ferric iron chelators, known as siderophores, to transport iron inside the bacterial cell. The vaccine consisted of multiple types of purified SRPs, of molecular weights of about 72-96kDa, extracted from the outer bacterial membrane. By engaging immunity against cell-surface SRP proteins, the vaccine was suggested to possibly restrict iron acquisition and thus competitively disadvantage the bacterium from finding a foothold in the gut. In the initial field study using three doses, efficacy was demonstrated to reduce fecal shedding (by 39% magnitude), reduce mucosal colonization (by 48%) and reduce hide contamination (by 70%). Like Econiche™, recommended usage is for three doses applied subcutaneously over the course of 8-10 weeks with an annual revaccination.

1.5 Plant-produced antibodies as an appealing intervention strategy

Rather than vaccines for active immunization, an appealing intervention strategy is the use of antibodies for passive immunization. Over the past five years, monoclonal

antibodies (mAbs) have become the highest grossing class of therapeutics in the global pharmaceutical market, valued at approximately US\$115.2 billion in 2018 with upside potential of \$300 billion by 2025 (Lu et al. 2020). mAbs are well recognized for their utility as diagnostics, immunotherapeutics and as research reagents such as for immunodetection and purification. As of December 2019, 79 therapeutic mAbs have been approved by the US FDA with considerable expected growth (Lu et al. 2020). The increasing demand necessitates the continuous development of more efficient and more cost-effective production methods. Plants have progressively become more accepted as green bioreactors capable of scaling up production cost effectively. The concept of plant-produced antibodies dates back to 1989 in which light and heavy chain subunits of an IgG were co-expressed and shown to fold correctly, assemble and be functionally equivalent to its mammalian counterpart (Hiatt et al., 1989). Plant platforms are not only useful for correct eukaryotic protein folding and modification but also accrue the market benefits of low cost, high scalability, increased safety, and notably a potential for scale-up to accommodate existing agricultural infrastructure for the bulk manufacture of transgenic plants (Sack et al. 2015; Twyman et al. 2003). Although no plant-made antibodies are in commercial production yet, several noteworthy plant-made antibodies have emerged as milestones. For example, personalized (patient-specific) idiotypic single chain antibodies by Large Scale Biology Corporation (McCormick et al. 2008) and Denka Corp, have cleared phase I trials for treating patients with non-Hodgkin's lymphoma. A tobacco-expressed antibody against HIV-1 was the first plant-made antibody to obtain GMP-compliant status in Europe for use in a first-in-human phase I clinical evaluation and has been shown to be safe and well

tolerated (Ma et al. 2015). A heavy chain hybrid antibody formulation against *Streptococcus mutans*, CaroRX[®] (Planet Biotechnology Inc), was shown to prevent dental caries when applied oral topically (Weintraub et al. 2005) and was phase II evaluated in the US as well as is registered as a medical device in Europe (Walsh 2006). The 2014 outbreak of Ebola virus disease in West Africa in particular has brought attention to the platform when ZMAPP, a cocktail of three transiently expressed *Nicotiana benthamiana*-made chimeric antibodies, was shown to be able to neutralize Ebola virus disease in 100% of *Rhesus macaques* primates upon challenge and was one of the first experimental drugs to be used on human patients with Ebola (Olinger et al. 2012; McCarthy 2014; Qiu et al. 2014). With regards to the 2020 global pandemic of COVID-19, on March 12, 2020, Medicigo Inc announced the successful production of Virus-Like Particles, a synthetic vaccine, of the coronavirus in just 20 days after obtaining the SARS-CoV-2 gene while concurrently developing chimeric SARS-CoV-2 antibodies. Therefore, plant-produced antibodies have become a well-established industry and intervention strategy for various diseases.

1.6 Antibody formats

Antibodies are typically sub-categorized into full-length monoclonal antibodies, antibody fragments and antibody synthetics based on structural characteristics and have different nuances when it comes to production, functionalities and market advantages. The general structure of antibody formats discussed in this thesis are shown in Figure 1.

Structurally, a full-length mAb consists of a homodimer of a crystallizable fragment (Fc) attached to an antigen binding region, which consists of a variable fragment

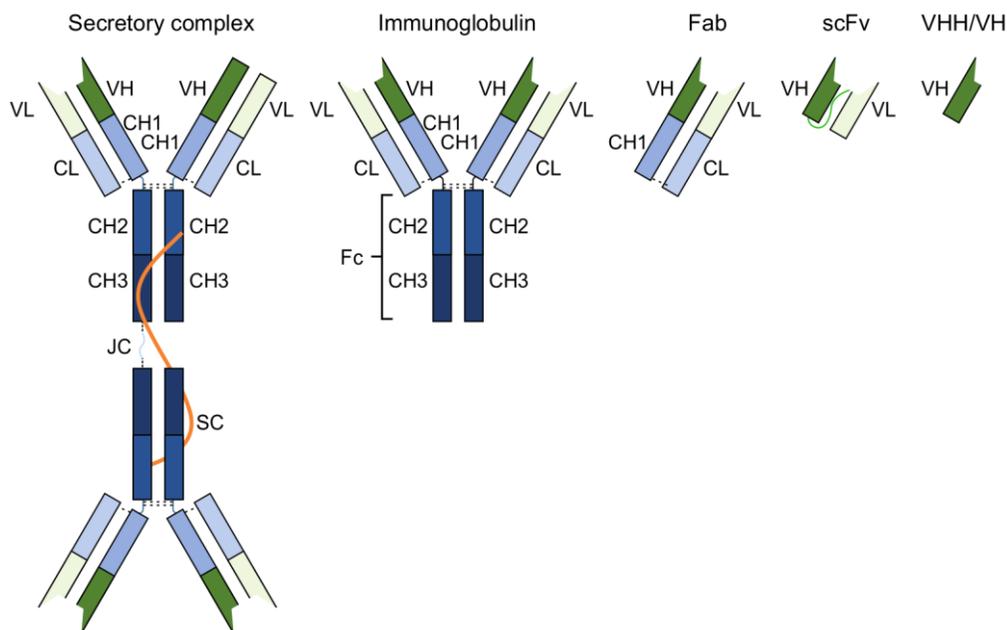


Figure 1. Schematic showing the generalized structure of some antibody formats produced in plants. VH: Variable heavy chain, VL: Variable light chain, CH: Constant heavy chain, CL: Constant light chain, JC: Joining chain, SC: Secretory component, Fc: Crystallizable fragment, Fab: Antigen binding fragment, scFv: Single chain variable fragment.

(Fv). The immunoglobulin molecule is distinctly modular and separate domains can be isolated to give distinct functionalities. As the name suggests, antibody fragments refer to specific portions of the immunoglobulin that have been selectively truncated to optimize pharmacological properties or efficiency of tissue penetration because of their smaller size. Fragments typically have a single antigen valency rather than the usual two for a standard immunoglobulin and because they lack the Fc component, may not be appropriate as therapeutics that rely on complement-mediated activation of effector functions such as IgG functionalities. There are three general categories of fragments, representing successive waves of antibody development: antigen binding fragments (Fab) composed of constant and variable domain of each of the heavy and light chains that form the paratope; single chain variable fragments (scFv) composed of the variable domain of the light chain fused

to the variable domain of the heavy chain via a linker; and single domain antibodies (sdAb), also known as variable heavy chain only (V_HH) fragments, composed of a single light chain variable region or heavy chain variable region. Notably, the variable heavy domain of the camelid antibody, also known as a single-domain antibody (VHH) or nanobody, is the smallest antibody derivative that remains functional in terms of antigen binding despite lacking light chains (Muyldermans et al. 2009).

Antibody synthetics refer to engineered versions of native antibodies such as by fusions, truncations or mutagenesis to produce proteins with altered properties such as yield, stability or immunogenicity. Synthetic fusions between an antigen binding fragment and an Fc is an appealing strategy that simplifies production to a single gene required for expression (De Greve et al. 2020). V_HH's in particular are amenable to Fc fusions because both fusion partners fold independently, do not require assembly as do Fabs and are more robust than scFvs.

Table 2. Relative comparison of properties across antibody production platforms. Ig: Immunoglobulin. Adapted from Sabalza et al. 2014

	Plant cells	Plants	Mammalian cells	Bacteria
Production cost	Medium	Low	High	Medium
Purification cost	Medium	High	High	High
Production time	Short	Very long	Long	Very short
Scale-up capacity	Medium	Very high	Low	Medium
Antibody format	Fragments; full-length Ig's; secretory	Fragments; full-length Ig's; secretory	Fragments; full-length Ig's	Fragments only
Contamination risk	Very low	Very low	Very high	Medium
Glycosylation fidelity	Plant-specific	Plant-specific; 'humanized'	Correct	None
Relative yield	Medium	Medium	High	Very high

1.7 Comparison to other production platforms

Relevant examples of currently used antibody production platforms include mammalian cell lines, process-scale microbial fermentation, plant cell cultures and whole plants. The relative strengths and weaknesses of mammalian cells, microbial fermentation, plant cell culture and plants have been summarized in Table 2.

Microbial platforms have emerged as promising systems for antibody fragment production with high yields and low production times. However, glycosylation is not feasible and expression of inactive misfolded proteins as aggregates or inclusion bodies is common (Gupta and Shukla 2017). Unlike full-length antibodies, antibody fragments do not require human-like post-translational modifications and therefore are amenable to expression in a prokaryotic system. Additionally, acetate and other endotoxins can accumulate as by-products that hinder growth and pose potential safety concerns (Brigham et al. 2011; Wong et al. 2008).

Mammalian cells are currently considered the ‘gold standard’ production platform for most applications because of high product quality, and are GMP-compliant (Wurm 2004). However, scale up is limited and there is concern about the possibility of contamination with mammalian pathogens. Several studies have suggested similar efficacy between plant and mammalian biosimilars. A tobacco-produced anti-rabies virus IgG was demonstrated to be similarly effective at neutralization *in vivo* as a post-exposure prophylaxis in hamsters as its commercially approved mammalian counterpart or human-derived rabies antibodies (Ko et al. 2003). A maize-produced broadly neutralizing anti-

HIV IgG activity was also shown to be similar to that of its counterpart produced in CHO cells when applied as a vaginal microbicide (Ramessar et al. 2008).

Plant cell cultures have also been active area of development because of the high level of containment and sterility of conditions. Notably, in 2012, carrot cells of *Daucus carota* L. (carrot) produced by Protalix Biotherapeutics were the first ever biotherapeutic-producing plant species to be approved by the FDA for human use (Shaaltiel et al. 2015). This sets a convenient precedent for market approval and the production of antibodies in this system may find an easier time of regulatory approval. The same group have also developed a BY-2 plant cell-expressed and delivered anti-TNF synthetic consisting of the human TNF receptor fused to an IgG Fc scaffold for the treatment of ulcerative colitis (Ilan et al. 2017) that has cleared phase I clinical trials (Almon et al. 2017). When orally administered, the fusion was safe and effectively induced anti-inflammatory immunomodulation. Still, the use of suspension cells results in the loss of all the agronomic and scalable economic advantages unique to plant production systems.

A techno-economic analysis of antibody production in a *Nicotiana* platform estimated a >50% reduction in capital investment and cost of goods sold as compared to production in a mammalian cell platform (Nandi et al. 2016). Additionally, it has been calculated by Planet Biotechnology Inc., a company working on producing antibodies in plants, that the costs of a plant-produced IgA are approximately 5% of the same IgA produced in mammalian cells (Daniell et al. 2001; Frenzel et al. 2013). Although plant platforms have an advantageous economy of scale, the downstream processing steps to obtain purified homogenous antibodies are comparably just as expensive as mammalian

cells or microbial fermentation. However, in the case of enteromucosal passive immunization, antibodies can be stored in lyophilized plant tissue (Chan and Daniell 2015; Tokuhara et al. 2013) and orally delivered as edible, unprocessed plant material removing costs associated with protein purification, vaccine formulation, and cold storage (Fischer et al., 2004). For passive immunization in animals in particular, the advantageous economy of scale and prospect of oral delivery is especially useful where bulk amounts of antibodies with repeated high doses are often required per animal and there are safety concerns associated with parenteral or intravenous delivery (Topp et al. 2016).

1.8 Plant expression systems

A variety of different plant species and tissue types have been investigated for antibody production (Table 2). Due to environmental risk and risk to the food chain, there is consensus now to use transient expression and cell culture which can both be done with high levels of containment (Menassa et al. 2012). The fast production time (~8days) of transient expression via agroinfiltration in particular is amenable to screening with high throughput and therefore allows quick optimization and troubleshooting of constructs. Transient expression via agroinfiltration using either traditional binary vectors (Vézina et al. 2009), with MagnICON vectors (Giritch et al. 2006) or with the use of cowpea mosaic vector hypertranslatable deleted RNA2 (Sainsbury and Lomonosoff 2008) has so far given the highest yield across general transformation methods. Great strides have been made in improving expression with various virus based expression systems like magnICON® (Gleba and Giritch 2011), Gemini (Huang et al. 2010b), Geneware (Pogue et al. 2010) and engineered vectors for *Agrobacterium*-infiltration, like the pEAQ system,

Table 3. Examples of plant-produced antibodies. Ig: Immunoglobulin. TSP: Total soluble protein. FW: Fresh weight

Pathogen/Disease/Purpose	Format	Expression host	Yield	Reference
Rabies; Human	Full-length IgG	<i>Nicotiana benthamiana</i> , leaves	0.07% TSP	(Ko et al. 2003)
Hepatitis-B virus; Human	Full-length IgG	Cell culture	0.2-0.6% TSP	(Yano et al. 2004)
Globodera rostochiensis nematode resistance; Tobacco	Full-length IgG	<i>Nicotiana benthamiana</i> , leaves	30-60 mg/kg FW	(Stevens et al. 2000)
Anthrax; Human	Full-length IgG	<i>Nicotiana benthamiana</i> , leaves	-	(Hull et al. 2005)
Rabies; Human	Full-length IgG	Cell culture	30 mg/kg FW	(Girard et al. 2006)
Botulinum neurotoxin; Human	Full-length IgG	<i>Nicotiana benthamiana</i> , leaves	20-40 mg/kg FW	(Almquist et al. 2006)
Skin cancer; Human	Full-length IgG	<i>Nicotiana benthamiana</i> , leaves	1.2 mg/kg FW	(Rodríguez et al. 2005)
HIV-1; Human	Full-length IgG	Cell culture	2.9 mg/kg FW	(Sack et al. 2007)
Breast/colorectal carcinoma; Human	Full-length IgG	<i>Nicotiana benthamiana</i> , leaves	30 mg/kg FW	(Brodzik et al. 2006)
Prevention of graft rejection; Human	Full-length IgG	<i>Nicotiana benthamiana</i> , Leaves and cell culture	-	(De Muynck et al. 2009)
Tumour-associated antigen tenascin-C; Human	Full-length IgG	<i>Nicotiana benthamiana</i> , leaves	50-100 mg/kg FW	(Villani et al. 2009)
Common cold; Human	Full-length IgG	<i>Nicotiana benthamiana</i> , leaves	-	Planet Biotechnology Inc.
Catalytic modulation	Full-length IgG	<i>Nicotiana benthamiana</i> , leaves	1.3% TSP	(Hiatt et al. 1989)
Herpes simplex virus; Human	Full-length IgG	<i>Glycine max</i> , <i>Oryza sativa</i> ; seeds	-	(Zeitlin et al. 1998)
Colon cancer; Human	Full-length IgG	<i>Nicotiana benthamiana</i> , leaves	-	(Verch et al. 1998)
<i>Porphyrous gingivalis</i> Periodontal disease	Full-length IgG	Cell culture	-	(Choi et al. 2016)
Hepatitis B virus; Human	Full-length IgG	<i>Nicotiana tabacum</i> ; seeds	6.5 mg/kg FW	(Hernandez-Velazquez et al. 2015)

HIV-1; Human	Full-length IgG	<i>Zea mays</i> ; seeds	30 mg/kg FW	(Rademacher et al. 2008)
Dental caries; Human	Full-length IgG (CaroRx®)	<i>Nicotiana benthamiana</i> , leaves	-	Planet Biotechnology Inc.
Colorectal cancer; Human	Full-length IgG (Cetuximab®)	<i>Zea mays</i> ; seeds	-	(Ludwig et al. 2004)
Cancer side-effects; Human	Full-length IgG (DoxoRx®)	<i>Nicotiana benthamiana</i> , leaves	-	Planet Biotechnology Inc.
Glioma; Human	Full-length IgG (Nimotuzumab®)	<i>Nicotiana tabacum</i> , leaves	-	(Rodríguez et al. 2005)
Respiratory syncytial virus; Human	Full-length IgG (Palivizumab ®)	<i>Nicotiana benthamiana</i> , leaves	-	(Zeitlin et al. 2013)
Common cold; Human	Full-length IgG (RhinoRX®)	<i>Nicotiana benthamiana</i> , leaves	-	Planet Biotechnology Inc.
Rheumatoid arthritis and B-cell non-Hodgkin's lymphoma; Human	Full-length IgG (Rituximab ®)	<i>Lemna minor</i> ; leaves	-	(Gasdaska et al. 2012)
Breast cancer and stomach cancer; Human	Full-length IgG (Trastuzumab®)	<i>Nicotiana benthamiana</i> , leaves	-	(Grohs et al. 2010)
Ebola; Human	Full-length IgG (ZMAPP®)	<i>Nicotiana benthamiana</i> , leaves	-	(Qiu et al. 2014)
Candida albicans fungal infection; Human	Full-length IgG; synthetic scFv-Fc fusion	<i>Nicotiana benthamiana</i> , leaves	50 mg/kg FW	(Capodicasa et al. 2011)
<i>Streptococcus mutans</i> ; Human	Full-length sIgA/G hybrid	<i>Nicotiana benthamiana</i> , leaves	-	(Ma et al. 1998)
<i>Salmonella enterica</i> ; Human	scFv fragment	<i>Nicotiana benthamiana</i> , leaves	42 mg/kg FW	(Makvandi-Nejad et al. 2005)
<i>Bothrops asper</i> venom; Human	scFv fragment	<i>Nicotiana benthamiana</i> , leaves	-	(Gomes et al. 2019)
Cytokinin modulation; <i>Nicotiana tabacum</i>	scFv fragment	<i>Nicotiana tabacum</i> , roots	-	(Gelova et al. 2018)
Tomato yellow leaf curl virus; Tomato	scFv fragment	<i>Solanum lycopersicum</i> ; leaves	-	(Safarnejad et al. 2008)
West Nile virus; Human	synthetic scFv-CH fusion	<i>Nicotiana benthamiana</i> , leaves	770 mg/kg FW	(Lai et al. 2014)

non-Hodgkins lymphoma; Human	synthetic scFv-Fc fusion	<i>Nicotiana benthamiana</i> , leaves	15-20 mg/kg FW	(Marusic et al. 2016)
Enterohemorrhagic <i>E. coli</i> toxin; Cow/humans	synthetic sIgG/A hybrid	<i>Arabidopsis thaliana</i> ; leaves	11 mg/kg FW	(Nakanishi et al. 2013)
Enterohemorrhagic <i>E. coli</i> ; Cow	synthetic VHH- sIgA	<i>Nicotiana benthamiana</i> , leaves	3% TSP	(Saberianfar et al. 2019)
Post-weaning diarrhea; Pig	synthetic VHH- sIgA, VHH-sIgG	<i>Arabidopsis thaliana</i> ; seeds	0.2%, 3% TSP	(Virdi et al. 2013)
Rotavirus; Human	VHH	<i>Oryza sativa</i> ; seeds	170mg/kg FW	(Tokuhara et al. 2013)

in which the T-DNA accommodates multi gene transfer and also bears the p19 viral silencing suppressor (Sainsbury et al. 2010; Sainsbury et al. 2009; Voinnet et al. 2003). Several groups, such as Medicago Inc., Fraunhofer IME, PlantForm Corp and Kentucky BioProcessing LLC have adopted this expression system and scaled up production with vacuum infiltration, automation and vertical farming to transform kilogram amounts of tissue per hour (D'Aoust et al. 2009; Pogue et al. 2010).

1.9 Glycosylation

Full length antibodies of all isotypes carry sites for N- and O- glycosylation both commonly recognized by both plant and animal systems (Cabanes-Macheteau et al. 1999). Although the core high mannose type of N-linked glycans added during ER passage are identical across plants and animals, subsequent complex glycans additions are different (Kallolimath and Steinkellner 2015). In general, plant glycans tend to be smaller and more heterogeneous than mammalian glycans and differ in the terminal sugar residues. There are three main differences in glycosylation between plants and animals. First, unique to plants is the presence of a β (1,2) xylose residue linked to the mannose residue of the glycan core. Second, a core fucose residue linked to the proximal glucosamine is positioned at α (1,3)

in plants but at α (1,6) in mammals (Strasser 2016). Third, plants lack β (1,4)-Gal and terminal α (2,6) sialic acid residues that are commonly found in mammalian proteins (Gomord et al. 2010).

Plant-specific glycans may alter serum half-life for antibodies intended for systemic protection (Bennett et al. 2018) and incompatible glycoforms have been associated with adverse immunogenic reactions in human patients (Reusch and Tejada 2015). For IgG, glycosylation is required for antibody-dependent, cell-mediated cytotoxicity and significantly extends the half life in the circulation (Cymer et al. 2018). Targeting the recombinant antibody for ER retention to prevent plant-specific glycosylation resulted in a decrease in serum half life and complement-dependent effector functions (Jefferis 2009). Therefore, to address concerns of immunogenicity, plant lines with ‘humanized’ glycans have been developed by knocking-out endogenous glycosyltransferases and/or knocking-in human glycosyltransferases to enable antibodies with a human-like glycan profile (Castilho et al. 2010; Kallolimath et al. 2018; Kallolimath and Steinkellner 2015; Strasser et al. 2009). The differences in glycosylation have been shown to not impact binding efficacy or specificity (Strasser 2016). Notably, some types of antibody fragments such as VHH’s or scFv’s are not glycosylated and thus depending on the end application, may be strategic choices for obtaining a more homogenous product. On the other hand, plants can synthesize largely homogenous complex N-glycans and are amenable to glycan engineering. For instance, targeted glycans-modification has been demonstrated for the seven N-glycosites of plant-produced human IgE to modulate Fc ϵ receptor affinity without sacrificing binding specificity (Montero-Morales et al. 2019).

Despite proof to the contrary, there still remains concern about the safety and efficacy of plant-based specific glycosylation, particularly with regards to potential immunogenicity, resulting in an uncertain environment for industry engagement yet spurring research to innovate around this regulatory bottleneck. This stringency has also shaped the direction of transformation methods for less contentious approaches to yield improvement such as by a keener interest in transient transformation (Huang et al. 2010a; Pogue et al. 2010; Sainsbury and Lomonossoff 2008) and plastid transformation (Bock 2007). Therefore, glycosylation is a relevant consideration for plant-produced antibodies because of potential effects on functionality, immunogenicity and regulation.

1.10 Plant-produced IgA as an oral passive immunotherapeutic for the control of EHEC

IgA is the predominant Ig class at mucosal surfaces such as the bronchial, genitourinary and digestive tracts (Corthesy 2010). In the gut lumen, secretory IgA interfaces with a variety of potentially pathogenic microorganisms and prevents their colonization. Because of its multivalent structure, secretory IgA can cross-link a number of large antigens with multiple epitopes and agglutinate them in the mucosa, a process known as immune exclusion (Corthesy 2013). Binding to pathogenic bacterial surface antigens inhibits their ability to dock and enter the cells of the intestinal epithelium and the agglutinated bacteria are then subsequently removed by peristaltic action (Corthesy 2003).

There are several examples of IgA production in edible plant tissue intended for passive enteromucosal immunization. A synthetic VHH-Fc fusion with IgA functionality produced in *Arabidopsis thaliana* seeds was effective in protecting piglets challenged with

enterotoxigenic E. coli, a related diarrheagenic species to EHEC (Virdi et al. 2013; Virdi et al. 2019). After oral administration of 20 mg/day, the VHH-Fc fusion protected against post-weaning diarrhea with progressive decline in shedding of bacteria by day 5, significantly lowered immune response biomarkers for pathogen exposure and significantly increased weight gain compared with the piglets receiving wild-type seeds (Virdi et al. 2013). In another example, an IgA against a rotavirus VP8 protein was produced in tomato fruits. Clarified extract was effective at preventing binding and neutralization of the rotavirus *in vitro* (Juarez et al. 2012).

A plant production platform is arguably the most suitable for producing recombinant IgA for veterinary applications because of the requirement for post-translational modification for proper folding and assembly, a high economy of scale which may be required considering the high prevalence and transmissibility of EHEC and lastly, the prospect of oral delivery as part of a feed formulation which may potentially be a safer and more cost effective delivery method (Wycoff 2005).

1.11 Research objectives

Towards the minimization of EHEC transmission, the development of an antibody for enteromucosal passive immunization is a promising pre-harvest intervention strategy. However, a higher yield requirement obstructs its transition from proof of principle to commercialization and thus strategies to improve its yield are also important considerations. To address these challenges, the objectives of this thesis are:

- 1) To assess the binding and neutralization of a synthetic antibody against the seven most prevalent strains of EHEC.

- 2) To identify and characterize engineered mutations of the antibody that enable improved yield without sacrificing functional efficacy
- 3) To enable targeting of the antibody to the thylakoid lumen for folding and assess its relative accumulation and efficacy.

2.0 PLANT-PRODUCED CHIMERIC VHH-SIGA AGAINST ENTEROHEMORRHAGIC *E. COLI* INTIMIN SHOWS CROSS-SEROTYPE INHIBITION OF BACTERIAL ADHESION TO EPITHELIAL CELLS

2.1 Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) has consistently been one of the foremost foodborne pathogen threats worldwide based on the past 30 years of surveillance. EHEC primarily colonizes the bovine gastrointestinal (GI) tract from which it can be transmitted to nearby farm environments and remain viable for months. There is an urgent need for effective and easily implemented pre-harvest interventions to curtail EHEC contamination of the food and water supply. In an effort to address this problem, Saberianfar et al. (2019) isolated single-domain antibodies (VHHs) specific for intimin, an EHEC adhesin required for colonization, and designed chimeric V_HH fusions with secretory IgA functionality intended for passive immunotherapy at the mucosal GI surface. The antibodies were produced in leaves of *Nicotiana benthamiana* with production levels ranging between 1 and 3% of total soluble protein. *in vivo* assembly of all subunits into a hetero-multimeric complex was verified by co-immunoprecipitation. Analysis of multivalent protection across the most prevalent EHEC strains identified one candidate antibody, VHH10-sIgA, that binds O145:Hnm, O111:Hnm, O26:H11, and O157:H7. Fluorometric and microscopic analysis also indicated that VHH10-sIgA completely neutralizes the capacity of the latter three strains to adhere to epithelial cells *in vitro*. This study provides proof of concept that a plant-produced chimeric secretory IgA can confer cross-serotype inhibition of bacterial adhesion to intestinal epithelial cells.

2.2 Introduction

Consumption of enterohemorrhagic *Escherichia coli* (EHEC) via contaminated food or water is associated with intestinal hemorrhage and osmotic dysregulation (Kandel et al. 1989). Each year, EHEC is estimated to affect approximately 230,000 people in the United States and is the fourth most frequently isolated food-borne pathogen from clinical stool samples (Hale et al. 2012). Approximately 73,000 EHEC infections are caused by the O157:H7 serotype which has consistently been the most prevalent and virulent EHEC serotype over the approximately 30 years of United States national surveillance (Hale et al. 2012). Six additional EHEC serogroups, O26, O45, O103, O111, O121, and O145, known as the “Big Six,” generally comprise >90% of non-O157 infections during any given year and have been traced to at least 22 human disease outbreaks in the United States since 1990 (CDC 2017).

The gastrointestinal (GI) tract of cattle is considered the primary reservoir of EHEC and can contaminate various food or water supplies via excreted fecal matter or after slaughter during processing of the carcass (Montenegro et al. 1990; Beutin et al. 1993). Indeed, cattle density has been identified as a primary risk factor for the incidence of local EHEC infections (Brehony et al. 2018). In accord with the “One Health” framework, virtually all strategic interventions to prevent EHEC transmission to humans have focused on minimizing colonization of cattle, thus reducing the risk of contamination from fecal shedding or at harvest. In cattle, EHEC principally adheres to and colonizes the lymphoid follicle-dense mucosa at the terminal rectum known as the rectoanal junction (Phillips et al. 2000; Naylor et al. 2003; Lim et al. 2007). The adhesin protein known as intimin

mediates intimate adherence of the bacterium with uninfected host epithelial cells by interacting with its cognate Translocated intimin receptor (Tir) and is a necessary prerequisite for further colonization (Kenny et al. 1997; Frankel et al. 1994).

The use and efficacy of recombinant secretory immunoglobulin A (sIgA) in passive mucosal immunotherapy is well established (Enriquez and Riggs 1998; Viridi et al. 2013; Nakanishi et al. 2017; Vanmarsenille et al. 2018). Because sIgA application can impart immediate, albeit transient, protection from a pathogen, it may be of value to beef producers and processors as a pre-harvest intervention for EHEC. In the GI tract, sIgA primarily functions to clear pathogens by immune exclusion: after binding to its target, glycans on the secretory component (SC) facilitate binding to the mucus lining of the GI tract enabling clearance of sIgA–pathogen complexes by peristalsis (Macpherson et al. 2008). A sIgA directed against intimin would thus be expected to neutralize colonization by blocking intimin from interacting with Tir, agglutinating EHEC in the mucus layer and then clearing it by subsequent peristalsis (Figure 2).

Structurally, sIgA consists of an IgA dimer linked by two additional chains: a 15-kDa joining chain (JC) that links the IgA Fc end-to-end (Krugmann et al. 1997) and a 70-kDa SC that coils around both Fc chains (Figure 1) (Bonner et al. 2007). A plant production platform is currently the most suitable for producing recombinant sIgA because of: 1) the requirement for glycosylation and disulfide bond formation for proper folding and assembly of sIgA subunits, 2) higher relative yields compared to other platforms and 3) the prospect of oral delivery (Wycoff 2005).

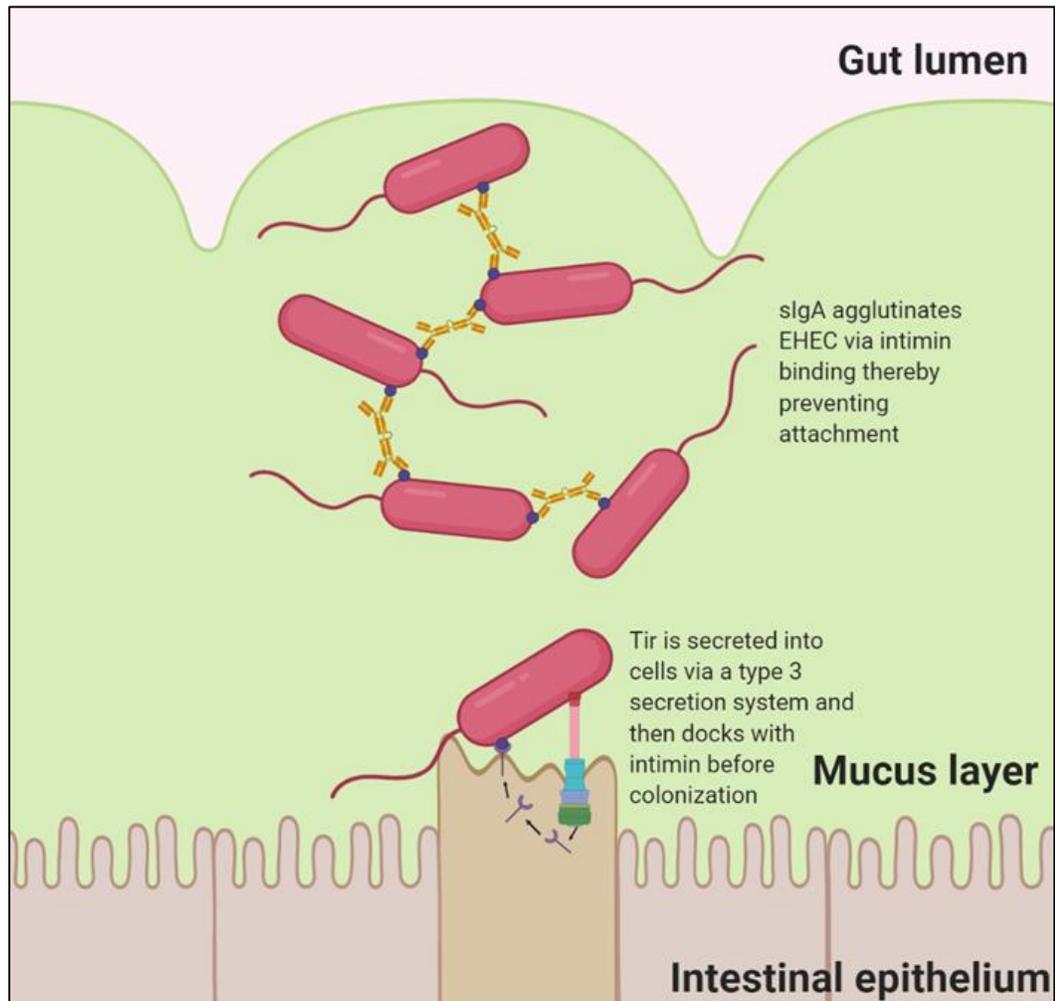


Figure 2. General schematic of the mechanism by which a secretory IgA would agglutinate EHEC cells to prevent colonization. Binding of secretory IgA inhibits EHEC's ability to dock and enter the cells of the intestinal epithelium by preventing the Tir-intimin interaction required for colonization. Cross-linked EHEC cells agglutinate in the mucosa and are shed by peristaltic action. (Figure created in Biorender.com)

With the intent of blocking the interaction of EHEC with the intestinal mucosa, Saberianfar et al. (2019) immunized a llama with the C-terminal 277 residues of intimin, which extend extracellularly from the bacterial cell and mediate the interaction with intestinal epithelial cells via binding to Tir (Frankel et al. 1994). They produced and panned a phage-displayed library of llama heavy chain only antibody variable domains (VHHs)

and identified four VHHs, denoted as VHH 1,3,9 and 10, that had nanomolar affinity to intimin γ , the main subtype associated with O157:H7. Of these, VHH 10 had superior binding affinity, as determined by ELISA and surface plasmon resonance assays, and was advanced for characterization. With passive mucosal immunotherapy and diagnostic development as end goals, they developed a chimeric antibody by fusing the isolated VHH to a bovine IgA Fc and co-expressing it with both JC and SC subunits to enable sIgA assembly and functionality. Unlike native mammalian sIgA which consists of four light chains, four heavy chains, one JC, and one SC, the chimeric antibody (VHH-sIgA) is composed of four VHH-Fc heavy chain-only subunits, one SC, and one JC (Figure 1). They demonstrated and optimized the production and correct assembly of the chimeric VHH-sIgA against intimin γ in *Nicotiana benthamiana* (Saberianfar et al. 2019).

In this collaborative project, I expressed and purified VHH10-sIgA and characterized its binding to and neutralization of the O157:H7 serotype as well as the “Big Six” serotypes. This study is notable because of the potential for development of an oral passive mucosal immunotherapeutic capable of multi-serotype protection, and as a diagnostic tool for detection of four of the seven most prevalent EHEC strains.

2.3 Materials and methods

Cloning and transient expression in *N. benthamiana*

The bovine Fc, JC, and SC sequences were obtained from the NCBI public database (ANN46383, NP_786967, and NP_776568, respectively). The VHHx-Fc, JC, and SC genes were synthesized by Bio Basic Inc. (Markham, ON, Canada), cloned into pEAQ-DEST-1 plant expression vectors (Sainsbury et al. 2009), and transformed into

Agrobacterium tumefaciens (EHA105). *N. benthamiana* plants were grown in a growth chamber at 22°C with a 16 h photoperiod at a light density of 110 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for 7 weeks, or in a greenhouse with natural light for 5 weeks before infiltration. Plants were fertilized with water soluble N:P:K (20:8:20) at 0.25 g/l (Plant Products, Brampton, ON, Canada). *Agrobacterium* cultures were prepared as previously described (Saberianfar et al., 2015). Transient expressions were performed either by injection (Miletic et al. 2015) or by vacuum infiltration for small-scale or large-scale transformations, respectively. Prior to vacuum infiltration, *Agrobacterium* transformed with expression vectors encoding either VHH1-Fc, VHH3-Fc, VHH9-Fc, VHH10-Fc, SC, JC, or p19 were sub-cultured from starter cultures and grown separately in Luria-Bertani (LB) broth at 28°C overnight. Each of the cultures bearing constructs encoding the VHHx-Fc genes was then combined with cultures carrying SC, JC, and p19. Trays of *N. benthamiana* plants were inverted and submerged into each of these co-cultures and placed into a vacuum chamber. To enable infiltration into the leaves, a pump was used to lower the pressure of the chamber to 85 kPa for 2 min and then immediately released. Plants were transferred back to the growth chamber until sampling.

Tissue sampling and protein extraction

Plant tissue was homogenized in a native buffer (1x PBS, pH 7.5, 0.1% Tween-20, 1 mM EDTA, 2% PVPP, 100 mM sodium ascorbate, 8 M sucrose, 1 $\mu\text{g}/\text{mL}$ leupeptin, 1 mM PMSF, 1 $\mu\text{g}/\text{mL}$ pepstatin A) in a 6 μL :1 mg tissue ratio. The homogenate was filtered through cheesecloth and subsequently centrifuged twice at 20,000xg for 20 min. Purification of the clarified extract was then performed using peptide M/Agarose

(Invivogen, San Diego, CA, United States, Cat. No. gelpdm-5) and anti-DYKDDDDK G1 affinity resin (GenScript, Piscataway, NJ, United States, Cat. No. L00432) according to the manufacturers' protocols.

Enterohemorrhagic *E. coli* binding assays

EHEC strains O26:H11, O45:H2, O103:H2, O145:Hnm, O121:H19, O111:Hnm, and O157:H7 were obtained from Dr. Michael Mulvey at the Public Health Agency of Canada, National Microbiology Laboratory, *E. coli* Unit, Enteric Diseases Program, Winnipeg, MB, Canada. EHEC strains were individually grown overnight in 5 ml of LB broth (Miller Formulation, Difco, Thermo Fisher Scientific, Ottawa, ON, Canada) at 37°C. The next day, 100 µl of the overnight culture was inoculated in 3 ml of LB broth and grown to an OD600 of 0.7–0.9. Cells were harvested from 1 ml of the culture by centrifugation at 13,000 × *g* for 5 min, rinsed three times in PBS for 5 min each time. The bacterial pellet was then resuspended in 1 ml of 2.5% paraformaldehyde (PFA) and incubated at 37°C for 10 min with gentle agitation (350 rpm). The excess PFA was rinsed by centrifugation at 13,000 × *g* for 5 min. The pellet was then resuspended in 200 µl of PBS-T and incubated overnight at 4°C. The next day, 20 µl aliquots of the cell suspension were prepared in separate tubes, centrifuged at 13,000 × *g* for 5 min, and resuspended in 20 µl of the primary plant-produced antibody treatments (100 ng/µl) in PBS-T, as well as PBS-T with no antibody as control, and incubated at 37°C for 90 min with gentle agitation (350 rpm). The primary antibodies were removed by centrifugation at 13,000 × *g* for 5 min, followed by three washes in PBS for 5 min each time. The cells were then resuspended in 20 µl aliquots of secondary antibody, rabbit anti-bovine IgG, IgM, IgA-FITC (1:40 dilution, Thermo

Fisher Scientific, Cat. No. SA1-36043), and incubated at 37°C for 1 h. The cells were washed and rinsed three times in PBS as described previously, and one final time in dH₂O. To stain the bacteria, the cells were resuspended for 2 min in 20 µl aliquots of DAPI (10 mg/ml solution diluted 1:1000 in dH₂O, Thermo Fisher Scientific, Cat. No. D1306), centrifuged at 13000 × g for 5 min, and resuspended in 20 µl of dH₂O. The cells were then transferred onto poly-L-lysine coated coverslips (Millipore Sigma, Cat. No. S1815) contained in a 24-well plate and centrifuge at 450 × g for 10 min. Coverslips were then dried and mounted onto glass slides with Aqua-Poly/Mount (Polyscience Inc., Warrington, PA, United States, Cat. No. 18606).

HEp-2 adherence inhibition assay

HEp-2 cells were grown in eight-well chamber slides in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Thermo Fisher Scientific, Toronto, ON, Canada) supplemented with 10% fetal bovine serum at 37°C in 5% CO₂ to ~80% confluency. EHEC strains O26:H11, O45:H2, O103:H2, O145:Hnm, O121:H19, O111:Hnm, and O157:H7 were individually grown overnight in 5 ml of LB broth at 37°C then subcultured into DMEM at a 1:50 dilution and incubated at 37°C in 5% CO₂ for 2 h. This subculture was further diluted at 1:10 in DMEM with and without 100 ng/ml of VHH10-Fc/SC/JC and then incubated with the HEp-2 cells at 37°C in 5% CO₂ for 3 h. The cultures were then washed with PBS to remove non-adherent bacteria and fixed using 2.5% PFA (Sigma) in PBS. Cells were then washed in PBS four times and blocked overnight in PBS containing 10% BSA and 0.1% Triton X-100. Cells were then hybridized with Alexa 647 phalloidin (Thermo Fisher Scientific, Cat. No. A22287) used to visualize actin in the HEp-2 cells and

donkey anti-rabbit Alexa 350 (Thermo Fisher Scientific, Cat. No. A10039) used to visualize EHEC cells. Cells were then washed in PBS and mounted using Aqua-Poly/Mount (Polyscience Inc., Warrington, PA, United States, Cat. No. 18606). To quantify adherence inhibition by relative fluorescence, the assay was adapted by growing the HEp-2 cells in 96-well black fluorometry plates that had been coated with poly D-lysine. Relative fluorescence was measured using a Synergy2 plate reader (Biotek) using the Gen5 v1.10 software (Biotek). Relative fluorescence of the donkey anti-rabbit IgG Alexa 350 antibody (Thermo Fisher Scientific, Cat. No. A10039) used to visualize EHEC cells was measured in each well at 37°C, with 5s intermediate shaking, excitation at 360nm, and emission at 460nm.

Confocal and fluorescence microscopy

To visualize binding of the VHH-sIgA to *E. coli* cells, FITC and DAPI sequential imaging was performed with an Olympus LSM FV 1200 or a Leica TCS SP2 CLSM. Samples were mounted in an Aqua-Poly/mount medium (PolySciences Inc, Cat No 18606-20) and images were acquired with a 100× oil objective lens. FITC was imaged by excitation with a 480 nm laser and detection at 520–540 nm. DAPI was imaged by excitation at 350 nm and detection at 455–465 nm. To visualize adherence to HEp-2 cells, a Leica TCS SP2 confocal microscope was used. Images were acquired with a 64× water objective lens. Alexa 647 phalloidin was imaged by excitation at 650 nm and detection at 660–680nm. The donkey anti-rabbit Alexa 350 antibody was visualized by excitation at 350 nm and detection at 455–465 nm.

2.4 Results

Plant-produced VHH10-sIgA binds EHEC strains O26:H11, O145:Hnm, O111:Hnm, and O157:H7

To determine if plant-produced chimeric VHH-sIgA antibodies bind to the seven most prevalent strains of EHEC, bacterial cells of O26:H11, O45:H2, O103:H2, O145:Hnm, O121:H19, O111:Hnm, and O157:H7 were incubated with VHH10-sIgA purified using anti-FLAG (binds the SC), then visualized using a secondary fluorescent antibody (rabbit anti-bovine-FITC) that binds the Fc and 4',6-diaminodino-2-phenylindole (DAPI) that stains bacterial cells. The confocal images showed consistent co-localization of FITC signal with strains O26:H11, O145:Hnm, O111:Hnm, and O157:H7 cells (Figure 3).

Plant-produced VHH10-sIgA reduces adherence of three EHEC serotypes to epithelial cells

Since intimin mediates the intimate attachment of EHEC to epithelial cells, I investigated if the binding of VHH10-sIgA to EHEC could neutralize the ability of bacteria to adhere to epithelial cells. HEp-2 cells were incubated with a culture of one of seven EHEC strains (O26:H11, O45:H2, O103:H2, O145:Hnm, O121:H19, O111:Hnm, and O157:H7) in the presence or absence of VHH10-sIgA, washed to remove any non-adherent bacteria, and then visualized by immunofluorescence microscopy. HEp-2 cells were visualized by fluorescent actin staining using rhodamine phalloidin (red) and EHEC cells using a donkey anti-rabbit Alexa 350-conjugated secondary antibody (shown in white).

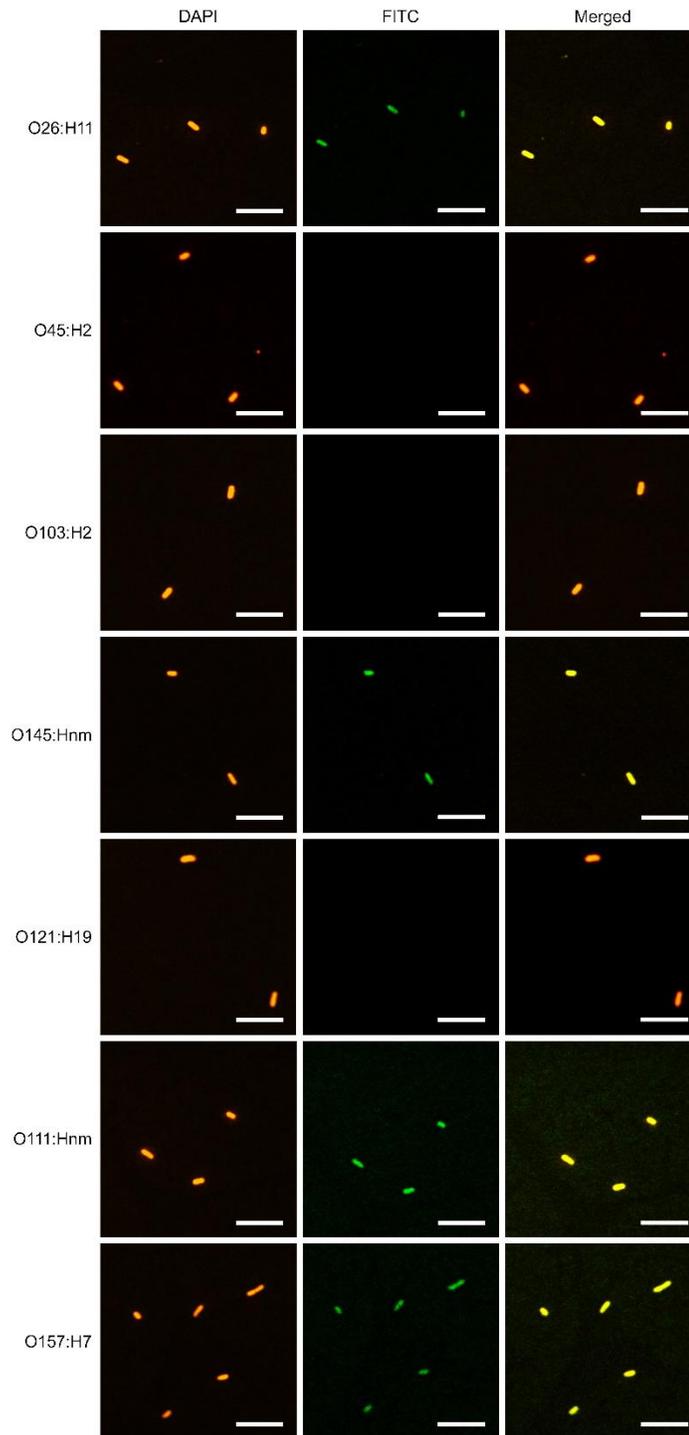


Figure 3. VHH10-sIgA binds EHEC O145:Hnm, O26:H11, O111:Hnm, and O157:H7. Shown are confocal images visualizing the binding of the seven most prevalent strains of EHEC with VHH10-sIgA. Binding is visualized by DAPI that stains EHEC bacterial cells (blue) and a FITC-conjugated antibody that hybridizes to the Fc chain of VHH10-sIgA (green). A merged image shows an overlay of the blue and green channels used to visualize DAPI and FITC, respectively, to assess co-localization. Size bar = 10 μ m

Compared to the respective positive controls of HEp-2 cells and EHEC only, the addition of VHH10-sIgA seemed to abrogate the adhesion of EHEC strains O26:H11, O111:Hnm, and O157:H7 to HEp-2 cells, while it seems to somewhat reduce adhesion of EHEC strain O145:Hnm to HEp-2 cells (Figure 4). To quantify the neutralization capacity of VHH10-sIgA, I adapted the adhesion assay for fluorometry and measured the relative fluorescence of HEp-2 cells incubated with a culture of each of the seven EHEC strains with and without VHH10-sIgA. The addition of VHH10-sIgA afforded complete protection, that is, it reduced the relative fluorescence caused by adherent bacteria for strains O26:H11, O111:Hnm, and O157:H7 to background levels, and somewhat reduced the relative fluorescence caused by adherent bacteria for strain O145:nm, although this effect was not statistically significant ($p = 0.09$ in a T-test; Figure 4).

I performed a multiple sequence alignment and derived a neighbor-joining tree of Int277 across all seven strains and found that EHEC strains O157, O111, O145, and O26 grouped together based on sequence similarity, while O45, O103, and O121 were more disparate in sequence (Figure 5). With the exception of O145, this is in accord with VHH10-sIgA being able to bind and neutralize O26, O111, and O157 but not bind and neutralize O45, O103, and O121.

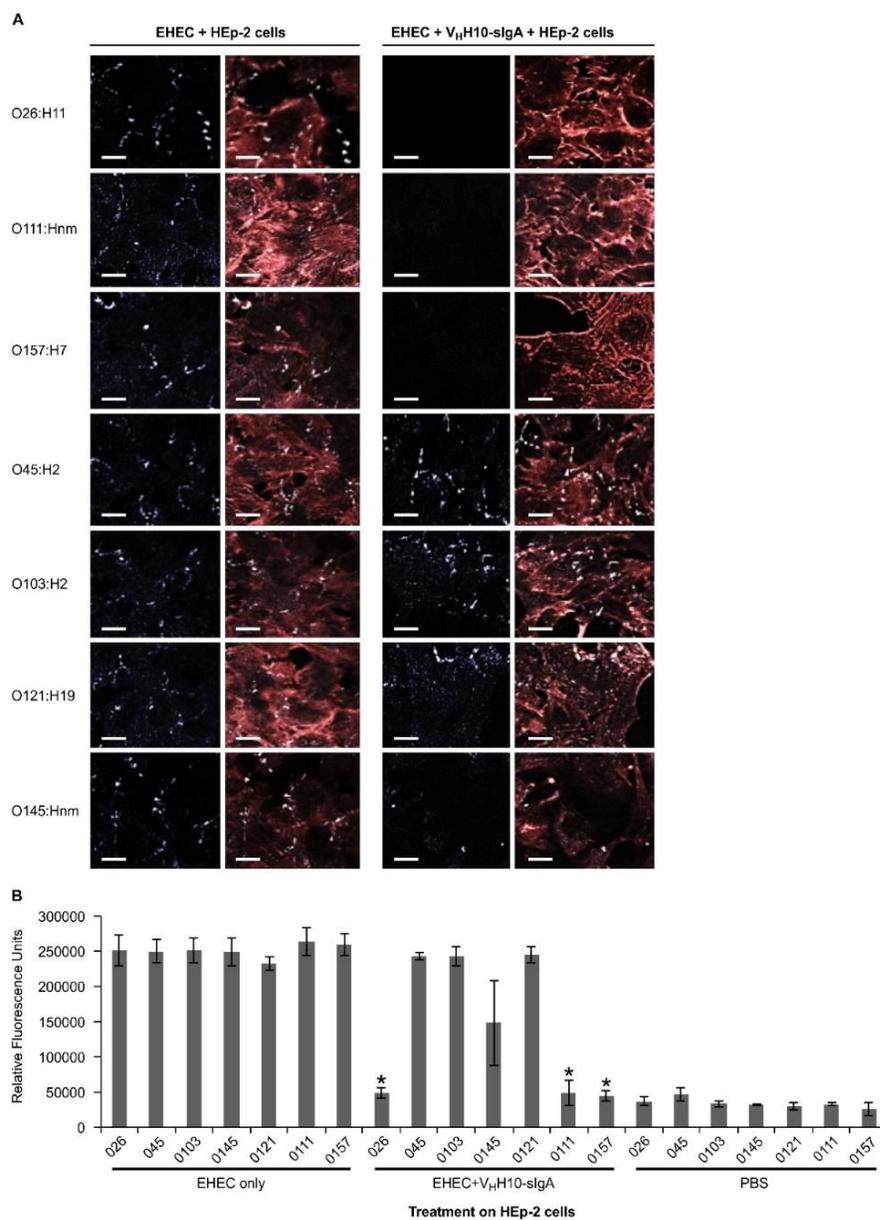


Figure 4. VHH10-sIgA neutralizes EHEC O26:H11, O111:Hnm, and O157:H7 adhesion to HEp-2 cells. (A) Neutralization was visualized by immunolabelling EHEC cells with a donkey anti-rabbit secondary antibody (white) as well as the actin cytoskeleton of HEp-2 cells using rhodamine phalloidin (red). Shown are merged images of the red and white channels for either HEp-2 cells incubated with EHEC alone (left panel) or with EHEC and VHH10-sIgA (right panel). (B) VHH10-sIgA reduces fluorescence of O26:H11, O111:Hnm, and O157:H7 to background levels. Shown is the relative fluorescence of EHEC strains that have been immunolabeled, are adherent on HEp-2 cells, and either incubated on HEp-2 cells alone or in combination with VHH10-sIgA. As a negative control, HEp-2 cells were incubated with PBS instead of a bacterial strain or antibody. * indicates a significant reduction of the amount of immunolabeled adherent bacteria as determined by a one-tailed unpaired homoscedastic T-test between an EHEC strain alone versus the same EHEC strain with VHH10-sIgA added ($p < 0.05$, $N = 3$ biological replicates). Error bars indicate standard errors of the means. Size bar = 10 μ m

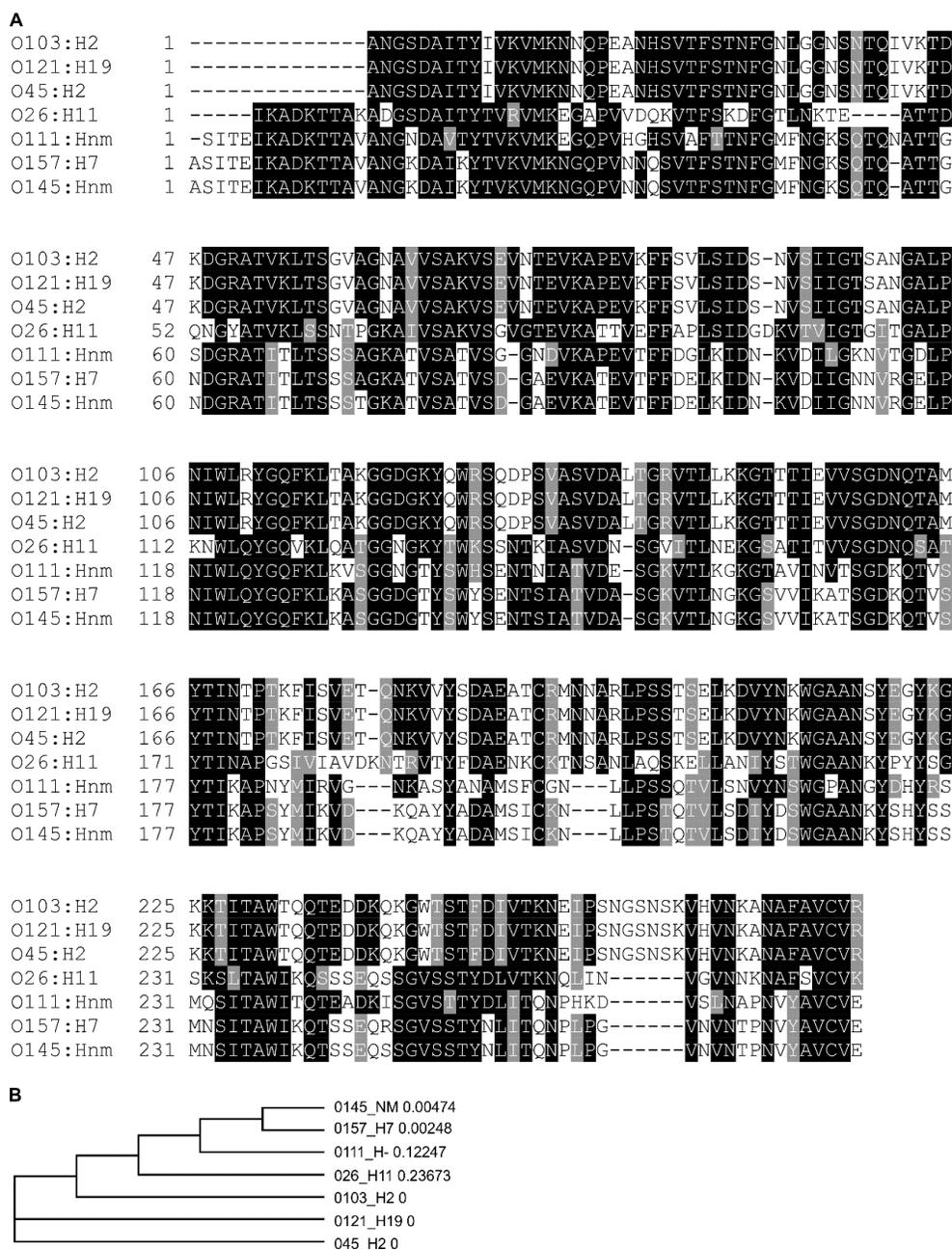


Figure 5. Sequences for Int277 are similar across EHEC strains O157, O111, O26, and O145. (A) Multiple sequence alignment using Clustal Omega default settings of Int277 protein sequence for the seven tested EHEC strains (B) Phylogenetic tree using a neighbor joining method to cluster the aligned Int277 sequences based on similarity.

2.5 Discussion

All EHEC strains use a highly conserved type III secretion system to enable colonization of intestinal epithelial cells. Intimate adherence mediated by intimin docking to its translocated cognate receptor is a necessary prerequisite for invasion and virulence (Dziva et al., 2004; Buttner, 2012). Given that the results indicate that VHH10-sIgA prevents intimate adherence for strains O26:H11, O111:Hnm, and O157:H7, it is tempting to speculate that this protective effect will also be observed when used in animal trials. Although VHH10-sIgA was able to bind O145:Hnm, fluorometry and confocal images of the adhesion assay suggested a compromised ability to neutralize. It is possible that VHH10-sIgA can partially bind O145:Hnm but not sufficiently to prevent intimate adherence to epithelial cells. Regarding the differential capacity of VHH10-sIgA to bind and neutralize across strains, I speculate that this may be due to sequence variability across the C-terminal 277 residues of the intimin protein. Although the transmembrane and intracellular residues are strongly conserved in native intimin, the extracellular Int277 region is highly variable and is likely shaped by selection pressures of the host immune system. Despite O145 being similar in sequence to O157, weaker binding and neutralization of O145 may be due to sequence variability at a local epitope to which VHH10 binds rather than sequence conservation of Int277 as a whole. (Saberianfar et al. 2019) showed by surface plasmon resonance and ELISA that both the plant-produced VHH-Fc chain as well as the assembled VHH-sIgA complex have the same binding affinity as the monomeric VHH produced in *E. coli*, suggesting that binding is modularly mediated and retained via the VHH following Fc fusion and assembly with the SC and JC. Consistent

co-localization was observed for VHH10-sIgA with strains O26:H11, O145:Hnm, O111:Hnm, and O157:H7 cells by immunofluorescence confocal microscopy. Coverage was observed across the entirety of the cells unlike previous reports of partial binding for rat- and chicken-produced antibodies against EHEC (Cook et al., 2007), suggesting that intimin is abundantly embedded across the entire cell surface membrane of these four strains and is accessible to VHH10-sIgA.

The finding that VHH10-sIgA offers multivalent protection against EHEC O26:H11, O111:Hnm as well as O157:H7 is notable because the vast majority of previously developed therapeutics against EHEC have focused on O157 only, despite the clinical relevance of the “Big Six” strains. The current incidences across the United States of O26, O111, and O157 are 206, 125, and 807 per 100,000 individuals, respectively (CDC, 2017). Although non-O157 strains are individually less prevalent, the collective contribution of non-O157 strains to GI illness has recently been of growing concern, particularly since surveillance data indicated a 41% increase in the average annual incidence of infection of non-O157 strains over the last 5 years across the United States. The majority of diagnostic, intervention, and awareness strategies have historically been O157-specific (Gill and Gill, 2010; CDC, 2017). O26 and O111 currently account for 25.5 and 15.5% of non-O157 EHEC infections, respectively (CDC, 2017).

In conclusion, I have demonstrated a plant-based chimeric antibody to show neutralization competency against three EHEC strains. Further work testing the efficacy of VHH10-sIgA in live animals will hopefully confirm its ability to prevent EHEC colonization and shedding as well as its utility for fast acting prevention and intervention.

Because of its multivalency, VHH10-Fc may also be useful if developed as a diagnostic reagent for detecting O26, O111, O145, and O157 in food, the environment, colonized animals, and in infected individuals. Currently, there are no EHEC diagnostics available on the market that can detect both O157 and non-O157 strains despite their clinical relevance. I am optimistic that either of these directions for development will be of value in minimizing EHEC contamination of food and the environment.

Addendum

VHH10 was identified at a later date after VHHs 1, 3 and 9. Because VHH10 was shown to have superior binding affinity by surface plasmon resonance (Saberianfar et al. 2019), it was chosen in lieu of the other three previously identified VHHs to be advanced toward pathogen binding and neutralization assays for publishing the article used in Chapter 2. However, by that time, I had already advanced the previously superior VHH9 in cloning the constructs used in Chapters 3 and 4. Both VHHs 9 and 10 show similar patterns of cross-serotype neutralization and are considered high affinity binders with dissociation constants (K_D) of 1.1 nM and 0.1nM respectively. Although I realize that this detracts from the cohesiveness of this thesis, Chapter 2 is nonetheless instructive in introducing the original design and production of the VHH-Fc antibodies and for transitioning the concept to the yield improvement strategies discussed in Chapters 3 and 4.

After Chapter 2 was published, I had doubts about the neutralization data for the O145 strain that I had used. The relative fluorescence numbers in the fluorometric neutralization assay for O145 seemed out of step with the rest of the data (Figure 4) and the microscopy images suggested that the antibody was binding to O145 but not neutralizing it. Furthermore, upon examination of the amino acid sequences (Figure 5), O145 intimin was the most similar in sequence to O157 intimin, which was the antigen originally used for the animal challenge to identify the VHHs. Suspecting contamination, I ordered a new strain from a different supplier (ATCC, C625) and redid the neutralization assay. Both VHH10-sIgA, used in Chapter 2, and VHH9-Fc, used in Chapters 3 and 4, were

able to completely neutralize the new O145 strain (Figure 6). So, the disparity of neutralization competency across the seven strains does seem to be indicated by the phylogenetic grouping and the binding epitope can be speculated based on the sequence conservation of strains O157, O111, O26 and O145 versus strains O45, O121 and O103. Neutralization assays in Chapters 3 and 4 use the new O145 strain (C625).

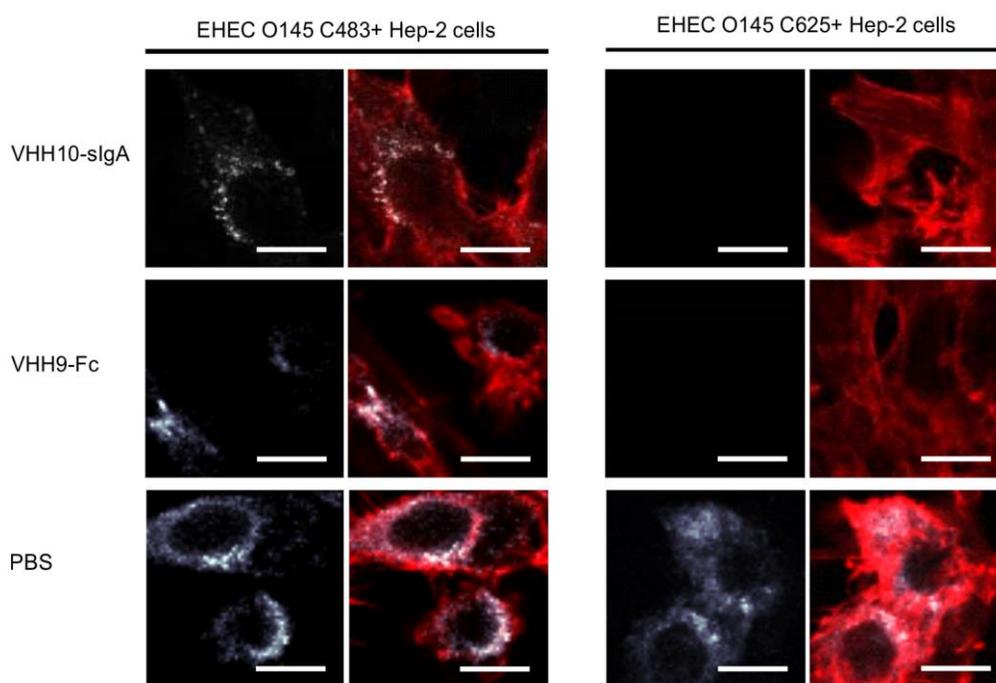


Figure 6. A differently sourced O145 strain (C625) is neutralized by VHH10-sIgA and VHH9-Fc(5+1). Shown are confocal images of the previously used EHEC strain O145 (C483) and the subsequently obtained EHEC strain O145 (C625) (white) that have been incubated with Hep-2 cells (red) in the presence of PBS as a control, with VHH9-Fc or VHH10-sIgA. Cells that are immunolabelled (white) are intimately adherent on Hep-2 cells (red) after repeated washes and their absence suggests neutralization. Size bar = 20 μ m

Chapter 2 focused on characterizing the functionality of the VHH-sIgA complex because it was previously thought that the entire complex was needed for protection, as secretory IgA is the predominant functional unit in enteromucosal protection in mammals. Although VHH-sIgA's are effective, the requirement for multi-subunit assembly greatly reduces yield and increases product heterogeneity and thus recent design has shifted toward simplifying production to just the VHH-Fc immunoglobulin (Figure 1; De Greve et al. 2020) which has been demonstrated to be sufficient for neutralizing enterotoxigenic infection in the gastrointestinal tract of piglets (Viridi et al. 2019). So, although the secretory complex enables better avidity and better proteolytic protection, simplifying production to just the VHH-Fc is a more practical design choice, especially with yield as a priority.

Given the importance of yield, Chapter 3 explores the use of protein engineering principles to improve the yield of the VHH-Fc. Because the Fc is considered a stabilizing scaffold for a variety of different therapeutics (Ning et al. 2019), my general approach was to design around a stabilized Fc that could potentially have other applications beyond anti-EHEC VHH fusions. With this in mind, I hypothesized that I could identify key amino acid mutations in the Fc that would enable improved yield upon fusion to an anti-EHEC VHH without sacrificing its efficacy for cross-serotype neutralization.

3.0 A RATIONALLY DESIGNED BOVINE IGA FC SCAFFOLD FOR ENHANCED *IN PLANTA* ACCUMULATION OF A VHH-FC FUSION THAT BINDS ENTEROHEMORRHAGIC *E. COLI*

3.1 Abstract

To improve the yield of a synthetic antibody intended for enteromucosal passive immunization in cattle herds, I employed three rational design strategies, 1) supercharging, 2) introducing *de novo* disulfide bonds and 3) reducing Gibbs' free energy, on a bovine IgA Fc chain. I fused the mutagenized Fc to a single domain antibody (VHH) that binds Enterohemorrhagic *E. coli* (EHEC) and screened for accumulation levels after transient transformation into *Nicotiana benthamiana* leaf tissue. I have identified and characterised five supercharging and one disulfide mutant that, in comparison to native, all individually improve accumulation. Pyramiding of these mutations is associated with an incremental increase of accumulation for the Fc alone as well as when fused to a VHH that binds EHEC. Co-immunoprecipitation experiments suggest that the capacity to assemble with a joining chain and secretory component into a secretory IgA form is retained after introducing the stabilizing mutations. Immunofluorescence microscopy indicated that the cross-serotype pattern of binding efficacy and neutralization is retained in the engineered fusion similar to native. These results collectively suggest that an Fc chain can be rationally designed for improved accumulation of a VHH-Fc fusion without sacrificing efficacy.

3.2 Introduction

Fragment crystallizable (Fc)-fusion proteins, also known as Fc chimeric proteins or Fc-tagged proteins, comprise the heavy chain domains CH2 and CH3 of an immunoglobulin (Ig) that have been genetically linked to a protein of interest, typically an antigen or a small scale binder. The Fc independently folds and can improve the solubility and stability of its fusion partner upon *in vivo* expression (Czajkowsky et al. 2012). Additionally, the Fc domain homodimerizes with itself via an interchain disulfide bond allowing an increase in avidity for a fused partner (Bastian et al. 1992). Fc fusion proteins and monoclonal antibodies comprise 15 and 48% respectively of all recently approved biologics by the FDA (2011-2016), with significant market increases forecasted for 2016 to 2025 (Lagasse et al. 2017). Since the approval of Etanercept (Enbrel), the first Fc fusion, by the US Food and Drug Administration (FDA) in 1998, for the treatment of rheumatoid arthritis and psoriasis, sales of fusion proteins have dramatically increased and are now among the top four most lucrative classes of therapeutics (Ecker et al. 2015). Although the market consists predominantly of IgG subtype Fc fusions, there has been much interest in developing an alternative IgA-based Fc scaffold for the control of mucosal pathogens in *in vivo* applications such as passive immunization (Bakema et al. 2011; Bakema and van Egmond 2011; Lohse et al. 2011). However, development has been hampered by technical difficulties because of native IgA's large size, tendency to aggregate and the requirement for post-translational modifications for proper folding and assembly (Reinhart and Kunert 2015). The more effective successful reports of IgA production have utilized truncated IgA

synthetics or utilized a plant platform for the complex folding required (Vanmarsenille et al. 2018; Viridi et al. 2013).

Over the last two decades, there has been much research focus on developing small size binders with stable scaffolds from a variety of sources. However, yield is arguably the greatest barrier for pushing these products to market. Based on a techno-economic analysis of a transient plant-based platform for monoclonal antibody (mAb) production, a model estimates that improvements to yield substantially decrease capital costs and the cost of goods sold (Nandi et al. 2016). Therefore, there is value in exploring upstream optimization strategies to offset production and purification costs.

To develop a stabilized IgA Fc chain that could improve yield of its fusion partner without impairing function, I investigated three rational design strategies, supercharging, introducing *de novo* disulfide bonds and reducing free energy, on a bovine IgA Fc chain. Conceptually, supercharging can enhance accumulation by reducing non-specific protein aggregation during the macromolecular crowding effect of recombinant protein production by providing small charge-charge repulsive forces on the protein surface (Lawrence et al. 2007). The introduction of novel disulfides at strategic locations can enhance accumulation by preventing exposure of the reactive hydrophobic interior normally susceptible to proteolysis or non-specific aggregation (Zabetakis et al. 2014). Lowering the free energy of the folded protein state can conceptually enhance kinetic stability by extending the required activation energy for the folded protein to transition to an unfolded state thereby reducing the rate by which the unfolded state irreversibly inactivates such as by aggregation, degradation, free disulfide exchange etc. (Chandler et al. 2020).

Enterohemorrhagic *Escherichia coli* (EHEC) has consistently been one of the foremost foodborne pathogen threats worldwide, conservatively estimated in causing 2.8 million acute illnesses annually (Majowicz et al. 2014a). It primarily colonizes the bovine digestive tract as a reservoir from which it can be transmitted via fecal shedding or during slaughter to ultimately compromise food and water safety (Beutin et al. 1993a; Montenegro et al. 1990a). Towards addressing this problem, we previously isolated camelid-derived single-domain antibodies (VHHs) specific for intimin, an EHEC adhesin required for colonization, fused them to a bovine IgA Fc and demonstrated that these chimeric VHH-Fc fusions could bind and neutralize the adherence of three of the most prevalent EHEC serotypes: O157, O26, and O111 (Saberianfar et al. 2019). I subsequently showed that these chimeric fusions neutralize a 4th EHEC serotype, O145.

In this study, I identified a stabilized rationally designed bovine IgA Fc chain with five mutations predicted to supercharge the protein, and one mutation predicted to introduce a novel disulfide respectively. After transient transformation into *Nicotiana benthamiana* leaves, these mutations, individually as well as when combined, enabled an improved and pyramided *in vivo* accumulation of both the Fc chain alone and a fused VHH partner. Co-expression of the engineered VHH-Fc fusion with a bovine joining chain and secretory component and subsequent co-immunoprecipitation suggests that the mutations do not impair correct folding and assembly of components to its secretory IgA form. I also demonstrate that the engineered Fc fusion does not compromise the VHH's function of binding and neutralizing EHEC strains O26, O111, O145 and O157. This study is notable because it provides proof of concept that an Fc chain may be rationally designed for

improved IgA yield in a plant platform without sacrificing efficacy or its ability to assemble into its secretory form.

3.3 Materials and methods

Design and selection of rationally designed Fc candidates

Although the crystal structure of human IgA Fc has been determined and is publicly available (pdb: 1IGA), bovine IgA Fc, which is 70% similar in sequence, has not yet been documented. Because the structure of Fc is generally well conserved across species, I used the I-TASSER online program (Zhang 2008) to predict the structure of bovine IgA Fc using the human IgA Fc as a threading template (Wu and Zhang 2008; Zhang 2008). Because the resulting predicted structure had a high confidence score of 1.35 (given a range of -5 to 2), I speculated that it may be sufficient for predicting rational design candidates.

Estimating of negatively supercharged Fc candidates was performed computationally by first ranking residues for solvent accessibility by their average number of neighboring atoms (within 10 Å) per side-chain atom (AvNAPSA) and then identifying highly polar solvent-exposed Asn and Gln residues for mutation to their negatively charged counterparts, Asp and Glu respectively (Schrodinger 2010). For visualizing the multimeric complex, the VHH-Fc sequence was submitted as a dimer to the SPRING server which uses a template-based threading algorithm across the PDB library to predict structure and assembly (Guerler et al. 2013)

Disulfide candidates were selected by manual inspection of the model in PyMol (Schrodinger 2010) based on residue proximity (under 5 Å) or by ranked selection of ΣB -factor, a measure of dynamic mobility for each atom, in the DisulfidebyDesign 2.0 software

(Dombkowski et al. 2013). To retain the functionality of the native Fc, native disulfide sites were avoided (interchain: 16-18, 205-207, 199-201, 13-15; intrachain: 100-102, 271-273, 412-414, 601-603; tailpiece to JC: 718-720; 235-237; free S-H: 235-237).

Lowered Gibbs free energy candidates were chosen using the FoldX online force field that uses an empirical method to estimate mutational effects on protein thermostability (Schymkowitz et al. 2005). The predicted structure from the I-TASSER online program (Zhang et al. 2008) was used as the template for mutation. The structure was first minimized using the 'RepairPDB' command. Then, each residue was sequentially screened by substituting for each of the other nineteen amino acids using the 'PositionScan' command. The predicted stabilizing mutations were then built using the 'BuildModel' command for free energy calculations. The outputted free energy estimations were output to and sorted in Excel and twelve candidates were selected based on most negative difference in free energy ($\Delta\Delta G$) with a cut-off value of $-1.5 \text{ kcal mol}^{-1}$.

Cloning of rationally designed mutations and transient expression in plants

Rational design mutations were individually made to the native bovine IgA Fc sequence (ANN46383)(Chapter 2) using an *in vitro* single primer site-directed mutagenesis method (Huang and Zhang 2017). Combination of mutations was done using a multi-site-directed mutagenesis method (Liang et al. 2012). Genetic fusion to an anti-EHEC VHH (denoted as VHH9 identified previously in Chapter 2) was done using a sequence and ligation independent cloning (SLIC) method (Li and Elledge 2007). All cloning was confirmed by sequencing.

To enable expression in leaf tissue, each construct was cloned into an in-house pCaMGate plant expression vector (Pereira et al. 2014) using the Gateway® cloning kit (Thermo-fisher Scientific Inc, Waltham, MA, USA). The pCaMGate vector attaches an N-terminal Xpress tag for protein stability, a C-terminal c-myc tag for detection and purification as well as a C-terminal KDEL tag for retrieval to the ER of the plant cell. Vectors were then transformed into *Agrobacterium tumefaciens* (EHA105) and transformed bacteria were selected for using kanamycin and rifampicin antibiotics. Cultures were resuspended into infiltration media consisting of Gamborg's B-5 medium solution (3.2 g/L Gamborg's B5 salts with vitamins, 20 g/L sucrose, 10 mM MES, pH 5.6, 200 μ M acetosyringone). Each construct was co-infiltrated with a vector carrying p19, a suppressor of post-transcriptional gene silencing from *Cymbidium* ringspot virus (Silhavy et al. 2002), each at a final OD of 0.3. Transient expression was performed by syringe infiltration into leaf tissue of *N. benthamiana* plants. Plants were grown in a growth chamber at 22°C with a 16 h photoperiod at a light intensity of 110 μ mol m⁻² s⁻¹ for 7 weeks and fertilized with water soluble N:P:K (20:8:20) at 0.25 g/L (Plant products, Brampton, ON, Canada).

Protein extraction and western blotting

Pre-weighed leaf samples were frozen in liquid nitrogen and homogenized with silica beads (Bio Spec Products Inc., Bartlesville, OK, USA) for 2 min using a TissueLyser II (Retsch Inc., Newton, PA, USA). One mL of either a denaturing extraction buffer (1x PBS, pH 7.5, 4% SDS, 2% PVPP) or a native buffer (1x PBS, pH 7.5, 0.1% Tween-20, 1 mM EDTA, 2% PVPP, 100 mM sodium ascorbate, 8 M sucrose, 1 μ g/mL leupeptin, 1 mM

PMSF, 1 $\mu\text{g}/\text{mL}$ pepstatin A) was added per approximately one hundred mg of sample. All samples were vortexed on high speed for 30 s and centrifuged at 20,000 x *g* for 10 min to remove insoluble debris. Extracted proteins were combined with 1/5th volume of 5 x reducing loading buffer (0.3 M Tris-HCl pH 8.0, 5% SDS, 10% glycerol, 100mM DTT, 0.05% Phenol Red) heated at 90°C for 10 min, then loaded onto Express Plus 4-20 % gradient polyacrylamide gels (Genscript Inc., Piscataway, NJ, USA). Gels were run at 100 V for 100 min, then transferred to polyvinylidene difluoride (PVDF) membrane using the Trans-Blot Turbo transfer system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Blots were blocked overnight with 5% skimmed milk in tris-buffered saline, pH 7.5, and proteins of interest were probed with a mouse anti-c-myc antibody (diluted 1:1000; Genscript Inc., Piscataway, NJ, USA) and the One-Hour Basic western kit for mouse primary antibody (Genscript Inc., Piscataway, NJ, USA). Detection was performed using Amersham ECL western blot detection reagents (GE Healthcare, Mississauga, ON, Canada) or Enhanced Chemiluminescent detection solution (Biorad Laboratories Inc., Hercules, CA, USA) and a MicroChemi 4.2 imaging system with GelCapture acquisition software (DNA Bio-Imaging Systems Ltd., Jerusalem, Israel). For staining, membranes were rinsed in methanol followed by ultrapure water, stained using GelCode Blue (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 15 min, and destained in 50% methanol 1% acetic acid for 15 min. Quantification of accumulation was done by densitometry using a calibrated standard curve of an in-house-produced purified protein. Statistical significance for accumulation in tissue expressing the native and mutant Fc was determined using a one-way ANOVA with

three to five biological replicates. Post hoc comparisons were then performed on the accumulation means using the Tukey HSD test.

Recombinant protein purification

Plant extracts were prepared under native conditions as described above. Purification was performed using an anti-c-myc purification kit (MBL International Corp., Woburn, MA, USA) according to the manufacturers' protocols.

Enterohemorrhagic *E. coli* binding assays

EHEC strains O26:H11, O45:H2, O103:H2, O121:H19, O111:Hnm and O157:H7 were obtained from Dr. Michael Mulvey at the Public Health Agency of Canada, National Microbiology Laboratory, *E. coli* Unit, Enteric Diseases Program, Winnipeg, MB. EHEC strain O145 (C625) was obtained from the American Type Culture Collection supplied by Cedarlane Labs in Burlington, ON. Binding and neutralization assays were performed as previously described (Saberianfar et al. 2019).

3.4 Results

Selection of rational design candidates

To develop a more stable Fc chain that could act as a stabilization partner when fused to a VHH, I tested three different rational design strategies: 1) changing key surface residues to give a more electronegative net charge, 2) introducing *de novo* intrachain disulfide bonds and 3) residue changes based on a predicted lowering of Gibbs free energy (Table 4). A total of 24 mutants were assessed for improved yield of which five supercharging (N9D, N84D, N131D, Q175E, Q195E) and one disulfide (G196C/R219C) mutants were advanced for pyramiding and further characterization (Table 4).

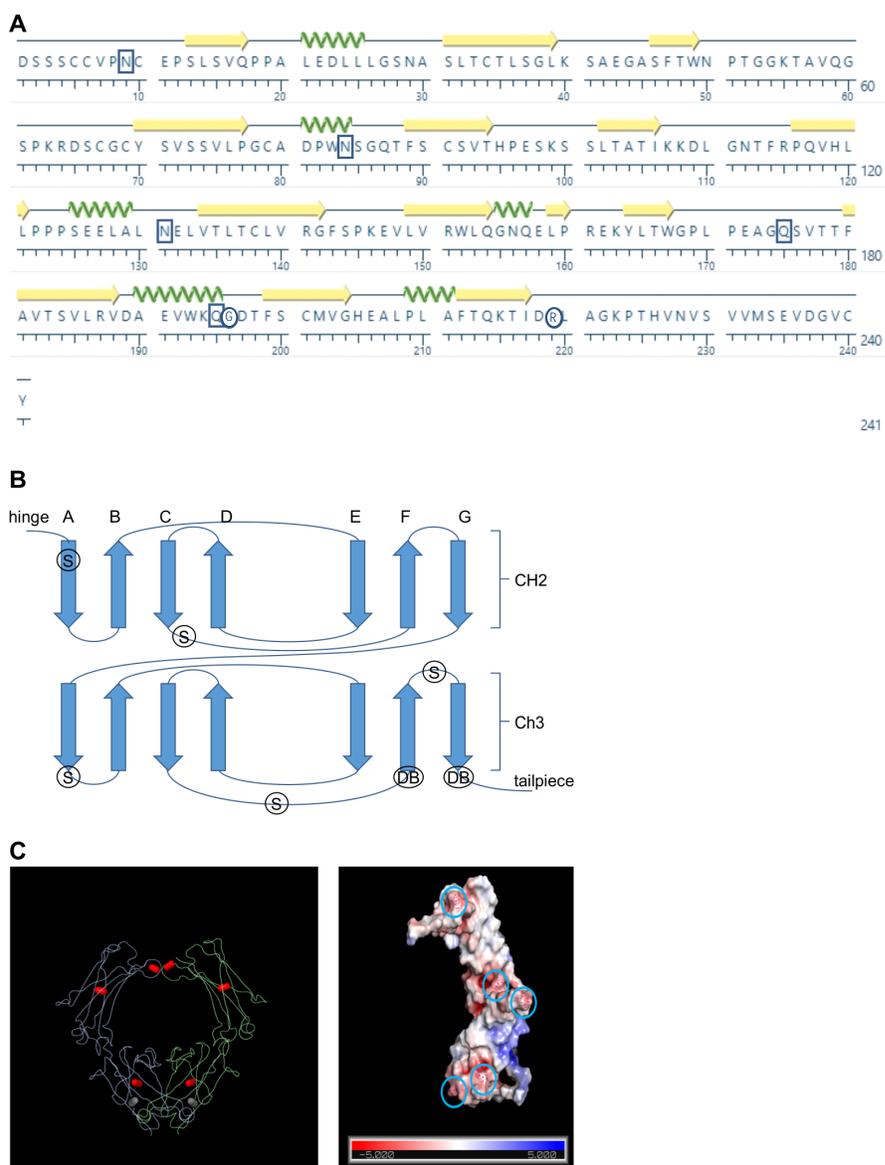


Figure 7. Schematics showing the positions of the rational design candidates. **A)** Amino acid sequence of the bovine IgA Fc sequence. Boxes indicate positions of the candidates for supercharging and circles indicate the positions of the candidates for *de novo* disulfide bonds **B)** Schematic showing the Greek key connectivity of the Fc's beta barrel structure. Arrows indicate beta strands, S indicates the positions of supercharging candidates and DB indicates the positions of *de novo* disulfide bond candidates **C)** Wire diagram of a dimerized Fc with native intra- and inter- chain disulfides colored in red and the *de novo* disulfide colored in grey **D)** Surface representation of the bovine Fc chain with predicted charge colored on a scale of red indicating more electronegative to blue indicating more electropositive. Circles indicate the positions of the supercharging candidates

Table 4. Rational design mutations tested for improved yield. Each mutational candidate was transiently expressed in leaves of *N. benthamiana* and yield assessed based on western blot densitometry of harvested leaf tissue.

Mutation	Strategy	Improved yield?
T113C/A207C	<i>de novo</i> disulfides	✗
K146C/T179C	<i>de novo</i> disulfides	✗
G196C/R219C	<i>de novo</i> disulfides	✓
E132C/D189C	<i>de novo</i> disulfides	✗
G86C/K108C	<i>de novo</i> disulfides	✗
K40C/(pre-existing cysteine)	<i>de novo</i> disulfides	✓
N9D	supercharging	✓
N84D	supercharging	✓
N131D	supercharging	✓
N156D	supercharging	✗
Q175E	supercharging	✓
Q195E	supercharging	✓
D218P	↓ Gibbs free energy	✗
C79D	↓ Gibbs free energy	✗
S71G	↓ Gibbs free energy	✗
Q175G	↓ Gibbs free energy	✗
D218P	↓ Gibbs free energy	✗
C79D	↓ Gibbs free energy	✗
S71G	↓ Gibbs free energy	✗
Q175G	↓ Gibbs free energy	✗
S74M	↓ Gibbs free energy	✗
G204W	↓ Gibbs free energy	✗
H95F	↓ Gibbs free energy	✗
E147Y	↓ Gibbs free energy	✗

Supercharging residue candidates were determined by modeling a predicted bovine IgA Fc structure using the I-TASSER and PyMol programs (Schrodinger 2010; Yang et al. 2015) and then selecting the most surface exposed asparagine and glutamine residues for mutating to their conservative but negatively charged counterparts: aspartic acid and glutamic acid respectively. The asparagines and glutamines were also selected based on non-involvement in native glycosylation (nucleotides 682-684, 85-87) or Fc α receptor

(Fc α R) binding (nucleotides 70-78, 349-351, 451-453, 457-459, 466-468, 472-474, 604-606, 613-642). The asparagines that were mutated are unlikely to be involved in N-glycosylation considering that the adjacent residues do not match the standard glycosylation motif (N-X-S/T where X is a non-proline residue) and crystallization studies of human IgA have not suggested otherwise. Of the four asparagines and two glutamines selected, the mutation of three asparagines to aspartic acid (N9D, N84D, N131D) and two glutamines to glutamic acid (Q175E, Q195E) improved yield.

For the selection of *de novo* intrachain disulfide bonds, the predicted Fc structure was modelled and the disulfide candidates chosen either by manual inspection of the molecule in PyMol or by ranked selection of estimated β factor, a measure of dynamic mobility for each atom, using the Disulfide by Design 2.0 software (Dombkowski et al. 2013). The secondary structure of the IgA Fc consists of a characteristic beta sandwich of seven anti-parallel beta strands for both its CH2 and CH3 domains. Both domains exhibit Greek key connectivity (ABED CFG) forming two distinct beta sheets that fold over each other (Figure 7B). For both domains, an intra-chain disulfide in the centre of the beta sheet stabilizes the tertiary structure. There are also interchain disulfides adjacent to the hinge area that enables dimerization of the Fc. Of the six disulfide pairs tested, two (K40C/pre-existing cysteine, G196C/R219C) improved yield. However, upon further examination of the model, we decide to not advance K40C because of the potential for distorting correct interchain disulfide formation at the hinge. The G196C/R219C disulfide candidate was manually chosen based on neighboring proximity (under 5 Å) with the intent of tethering the C-terminal end of strand G to the N terminal end of strand F (Figure 7B). The tailpiece

leading out from strand G is unstructured and contains a free S-H that natively forms a disulfide bond with the J chain in dimeric IgA. With the G196C/R219C mutation, I hypothesized that tethering the tailpiece to strand F could potentially stabilize the molecule by hiding any vulnerable hydrophobic regions that may become exposed as the strand becomes unstructured, and in so doing, prevent access by proteolytic enzymes. In particular, free floating unstructured tails are known to be preferentially loaded into the 26S proteasome apparatus for degradation (Inobe and Matouschek 2014). Thus, tethering the tailpiece by use of G196C/R219C could potentially prevent proteasome access.

The mutational candidates for reduced Gibbs free energy were selected based on the most negative estimated $\Delta\Delta G$ values beyond a cutoff of $-1.5 \text{ kcal mol}^{-1}$. Of the twelve candidates selected, none showed improved yield. The reason for this is unclear but may be related to my use of predicted structure for estimating stability in which the conformation is less accurate than the crystal structures used in FoldX's algorithm.

Rationally designed mutations improve Fc accumulation in transiently transformed leaf tissue

The rationally designed candidates were enabled by site-directed mutagenesis of the native bovine IgA Fc and confirmed by sequencing. Screening of ER-targeted wild type and mutant Fc was done by agroinfiltration of *N. benthamiana* leaves and semi-quantitative western blotting at four, six and eight days post-infiltration (dpi).

Compared to accumulation of native Fc, each of the supercharged Fc mutants showed a two- to four-fold improvement and persisted across the time course post infiltration (Figure 8A). Similarly, accumulation was enhanced by two- and 10-fold for the

de novo disulfide mutant compared to the native Fc across days four and eight respectively (Figure 8B). To test if these Fc mutants could also enhance accumulation as an Fc fusion protein, I fused each to a VHH that was identified from panning a phage display library with intimin of O157:H7 (Saberianfar et al. 2019). Similar to the comparison using Fc alone, each of the mutant VHH-Fc fusions showed a three to six-fold improvement in accumulation when compared to native (Figure 8D). To test if these mutations could be combined to further improve accumulation, I combined the mutations step-wise and measured accumulation in transformed leaf extract by western blot. Combining the mutations gave a pyramided increase in accumulation after transient expression. The Fc construct containing all five supercharging residues showed a seven-fold improvement in accumulation compared to native (Figure 8C). Adding the *de novo* disulfide to these five supercharging residues further improved accumulation to an approximately 32-fold improvement (Figure 8C). When fused to the anti-EHEC VHH, pyramiding the mutations on the Fc incrementally improved accumulation mutants with all five supercharging mutations plus the disulfide showing a twenty-fold improvement compared to native (Figure 8E). For consistency, day 8 post-infiltration was selected for extraction towards binding and neutralization assays. Because the engineered construct (5+1 Fc) containing all supercharging and disulfide mutations showed the most promise, I focused on it for further characterization.

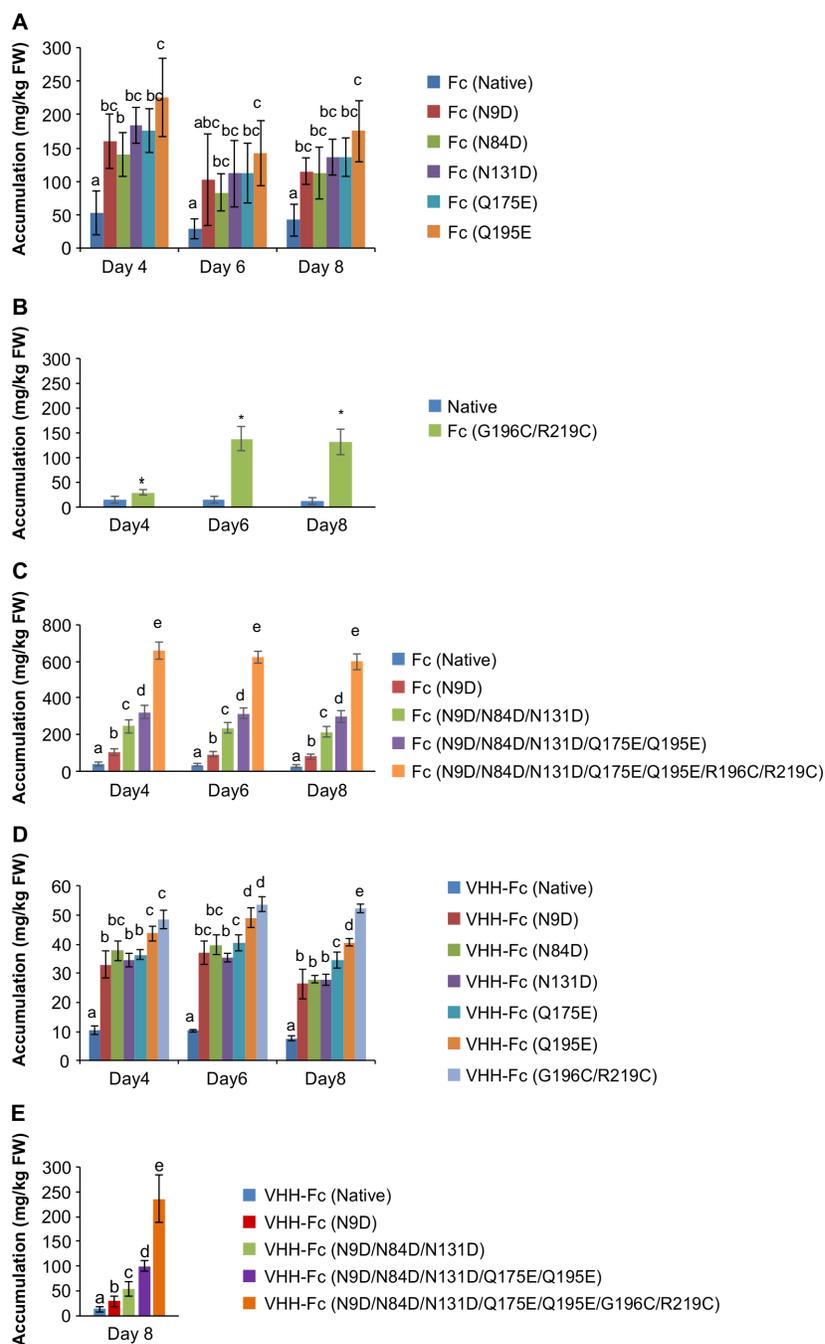


Figure 8. Time courses showing accumulation after transient transformation. Shown are **A**: Supercharging mutations on the Fc chain. **B**: Novel disulfides on the Fc chain **C**: Pyramiding of mutations on the Fc **D**: Fc with individual mutations fused to a VHH **E**: Fc with pyramided mutations fused to a VHH on day 8 dpi. * represents statistically significant difference from native as determined by a T-test. Letters denote significantly different treatments as determined by one way ANOVA and post-hoc Tukey HSD test. $P < 0.05$, $n = 3-5$ biological replicates. Error bars shown are standard error of the mean.

Engineered VHH-(5+1)Fc assemblies with other sIgA subunits *in vivo*

Structurally, secretory (sIgA) consists of an IgA dimer linked by two additional chains: a 15-kDa joining chain (JC) that links the Fc end-to-end (Krugmann et al. 1997) and a 70-kDa secretory component (SC) that coils around both Fc chains (Bonner et al. 2007). Although the VHH-Fc fusion lacks the light chains and CH1 domains found in native mammalian sIgA, assembly to the JC and SC is directed specifically via disulfide bond formation with the Fc. To determine if engineering of the VHH-Fc affected its ability to assemble with the SC and JC subunits, I co-expressed all three subunits and conducted immunoprecipitation experiments. Each subunit has a different tag (VHH-Fc-c-myc; SC-Flag; JC-HA) which allows us to find out if assembly occurs. Crude extracts were immunoprecipitated with the anti-FLAG antibody specific to the SC subunit, then separated and detected on a western blot probing for either anti-cmyc (VHH-Fc subunit), or anti-HA (JC subunit). Bands matching the predicted 44 kDa size of VHH-Fc were detected with anti-c-myc antibody in crude extract transformed with VHH-Fc, VHH-(5+1) Fc, co-expressed VHH-Fc/SC/JC and co-expressed VHH-(5+1)Fc/SC/JC, but no bands were detected in crude extract expressing only JC or SC (Figure 9A). After co-IP, ~44kDa bands were seen only in extracts co-expressing VHH-Fc/SC/JC and VHH-(5+1)Fc/SC/JC (Figure 9B). This indicates that both SC and VHH-Fc or SC and VHH-(5+1)Fc interact, and that the mutations in Fc did not hinder this interaction. Similarly, detection with anti-HA indicated bands of ~20kDa, matching the predicted size of JC, in crude extract transformed with JC, VHH-Fc/SC/JC and VHH-(5+1)Fc/SC/JC (Figure 9C). After Co-IP,

~20kDa bands were seen only for the co-expressed VHH-Fc/SC/JC and VHH-(5+1)Fc/SC/JC, indicating that SC and JC are present in the same complex (Figure 9D).

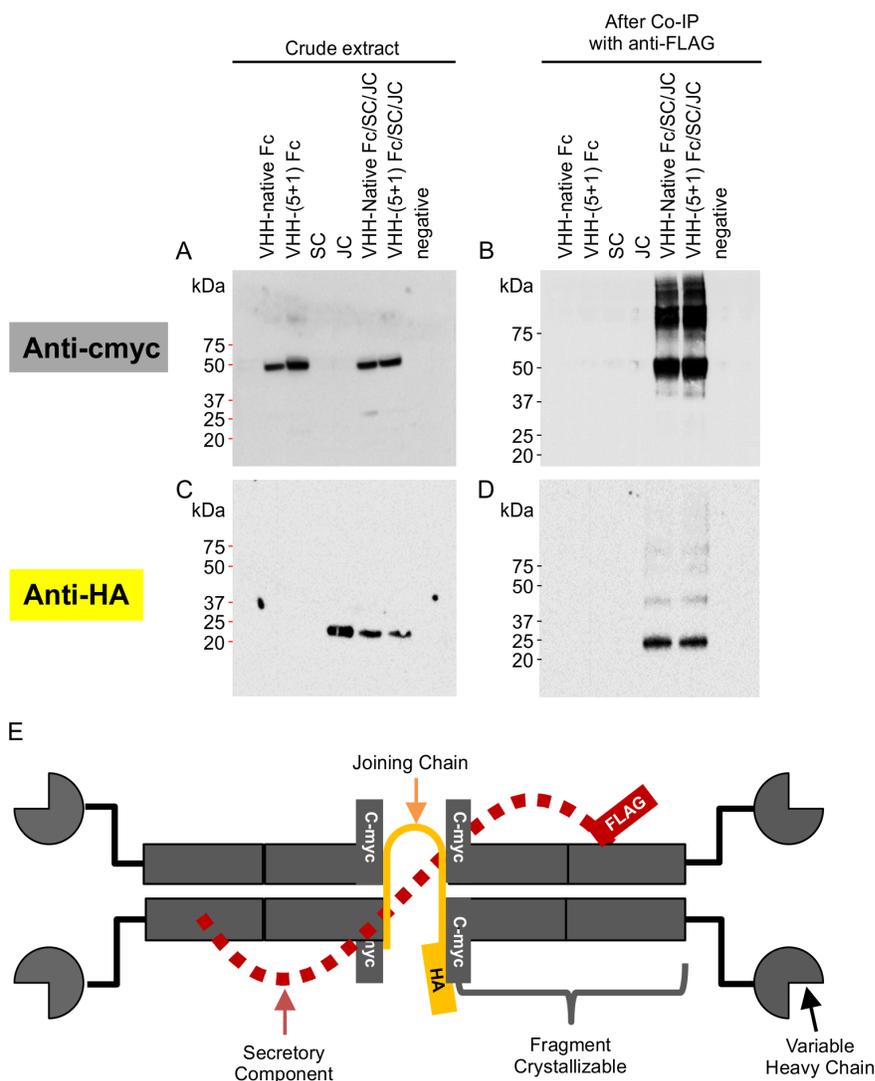


Figure 9. VHH-(5+1) Fc retains the ability to assemble with other secretory IgA subunits SC and JC. Shown are Western blots probed with either anti-cmyc (A,B), anti-HA (C,D) which correspond to differently tagged subunits VHH-Fc and JC respectively. Leaf issue was transformed with constructs of each subunit individually and also with combinations of VHH-Fc/SC/JC and VHH-(5+1) Fc/SC/JC for intended co-expression and assembly. Detection was done for both crude leaf extract (A,C) and for the eluent after the extract had been co-immunoprecipitated using an anti-FLAG column (B,D). A schematic of the assembled secretory IgA is shown (E) with VHH-Fc and C-myc tag shown in grey, SC with FLAG tag shown in red and JC with HA tag shown in yellow (Saberianfar et al. 2019)

Engineered VHH-(5+1)Fc retains the ability to bind EHEC strains O157, O26, O145 and O103

To determine if the rationally designed mutations impact VHH-Fc's pattern of cross-serotype binding against EHEC, I incubated either VHH-Fc or VHH-(5+1)Fc with the seven EHEC strains recognized as food adulterants, EHEC O26:H11, O45:H2, O103:H2, O145:Hnm, O121:H19, O111:Hnm or O157:H7. After washing and fixing with paraformaldehyde, I visualized bacteria with DAPI and VHH-Fc binding using a secondary fluorescent antibody (rabbit anti-bovine-FITC) that binds Fc. I saw consistent co-localization of FITC signal with strains O26:H11, O145:Hnm, O111:Hnm and O157:H7 cells for both VHH-Fc and VHH-(5+1)Fc (Figure 10). As a negative control, EHEC cells were also treated with PBS instead of antibodies and similarly stained but did not show fluorescence under FITC-related imaging conditions (480 nm excitation and 520–540 nm detection).

Engineered VHH-(5+1)Fc retains the ability to neutralize adherence of EHEC strains O157, O26 and O103 to HEp-2 cells

Intimin, the antigenic target of the VHH-Fc, mediates the intimate attachment of EHEC to epithelial cells. I previously showed that VHH-sIgA neutralizes EHEC's ability to intimately adhere to epithelial cells *in vitro* (Chapter 2). Here, I investigated if the VHH-Fc dimer alone is sufficient to neutralize EHEC's ability to adhere to epithelial cells, and if the rationally designed mutations impacted the VHH-Fc's pattern of cross-serotype protection. HEp-2 cells were incubated with a culture of one of seven EHEC strains (O26:H11, O45:H2, O103:H2, O145:Hnm, O121:H19, O111:Hnm and O157:H7) in the

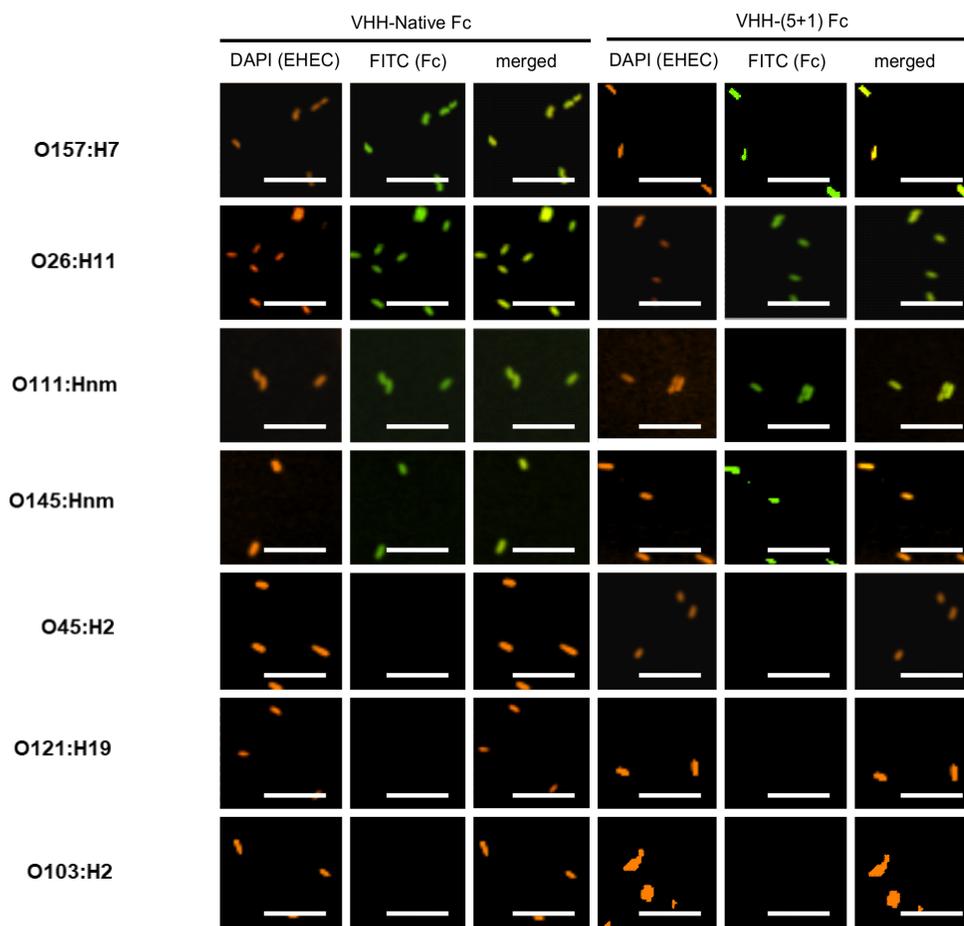


Figure 10. VHH-(5+1) Fc retains the same binding pattern across EHEC strains as VHH-Native Fc. Shown are confocal images of the seven most prevalent EHEC strains incubated with either VHH-Native Fc or VHH-(5+1) Fc. DAPI has been used to visualize EHEC cells (orange) and a FITC-conjugated antibody (green) has been used to immunolabel the Fc specifically. Size bar=10 μ m

presence or absence of either the either VHH-Fc or VHH-(5+1)Fc, washed to remove any non-adherent bacteria and then visualized by immunolabelling and confocal microscopy. HEp-2 cells were visualized by fluorescent actin staining using rhodamine phalloidin (red) and EHEC cells using a donkey anti-rabbit alexa 350 secondary antibody (blue). Compared to the respective positive controls of no VHH-Fc (+PBS treatment), the addition of either VHH-Fc or VHH-(5+1)Fc seemed to abrogate the adhesion of EHEC strains O26:H11, O111:Hnm, O145:Hnm and O157:H7 to HEp-2 cells (Figure 11). As a control to show that

neutralization is specifically mediated by the VHH, the Fc without an attached VHH was incubated with O157 and HEp-2 cells and showed a similar degree of adherent bacteria, based on microscopy and relative fluorescence, as did incubation without antibody. To quantify the relative neutralization capacity of the VHH-Fc compared to the VHH-(5+1)Fc, I adapted the adhesion assay for fluorometry and measured the relative fluorescence of HEp-2 cells incubated with a culture of each of the seven EHEC strains with and without either VHH-Fc or VHH-(5+1)Fc. The addition of either showed the same pattern of reducing the relative fluorescence caused by adherent bacteria for strains O26:H11, O111:Hnm, O145 and O157:H7 to background levels (Figure 12, Figure 12).

3.5 Discussion

Design

In this study, I successfully engineered a bovine IgA Fc to improve its *in vivo* accumulation alone or when fused to a VHH that binds EHEC without sacrificing the VHH's capacity for cross-serotype binding and neutralization. Two rational design approaches were successful for improving yield: supercharging specific surface residues and introducing a *de novo* disulfide bridge in the CH3 domain. The underlying idea behind both of these two designs is based on a number of previous proteomic observations that suggest that *in vivo* stability and solubility strongly correlate with protein abundance (Leuenberger et al. 2017; Walther et al. 2015). Although IgG has conventionally been

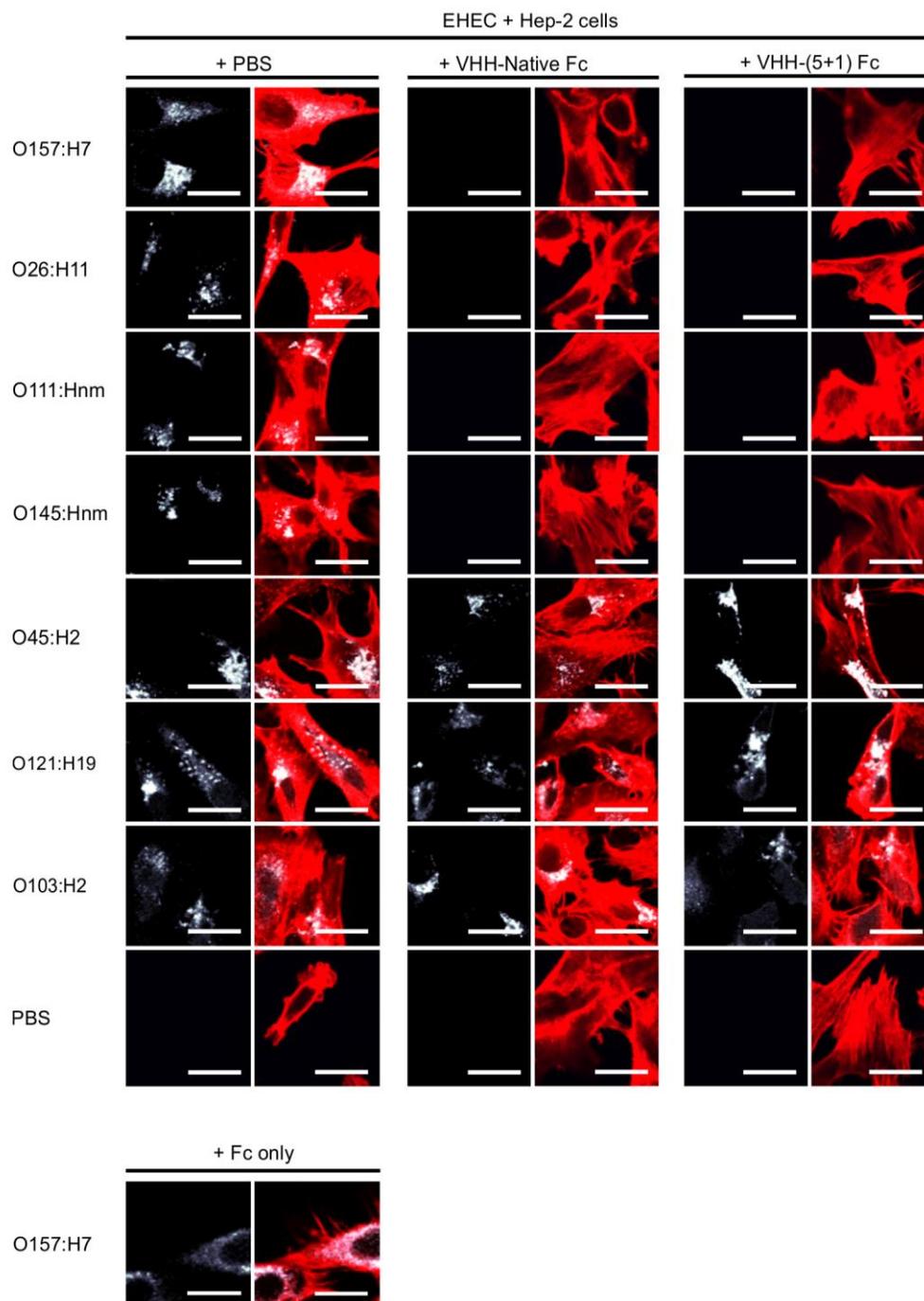


Figure 11. VHH-(5+1)Fc retains the VHH-Native Fc's pattern of cross-serotype protection by neutralizing bacterial levels of O26:H11, O11:Hnm, O145:Hnm and O157:H7. Shown are confocal images of the seven most prevalent EHEC strains (white) that have been incubated with Hep-2 cells (red) in the presence of PBS as a control, with VHH-Fc or with VHH-(5+1)Fc immunolabelled (white), are adherent on Hep-2 cells (red) and either incubated on Hep-2 cells in PBS, with VHH-Fc or with VHH-(5+1)Fc. As a control against non-specific Fc binding, O157:H7 was incubated with Fc only to confirm that neutralization was mediated through the VHH. Size bar = 20µm

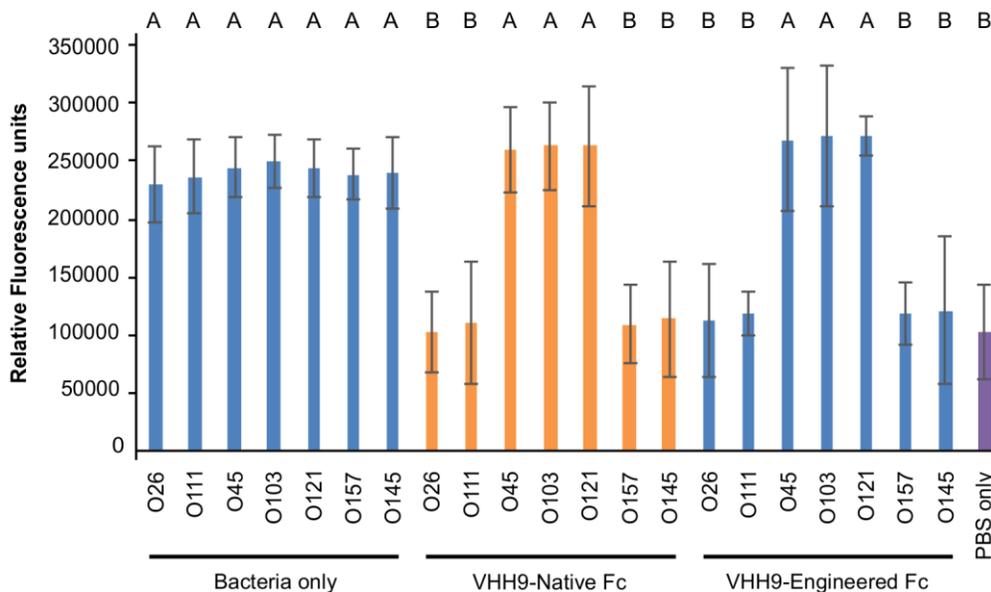


Figure 12. VHH-(5+1)Fc neutralizes bacterial levels of O26:H11, O11:Hnm, O145:Hnm and O157:H7 to baseline levels similarly to VHH-native Fc. Shown is the relative fluorescence of the seven most prevalent EHEC strains that have been immunolabelled, are adherent on Hep-2 cells and either incubated on Hep-2 cells in PBS, with VHH- Fc or with VHH-(5+1)-Fc and quantified by fluorometry. As a negative control, Hep-2 cells were incubated with PBS instead of a bacterial strain or antibody. Letters indicate a significant difference of the amount of immunolabelled adherent bacteria as determined by a one-way ANOVA with a post-hoc Tukey HSD test ($p < 0.05$, $N = 3$ biological replicates). Error bars indicate standard error.

used as the backbone for therapeutic Fc fusions, and engineering thereof, several previous studies have suggested the potential of an IgA-based Fc backbone for alternative effector systems as well as beneficial effects for the activity of the fused partner (Bakema et al. 2011; Bakema and van Egmond 2011; Dimitrov 2009; Lohse et al. 2011).

Conceptually, engineering for protein supercharging relies on an inverse relationship that exists between protein charge extent and aggregation propensity (Carballo-Amador et al. 2019). The concept of ‘resurfacing’ a protein has recently been of interest in the context of biobetter development because substitutions to protein surfaces are usually better tolerated than buried side chains and folding is driven primarily by the loss of solvation of the core hydrophobic residues (Chapman and McNaughton 2016; Dill

et al. 2008). Indeed, because surface side chains do not become buried and retain a similar environment in both a folded versus unfolded state, solvent-exposed amino acids are thought to contribute less energy towards stabilizing the folded state compared to buried core residues (Tokuriki et al. 2007). My initial motivation for supercharging the Fc was to explore if it could be a practical solution for the problem of heterologous mAb yield in plant platforms, which has been a primary limiting factor for advancing many therapeutics toward market. I introduced five negatively charged conservative substitutions predicted to change the net charge of the Fc from -5.30 to -10.29 (as calculated using PROPKA) (Olsson et al. 2011).

Yield improvement by rational design

My results indicate that each of these substitutions can improve *in vivo* accumulation of either the Fc or VHH-Fc by three- to four-fold. Previous studies using IgG Fc in bacterial systems have found similar success in promoting mAb stabilization by supercharging, particularly substitutions using aspartic and glutamic acid (Buchanan et al. 2013; Courtois et al. 2015; Dudgeon et al. 2012; Lee et al. 2013). This study is the first report demonstrating that this strategy can be successfully implemented for an IgA Fc in a plant platform.

I also designed a novel disulfide bridge in the CH3 domain of the Fc to fasten the C-terminal end of strand G to the N terminal end of strand F. My results showed that combining both the supercharging and disulfide mutations gave pyramidal improvement in accumulation to a 20-fold improvement. Given that combining these mutations did not seem to destabilize the protein, it is possible that there is room for improvement in

identifying the upper limit of mutation that can be accommodated in order to optimize accumulation.

Assembly into secretory complex

Co-immunoprecipitation of the differentially tagged subunits that had been coexpressed indicated that engineering of the Fc did not impact its capacity to assemble with the JC and SC subunits to form the secretory complex. Although the JC can double the avidity and the SC can protect the complex from proteolytic attack, full assembly comes at the cost of yield. (Saberianfar et al. 2019). Recently, an anti-F4 VHH-Fc monomer was shown to be effective in protecting piglets against F4 ETEC challenge suggesting that the assembled secretory format may not be necessary for protection (Virdi 2019).

Concluding remarks

In this study I demonstrated that rationally designing an IgA Fc for supercharged residues and *de novo* disulfides can impart a significant improvement in *in vivo* accumulation without sacrificing the binding efficacy of the VHH or the Fc's ability to structurally assemble with other secretory subunits. Because it folds independently from the VHH, this engineered IgA Fc scaffold may be useful as a modular tool for improving accumulation of other VHHs. Although plant-based IgA production is still an emerging field, I am optimistic that such a tool could be of value in overcoming the yield hurdles that have thus far hindered transition of these therapeutics to market.

Chapter thoughts and transition

Conceptually, the stable accumulation of a recombinant antibody is a function of both its amino acid sequence as well as its interaction with its proximal folding environment. In this chapter, I showed that altering the amino acid sequence at key residues can improve the yield of an anti-EHEC antibody without sacrificing efficacy. Indeed, the ideas behind the two successful rational design strategies tested, supercharging and *de novo* disulfides, are based on the protein's interaction with its folding environment. The supercharging strategy uses small charge-charge repulsive forces to deter non-specific aggregation as the antibody folds in the macromolecular crowded environment (Lawrence et al. 2007). The *de novo* disulfide strategy uses covalent bonds to sterically hinder access by proteolytic enzymes and shields a vulnerable hydrophobic interior (Zabetakis et al. 2014). Overall, considering the ideas behind these strategies and the successful identification of six stabilizing mutations in this chapter, the relationship between amino acid sequence and folding environment can be considered deeply interconnected, particularly with regards to experimental approaches aimed at improving *in vivo* stability.

Considering the importance of folding environment, Chapter 4 explores if targeting the antibody for folding in the thylakoid lumen of the chloroplast may impact yield. Compared to the conventional ER folding environment, the most relevant differences include: 1) an evolutionarily distinct system for disulfide bond formation (Karamoko et al. 2013) 2) lack of enzymes for N- or O- glycosylation (Schubert et al. 2002) and 3) a smaller range of proteolytic enzymes (Kieselback et al 2003). Thus, I hypothesized that it may be

possible to oxidatively fold aglycosylated antibodies in the thylakoid lumen, and that having a reduced range of exposure to proteolytic enzymes may mean a better yield.

4.0 A VHH-FC FUSION TARGETED TO THE THYLAKOID LUMEN BINDS AND NEUTRALIZES ENTEROHEMORRHAGIC *E. COLI* O157:H7

4.1 Abstract

VHH-crystallizable fragment (Fc) fusion proteins are synthetic third generation antibodies that are becoming increasingly in demand because of their value as diagnostics, research reagents and passive immunization therapeutics. Because ER-associated degradation and misfolding may potentially be limiting factors in the oxidative folding of antibodies in the ER, I sought to explore oxidative folding in an alternative subcompartment, the chloroplast thylakoid lumen, and determine its viability in a molecular farming context. I developed a set of in-house expression vectors for transient transformation of *Nicotiana benthamiana* leaves that target a VHH-Fc to the thylakoid lumen via either sec or tat import pathways. Compared to stromal, cytoplasm and Tat-import pathways, the Sec-targeted VHH-Fc showed superior accumulation, but 63% than that of the ER. Additionally, the introduction of a rationally designed *de novo* disulfide enhances *in vivo* accumulation when introduced into the Sec-targeted Fc fusion protein. *In vitro* immunofluorescent labelling assays on VHH-Fc and VHH-engineered Fc purified from Sec, Tat, and stromal pathways demonstrate that the antibody still retains VHH functionality in binding *E. coli* O157:H7 and neutralizing its adherence to human epithelial type 2 cells. These results overall provide a proof of concept that the oxidative folding environment of the thylakoid lumen may be a viable compartment for stably folding disulfide-containing recombinant VHH-Fc proteins.

4.2 Introduction

VHH-crystallizable fragment (Fc) fusion proteins are synthetic immunoglobulins (Ig) that are becoming increasingly in demand because of their potential value as passive enteromucosal immunization therapeutics (Harmsen and De Haard 2007). Structurally, they differ from conventional Ig's in that the antigen binding Fab fragment has been replaced by a single domain camelid-derived VHH that is smaller (~15kDa), more stable and does not require assembly with another subunit as is the case for conventional variable heavy and light chains (De Meyer et al. 2014). In mammals, the predominant Ig isotype associated with enteromucosal protection is the secretory IgA (sIgA) complex, which consists of two IgA's linked end-to-end via a joining chain subunit and an attached secretory component that coils around both Fc chains (Woof and Russell 2011). I have previously produced a VHH-sIgA in *Nicotiana benthamiana* leaf tissue by targeting it to the ER for folding and demonstrated its functionality *in vitro* in binding and neutralizing four serotypes of enterohemorrhagic *E. coli*, including the predominant strain O157:H7 (Saberianfar et al. 2019). A VHH-sIgA produced in *Arabidopsis thaliana* has also been demonstrated to be effective in passively immunizing weaned piglets against enterotoxigenic *E. coli* when fed 20 mg VHH-sIgA encapsulated in seeds per day and reduced bacterial shedding to beneath detection levels after four days (Viridi et al. 2013). Although VHH-sIgA's are effective, the requirement for multi-subunit assembly greatly reduces yield and increases product heterogeneity and thus recent design has shifted toward simplifying production to just the VHH-Fc fusion protein (De Greve et al. 2020). A yeast-produced food-admixed VHH-Fc protein was demonstrated to be sufficient for preventing

post-weaning diarrhea by neutralizing enterotoxigenic infection in the gastrointestinal tract of piglets (Virdi et al. 2019). Although the VHH can bind independently, fusion of the Fc to the VHH has been demonstrated to improve accumulation substantially in *A. thaliana* seeds (De Buck et al. 2013).

Over the past twenty years, plants have become a platform of choice for producing complex IgA antibodies and related synthetics in part because of the requirement of disulfide bond formation for proper folding and assembly (Vasilev et al. 2016). In plant cells, the oxidative folding of proteins with disulfide bonds is mainly localized to either the endoplasmic reticulum, the mitochondrial intermembrane space or the chloroplast thylakoid lumen (Onda 2013).

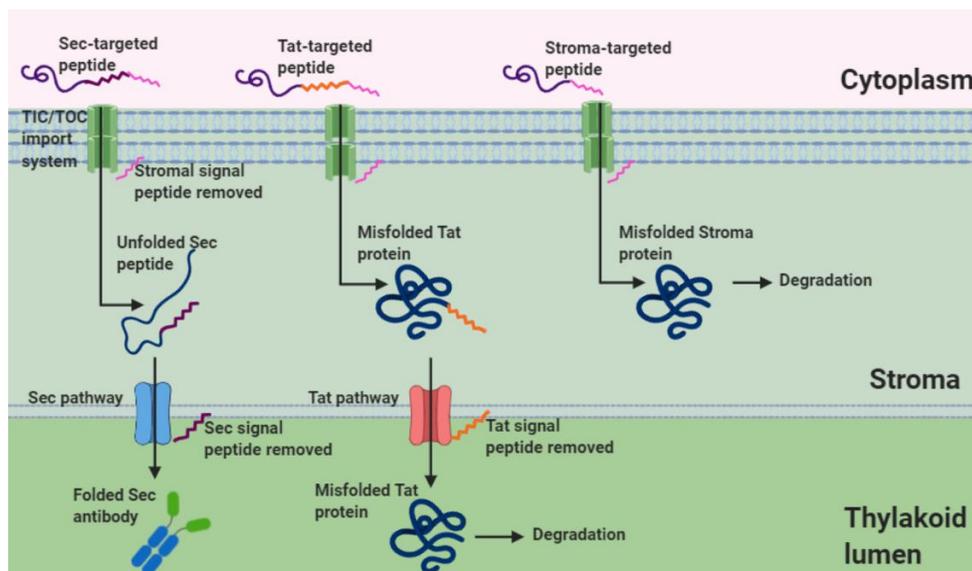


Figure 13. General schematic showing expected scenarios of targeting the antibody with Sec, Tat and stromal signal peptides. Although both Sec and Tat signals import an attached peptide into the thylakoid lumen, the Sec peptide is imported as an unfolded substrate which folds in the lumen while the Tat peptide folds first in the stroma and then is imported into the lumen. On the other hand, a stroma signal will target a peptide for both folding and accumulation in the stroma. Based on the idea that disulfide formation is exclusive to the lumen, I hypothesized that only Sec targeted antibodies would accumulate well and retain functionality. I included the Tat and stromal targeted antibodies as additional variables to provide a better understanding of the folding and accumulation environments of the stroma versus the lumen. (Figure created in Biorender.com)

Chloroplasts are structurally complex organelles that consist of a double membrane enclosing a soluble stromal phase (Kirchhoff 2019). Within the stroma, an independent membrane system known as the thylakoid is embedded with the chlorophyll-containing photosystems responsible for photosynthesis and further encloses an additional luminal phase (Figure 13; Johnson and Wientjes 2019; Pottosin and Shabala 2016). Protein transport across these membranes is differentially regulated and each of these compartments can be considered to have a different proteomic profile and contain different folding environments (Lee et al. 2017). Proteins that are encoded by the nucleus, synthesized in the cytosol and localized in the thylakoid lumen require an N-terminal bipartite transit peptide consisting of two signals in tandem: a signal to enter the TIC/TOC import system of the outer double membrane followed by a thylakoid targeting signal that may comprise one of two functionally distinct pathways, known as the secretory (Sec) and twin-arginine translocation (Tat) pathways (Fernandez 2018; Johnson and Wientjes 2019; Palmer and Stansfeld 2020; Pottosin and Shabala 2016). The Sec pathway bears homology to the bacterial secretory pathway and actively transports the unfolded pre-protein bound to the SecA chaperone through a Sec membrane complex (Ries et al. 2020). On the other hand, protein transport via the Tat pathway uses a transthylakoidal proton gradient as its energy source and can mediate transport of fully folded globular proteins across the thylakoid membrane (Palmer and Stansfeld 2020). The Tat pathway is also unique in having a protein proofreading ability that targets misfolded proteins for degradation (Robinson et al. 2011). In the thylakoid lumen, a single chimeric protein, lumen thiol oxidoreductase I (LTO1), performs both *de novo* formation and transfer of disulfides to proteins that undergo

oxidative folding (Karamoko et al. 2013). Folding of both the Fc and the VHH requires the formation of intra-chain disulfide bonds as a structure-stabilizing modification to prevent denaturation and reduce susceptibility to proteolysis (Vincke and Muyldermans 2012; Woof and Russell 2011). Structurally, the Fc chain consists of two distinct domains, CH2 and CH3, each comprising two anti-parallel beta sheets that are connected at the centre by an intra-chain disulfide bond. The Fc also homodimerizes with itself via an interchain disulfide bond found at the N-terminal end of the CH2 domain. The VHH is predicted to contain an intrachain disulfide (Wesolowski et al. 2009), and when produced in bacteria, requires targeting to the periplasm for correct folding (Henry et al. 2017). Because the Sec and Tat pathways differ in trafficking the unfolded and folded protein cargo respectively, I hypothesized that the interchain and intrachain disulfide formation of the VHH-Fc would be exclusive to the Sec pathway and may be evident in its stable accumulation and dimerization.

The oxidizing environment of the thylakoid lumen has conventionally been considered the only site for oxidatively folding chloroplast proteins that require disulfide stabilization because of the control of a trans-thylakoid redox pathway (Karamoko et al. 2013). However, a few studies have suggested that proteins requiring disulfide formation may unexpectedly fold and be biologically active in the reducing environment of the stroma (Bally et al. 2008; Daniell et al. 2001; Mayfield et al. 2003; Staub et al. 2000). These discrepancies may possibly be explained by spontaneous disulfide formation in the stroma or activity by the membrane embedded LTO1 even though it is known to be oriented toward the interior of the lumen (Kieselbach and Schroder 2003; Lu et al. 2013). To

differentiate between the ability of the stroma and thylakoid lumen to properly introduce disulfide bonds and fold a VHH-Fc protein, I targeted it to both compartments.

In this study, I have compared accumulation levels of a VHH-Fc that has been transiently targeted to the chloroplast thylakoid compartment via Sec and Tat pathways or to the chloroplast stroma in *N. benthamiana* leaf cells. I have also demonstrated that the VHH-Fc targeted to the thylakoid or stromal compartments is functional in binding and neutralizing adherence of *E. coli* O157:H7 to epithelial cells. This study is notable because it provides a proof of concept that the folding environment of the thylakoid lumen is conducive for accumulating a VHH-Fc fusion that retains IgA functionality in binding and neutralizing its target, and opens the way for producing transplastomic plants targeting such an antibody for folding and accumulation in the thylakoid lumen.

4.3 Methods

Cloning

Both Sec and Tat sequences (Accession #: NP_001318791 and NP_001321139 respectively) were obtained from a previous proteomics study that isolated and sequenced multiple luminal proteins in *Arabidopsis thaliana* (Schubert et al. 2002). The Tat targeting sequence corresponds to the N-terminal 71 amino acids of a FKBP-type peptidyl-prolyl cis-trans isomerase (At1g20810). The Sec targeting sequence corresponds to the N-terminal 75 amino acids of a thylakoid luminal 15.0 kDa protein 2 (At5g52970). Cleavage sites of the targeting peptides were predicted using the ChloroP and TargetP online tools (Almagro Armenteros et al. 2019; Emanuelsson et al. 1999) (Figure 14). Sequences were synthesized by BioBasic and then cloned using a ligation independent method (Li and

Elledge 2007) into an in-house developed cytosolic expression vector (Pereira et al. 2014). The VHH-Fc construct was developed previously and consists of an anti-EHEC VHH9 (Saberianfar et al. 2019) fused to a bovine Fc (ANN46383) (Chapter 3). The VHH-Fc was cloned into this adapted vector by Gateway cloning and the reading frame was confirmed by sequencing.

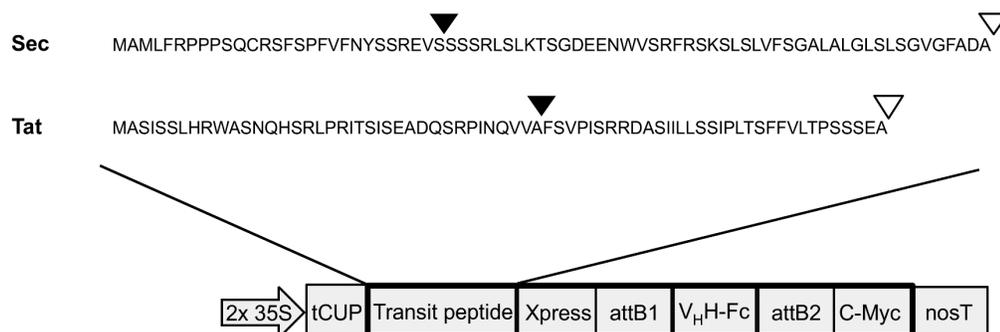


Figure 14. Construction of thylakoid expression vectors. Transit peptide sequences used for thylakoid targeting via either Sec or Tat pathways. Triangles indicate predicted cleavage sites after entry into stromal (black triangles) and thylakoid (white triangles) compartments. 2x35S: double-enhanced promoter from Cauliflower Mosaic Virus 35S gene; tCUP: translational enhancer from a tobacco cryptic upstream promoter; attB1/attB2: cloning sites used for Gateway™ cloning; nosT: nopaline synthase transcription terminator; Xpress/C-Myc: detection/purification tags

Visualizing subcellular compartment localization

GFP was fused to the Fc sequence by sequence and ligation independent cloning. The fusion was then cloned into Sec, Tat, and chloroplast stroma expression vectors and each was agroinfiltrated along with p19 into leaves of *N. benthamiana*. Tissue was harvested after two days, mounted in Aqua-Poly/Mount (Polyscience Inc., Warrington, PA, USA, Cat. No. 18606) and visualized using a 60× water immersion objective lens and an Olympus LSM FV 1200 confocal microscope for Sec and stromal samples or a Leica TCS SP2 CLSM confocal microscope for Tat samples. Samples were excited at 488 nm using a

multi-argon laser set at 5% and emission was detected at 500–545 nm for GFP and at 630–690 nm for chlorophyll.

Protein extraction and western blot

Pre-weighed leaf samples were frozen in liquid nitrogen and homogenized with silica beads (Bio Spec Products Inc., Bartlesville, OK, USA) for 2 min using a TissueLyser II (Retsch Inc., Newton, PA, USA). One millilitre of a denaturing extraction buffer (1x PBS, pH 7.5, 4% SDS, 2% PVPP) was added per approximately one hundred milligrams of sample. All samples were then vortexed on high speed for 30 s and centrifuged at 20,000 x g for 10 min to remove cell debris. Soluble protein samples were combined with either a 5 x reducing loading buffer (0.3 M Tris-HCl pH 8.0, 5% SDS, 10% glycerol, 100mM DTT, 0.05% Phenol Red) or a 5 x non-reducing buffer (0.3 M Tris-HCl pH 8.0, 5% SDS, 10% glycerol, 0.05% Phenol Red), heated at 90°C for 10 min, then loaded onto Express Plus PAGE 4-20% gradient gels (Genscript Inc., Piscataway, NJ, USA). Gels were run at 100 V for 100 min, then transferred to polyvinylidene difluoride (PVDF) membrane using the Trans-Blot Turbo transfer system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Blots were blocked overnight with 5% skimmed milk in tris-buffered saline, pH 7.5, and proteins of interest were probed with a mouse anti-c-myc antibody (Genscript Inc., Piscataway, NJ, USA) and the One-Hour Basic Western kit for mouse primary antibody (Genscript Inc., Piscataway, NJ, USA). Detection was performed using Amersham ECL Western Blot detection reagents (GE Healthcare, Mississauga, ON, Canada) or Enhanced Chemiluminescent detection solution (Biorad Laboratories Inc., Hercules, CA, USA) and a MicroChemi 4.2 imaging system with GelCapture acquisition software (DNA Bio-

Imaging Systems Ltd., Jerusalem, Israel). Quantification of accumulation was done using densitometry using a calibrated standard curve of an in-house produced purified protein. Statistical significance was determined using a two-tailed unpaired T-test on 3-5 separate plants that constitute biological replicates.

Recombinant protein purification

Leaf tissue was extracted in a native buffer and supernatant collected after two rounds of centrifugation at 22,000xg for 20 min each. The recombinant protein in the clarified extract was then immunoprecipitated using an anti-c-myc purification kit (MBL International Corp., Woburn, MA, USA) according to the manufacturer's protocol.

***E. coli* O157:H7 binding assay**

E. coli strain O157:H7 was obtained from Dr. Michael Mulvey (Public Health Agency of Canada, National Microbiology Laboratory, *E. coli* Unit, Enteric Diseases Program, Winnipeg, MB). Binding assays were performed as previously described (Saberianfar et al. 2019). A summary is as follows. A culture of *E. coli* O157:H7 was grown overnight, pelleted, rinsed in PBS, then fixed in 4% paraformaldehyde. The fixed cells were incubated with 2 µg purified VHH-Fc for 1 hour. After blocking, the cells were then hybridized to a FITC-conjugated secondary antibody. To stain the bacteria, the cells were resuspended in DAPI. FITC and DAPI sequential imaging was then performed by confocal microscopy with a 64x water lens and an Olympus LSM FV 1200 confocal microscope. FITC was imaged by excitation with a 480 nm laser and detection at 520–540 nm. DAPI was imaged by excitation at 350 nm and detection at 455–465 nm. Images were acquired with a 64x water objective lens.

HEp-2 adherence inhibition assay

Inhibition assays were performed as previously described (Saberianfar et al. 2019). A summary is as follows. HEp-2 cells (ATCC) were grown to ~80% confluency. *E. coli* strain O157:H7 was grown overnight and then subcultured to a 1:10 dilution in DMEM with and without 2 µg VHH-Fc and then incubated with the HEp-2 cells at 37°C in 5% CO₂ for 3 hours. The cultures were then washed with PBS to remove non-adherent bacteria, fixed, washed repeatedly, blocked and then hybridized with Alexa 647 phalloidin (Thermo Fisher Scientific Cat. No.A22287) to visualize actin in the HEp-2 cells, and donkey anti-rabbit Alexa 350 (Thermo Fisher Scientific Cat. No.A10039) to visualize EHEC cells. To visualize adherence to HEp-2 cells, imaging was performed using confocal microscopy. Alexa 647 phalloidin was imaged by excitation at 650 nm and detection at 660–680nm. The donkey anti-rabbit Alexa 350 antibody was visualized by excitation at 350 nm and detection at 455–465 nm.

4.4 Results

Subcompartment targeting influences accumulation and dimerization patterns of the VHH-Fc fusion

The VHH-Fc was cloned into five separate plant expression vectors that permit targeting of the protein to the chloroplast thylakoid via Sec or Tat pathways, the chloroplast stroma, the ER or the cytoplasm. After transiently transforming leaves of *N. benthamiana*, tissue was harvested and crude extract separated by SDS-PAGE in either a reducing buffer or a non-reducing buffer. Detection by Western blot using an anti-c-myc antibody showed accumulation of the VHH-Fc in the thylakoid lumen via both pathways, in the stroma, and

in the ER, but lacked sufficient signal for detection in the cytoplasm (Figure 15). Under non-reducing extraction conditions, the VHH-Fc is detected predominantly as an 88kDa band matching the predicted size of the VHH-Fc dimer. Total accumulation is highest in the ER at 51.1 mg/kg fresh weight (FW), followed by the thylakoid via Sec-targeting at 30.5 mg/kg FW. Accumulation in the stroma and thylakoid via Tat-targeting are substantially lower at 6.6 mg/kg FW and 5.4 mg/kg FW respectively. Under reducing extraction conditions of the same samples, an enriched band at 44kDa is detected matching the predicted size of the VHH-Fc monomer for the ER, stromal, thylakoid via Sec and thylakoid via Tat compartments suggesting that the VHH-Fc dimer in these compartments is stabilized by an interchain disulfide bond.

Sec- and Tat-targeted GFP-Fc localize in the thylakoid similarly

To verify that the tat and sec transit peptides indeed target the VHH-Fc to the thylakoid compartment, I tracked subcellular localization of the VHH-Fc by fusing GFP to the Fc chain in each of the expression vectors. Visualization by confocal microscopy showed the Sec and Tat-targeted GFP-tagged protein to consistently colocalize with chlorophyll, which accumulates in the thylakoid and autofluoresces at ~735nm (Figure 16). On the other hand, the construct targeting the recombinant protein to the stroma showed a very distinct pattern surrounding the thylakoid grana, and into stromules. Therefore, the sec and tat transit peptides I identified indeed target the recombinant protein to the thylakoid.

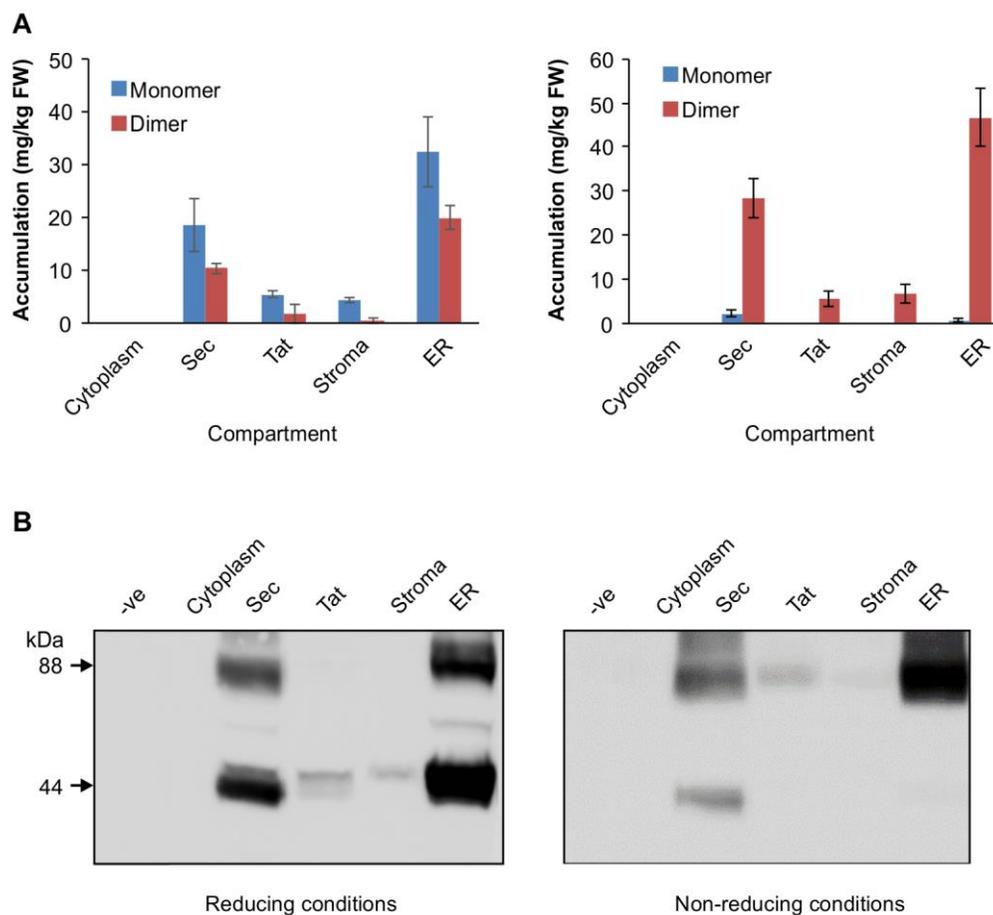


Figure 15. Accumulation profiles of VHH-Fc targeted with cytoplasm, Sec, Tat, stromal and ER signals. A) Bar chart comparing VHH-Fc accumulation levels across cytoplasm, Sec, Tat, stromal and ER signals extracted in reducing or non-reducing conditions. N=3 biological replicates. Error bars shown are standard error. B) Representative Western blot showing relative accumulation of VHH-Fc across compartments in reducing and non-reducing conditions.

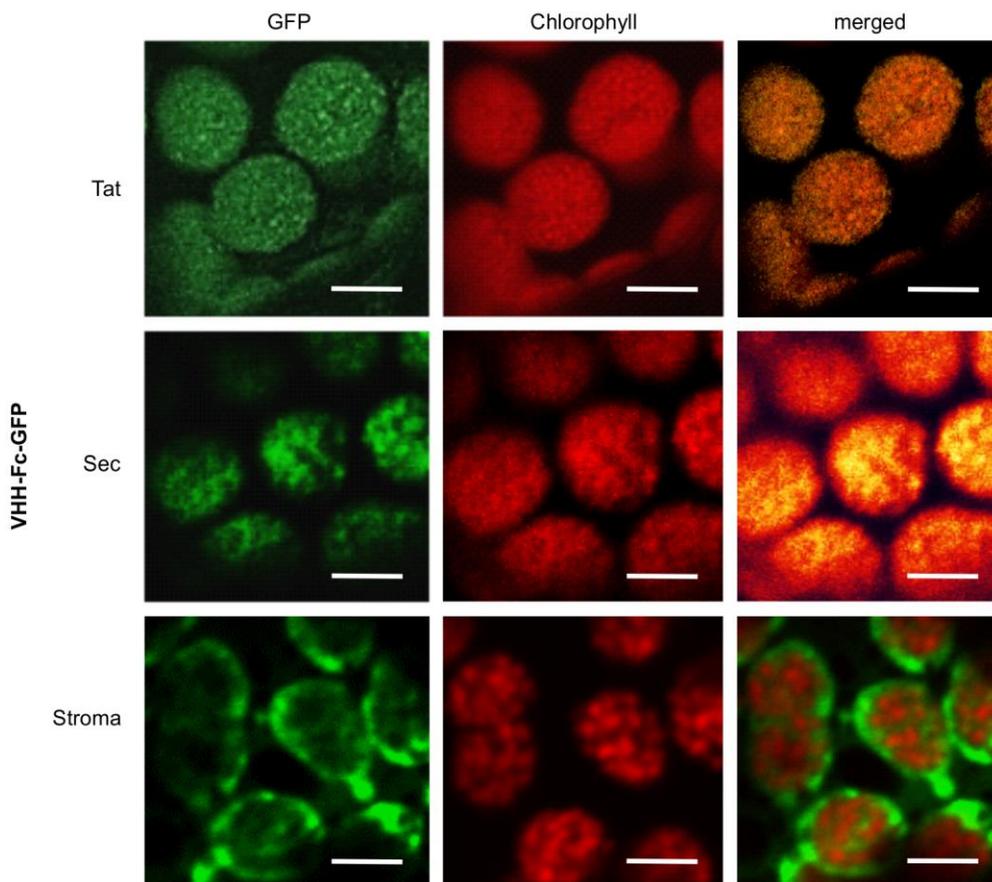


Figure 16. Sec and tat transit peptide target the recombinant VHH-Fc-GFP to the thylakoid chloroplast sub-compartment. Confocal images visualizing GFP-tagged VHH-Fc (green) targeted to the chloroplasts with either Sec, Tat or stromal signals. Chlorophyll (red) indicates the locations of the thylakoid grana. Fluorescence was sequentially captured, and the merged images show co-localization of GFP and chlorophyll (yellow). Size bar = 10 μ m

Sec-targeted VHH-Fc fusions with an engineered disulfide show improved yield

In the previous chapter, I identified by rational design a residue pair (G196C/R219C) on the Fc that if mutated to cysteines would form a *de novo* disulfide bond that enabled an improvement in yield of the molecule. The pair forms an intrachain disulfide between strand G and strand F on the CH3 domain in oxidative folding conditions (Figure 17). To determine if the oxidative folding of the thylakoid can recapitulate the

yield-improving effects of an engineered disulfide bond, I targeted the VHH-Fc fusion carrying the G196C/R219C mutation to the thylakoid lumen via the Sec pathway and measured accumulation by Western blot after agroinfiltration. Similar to expression in the ER (Chapter 3), the engineered VHH-Fc showed a significant yield improvement (Figure 17), suggestive of the ability of the thylakoid lumen to incorporate *de novo* disulfide bonds.

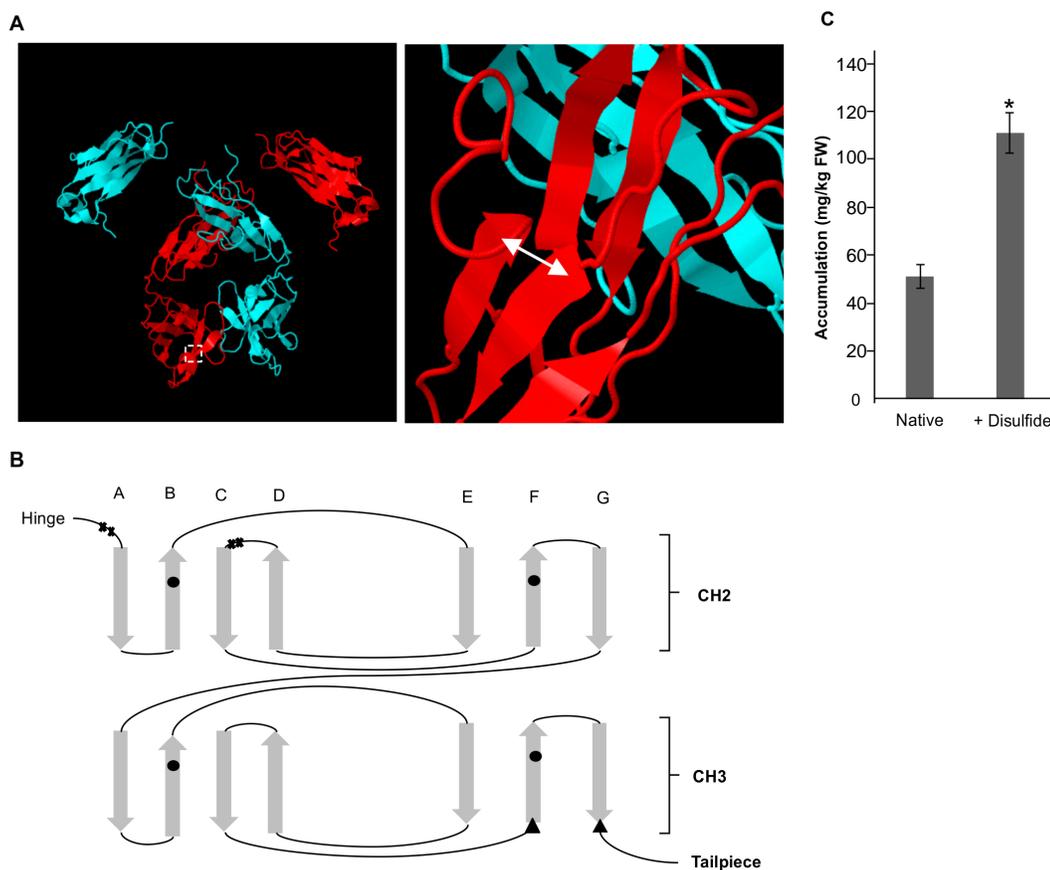


Figure 17. A *de novo* disulfide bond enhances accumulation for a Sec-targeted VHH-Fc. A) Structural model and Greek key connectivity figure showing the relative positions of native disulfides and the introduced disulfide in the Fc. The model shows the backbone of two dimerized Fc chains (blue and green). Red indicates the position of native disulfides and grey indicates the position of the introduced disulfides. In the connectivity figure, X's indicate cysteines involved in interchain disulfide formation, circles indicate cysteines involved in intrachain disulfide formation and triangles indicate introduced cysteines for *de novo* disulfide formation. B) Bar chart showing accumulation of Sec-targeted native VHH-Fc and a VHH-Fc with an added disulfide. *indicates statistical significance as determined by a T-test with $p < 0.05$, $n = 3$ biological replicates. Error bars shown are standard error of the mean.

Sec, Tat, and stroma- targeted VHH-Fc fusions bind O157:H7

I previously demonstrated that the ER-targeted VHH binds to intimin, an integral outer membrane protein of *E. coli* O157:H7 (Chapter 2). To determine if the thylakoid-targeted VHH-Fc retained the ability to bind *E. coli* O157:H7, purified VHH-Fc from each compartment was incubated with the pathogen then fixed in paraformaldehyde, washed and probed for immunofluorescence using a FITC labelled anti-c-myc secondary antibody. Visualization by confocal microscopy showed consistent co-localization between DAPI-stained bacterial cells and the FITC-labelled VHH-Fc for the thylakoid via Sec, thylakoid via Tat, and stromal compartments, indicating that the chloroplast-targeted VHH-Fc retains the ability to bind intimin on EHEC surfaces (Figure 18). As a negative control, O157:H7 cells were also treated with PBS containing 0.1% Tween-20 (PBS-T) instead of the VHH-Fc and similarly stained but did not show fluorescence under FITC-related imaging conditions (480 nm excitation and 520–540 nm detection). This result suggests that the VHH is folded correctly when Sec, Tat and stroma targeted regardless of the status of the Fc.

Sec, Tat and stroma-targeted VHH-Fc fusions can neutralize O157:H7's adherence to HEp-2 cells

Given that intimin mediates the attachment of *E. coli* O157:H7 to intestinal epithelial cells, I tested if thylakoid targeting of the VHH-Fc impacted its ability to neutralize the bacterium from adhering to epithelial cells by blocking intimin. HEp-2 cells were incubated with *E. coli* O157:H7 in the presence or absence of purified VHH-Fc from each of the compartments. Cells were then washed to remove non-adherent bacteria, fixed

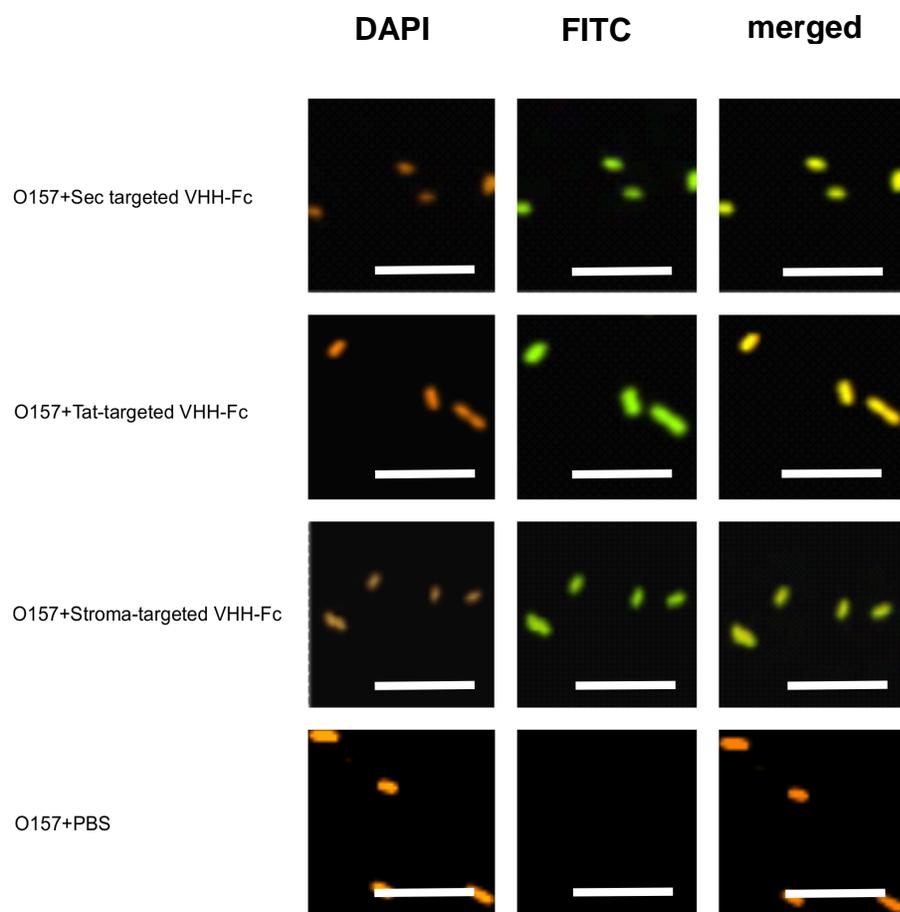


Figure 18. VHH-Fc targeted with either Sec, Tat or stromal transit peptides can bind O157:H7 cells. Shown are confocal images of VHH-Fc targeted with either Sec, Tat or stromal signals incubated with EHEC O157:H7. DAPI has been used to visualize EHEC cells (orange) and a FITC-conjugated antibody (green) has been used to immunolabel the Fc specifically. Size bar=10 μ m

in paraformaldehyde and incubated with immunofluorescent labels. Human epithelial type-2 (HEp-2) cells were visualized by fluorescent actin staining using rhodamine phalloidin (shown in red) and O157:H7 cells visualized using a donkey anti-rabbit alexa 350 secondary antibody (shown in white). Compared to the control lacking VHH-Fc, and to the control Fc lacking the VHH, the addition of purified VHH-Fc from any of the compartments seems to abrogate adhesion of any labelled *E. coli* O157:H7 to the incubated

HEp-2 cells as visualized using confocal microscopy (Figure 19). This indicates that indicating that the chloroplast-targeted VHH-Fc retains the ability to neutralize EHEC from colonizing epithelial cells and that the inhibition of adhesion is mediated by the VHH and not by non-specific interactions of the Fc moiety of the antibody, as expected.

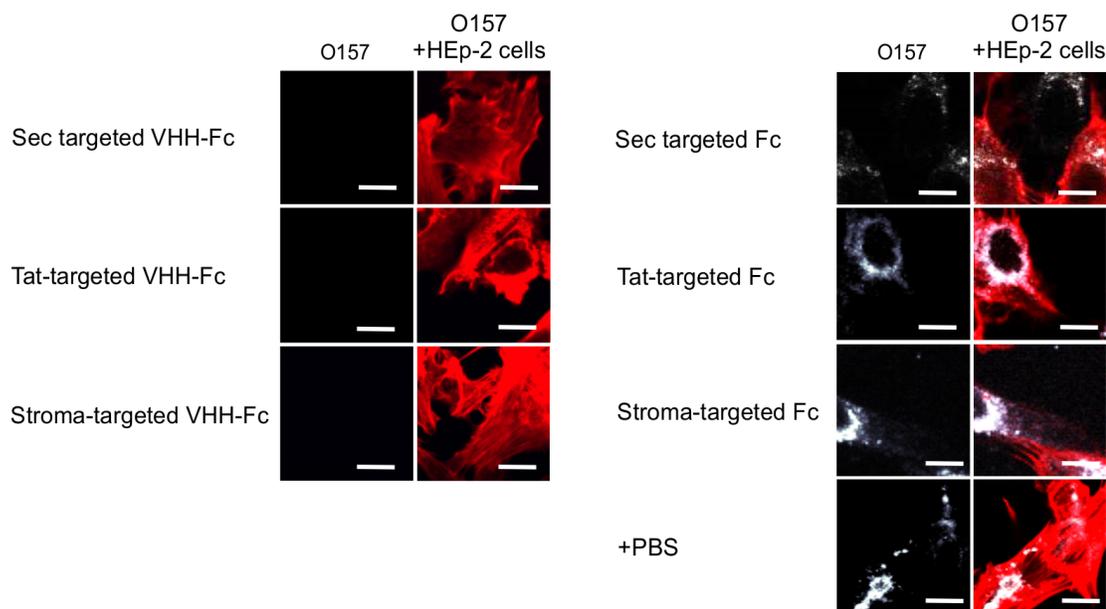


Figure 19. VHH-Fc targeted with either Sec, Tat or stromal transit peptides can neutralize adherence of O157:H7 to HEp-2 cells. Shown are confocal images of O157:H7 (white) that has been incubated with Hep-2 cells (red) in the presence of either VHH-Fc targeted to Sec, Tat and stromal compartments or Fc as a negative control targeted to the same compartments. Size bar = 20 μ m

4.5 Discussion

Utility of a thylakoid targeting system for antibodies

The utility of a plant platform for folding and assembling IgA antibodies and related synthetics in the ER is well established. However, in a previous study exploring how recombinant antibodies influence the endogenous proteome, a genome-wide Tiling array suggested that ER-targeted VHH-IgG Fc fusions in *A. thaliana* seeds generated an unfolded protein response (De Wilde et al. 2013). Because the thylakoid has a different proof reading

system for folding than the ER, there may be value in exploring it as an alternative oxidative folding compartment for antibody folding because of the potential to avoid ER associated degradation as a limiting factor. In this study, I explored the possibility of producing a synthetic IgA in the thylakoid lumen as a viable yield optimization strategy within the context of molecular farming. Overall, the results suggest that the VHH-Fc fusion seems to fold correctly and assemble with the requisite intra- and inter- chain disulfides as well as retains binding and neutralization efficacy. Although accumulation was not found to be higher than the ER, purified VHH-Fc protein from both stromal and lumenal fractions retained binding and neutralization efficacy. Notably, the Sec-targeted VHH-Fc fusion accumulated significantly better than stromal and Tat-targeted fusions, albeit at approximately 60% of the ER-targeted VHH-Fc fusion. Nonetheless, previous experience has suggested that robust accumulation for a transiently expressed, chloroplast targeted recombinant protein is indicative of high yields upon developing a stable transplastomic line for that protein. Transplastomically-expressed recombinant proteins tend to be of higher yield than when nuclear-expressed due to the polyploidy of the chloroplast genome and the lack of silencing and positional interaction effects (Bock 2007; Daniell et al. 2009). The use of stably-transformed chloroplasts presents several unique advantages as a molecular farming strategy, notably maternal inheritance of the chloroplast genome which virtually eliminates the prospect of gene escape to the environment by pollen (Kumar et al. 2004). Additionally, the recombinant proteins are encapsulated by chloroplast membranes and are effectively isolated from cellular proteases which are more abundant and diverse than those found in the chloroplast. The proteome and protease

profile of the thylakoid lumen in particular is substantially more limited in comparison (Kieselbach and Schroder 2003). Chloroplast-based expression might also facilitate alternative purification methods because intact chloroplasts can be easily isolated from crude extracts by low-speed centrifugation (Kubis et al. 2008). If yields are high enough, there may be value in scaling up production of biomass that could then be administered orally to animals for enteric protection against EHEC without the need for purification.

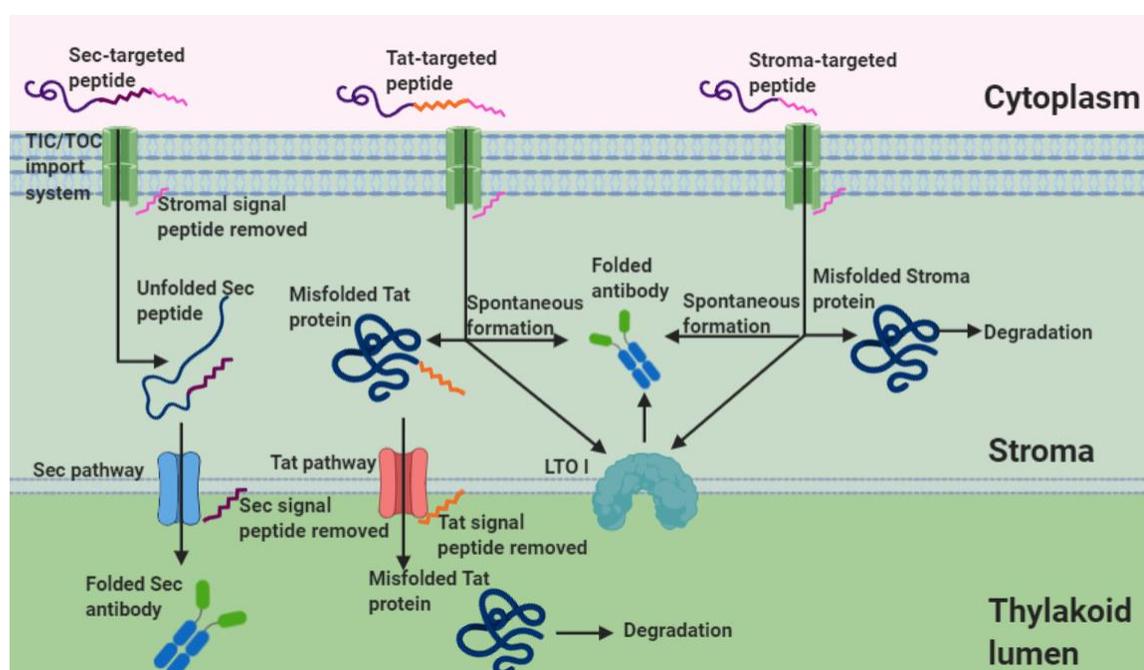


Figure 20. Schematic of Sec, Tat and stromal import of an antibody. Given that Tat and stromal imported antibodies retain functionality (Figure 18; Figure 19) and show dimerized banding under non-reducing conditions (Figure 15), this suggests disulfide formation in the stroma despite its reducing environment. Shown are two possible mechanisms of disulfide formation, interaction with LTO I and spontaneous formation, that may account for disulfide formation of tat and stroma targeted antibodies. (Figure created in Biorender.com)

Disulfide formation in the chloroplast

Under non-reducing conditions, banding corresponding to the VHH-Fc dimer was unexpectedly detected for the stromal and Tat-targeted compartments. Disulfide formation has conventionally been thought to be exclusive to the oxidative folding environment of

the thylakoid. However, a few recent studies have suggested that disulfide formation is possible in the reducing environment of the stroma for recombinant proteins, though it tends to be at much lower levels and the mechanics of which remain uncertain. For example, human growth hormone (Staub et al. 2000), cholera toxin B (Daniell et al. 2001), a recombinant alkaline phosphatase A (Bally et al. 2008), aprotinin (Tissot et al. 2008) and zeolin (De Marchis et al. 2011) all require disulfide bond formation for folding and are nonetheless biologically active when either expressed in or targeted to the stroma. Notably, Bally et al. (2008) have shown not only that the chloroplast stroma supports the formation of an active alkaline phosphatase A enzyme but also that sorting of the alkaline phosphatase to the thylakoid lumen leads to larger amounts and more active enzyme. If accumulation can be assumed to be a correlative measure of how well folded a protein is, then the higher accumulation observed for the Sec-targeted VHH-Fc suggests that the oxidative folding environment in the thylakoid lumen is conducive for proper folding of the VHH-Fc with the requisite disulfide stabilization. In contrast, both the stromal targeted and Tat-targeted VHH-Fc have similarly low accumulation levels and may be due to the suboptimal folding environment of the stroma. Similarly, the lack of detectable signal in the cytoplasm may be due to the inability of the VHH-Fc to fold sufficiently, particularly for the intrachain disulfides that are needed to stabilize the characteristic beta sandwich CH domains (Kumar et al. 2020). Several others have reported successfully expressing a VHH in the chloroplast. A nuclear-expressed and stroma-targeted VHH was shown to be effective in potato plants in modulating enzyme function endogenously, albeit at a very low accumulation of 0.03% TSP (Jobling et al. 2003). Similarly, a transplastomically produced stromal VHH in

tobacco retained binding efficacy against albumin lysozyme, a causative agent in proteinuria, but was produced at levels too low to be quantified and caused a semi-lethal pale-green seedling phenotype (Magee et al. 2004). Our results similarly show low accumulation levels in the stroma and I speculate that these low levels may reflect the relatively low occurrence of spontaneous disulfide formation despite the reducing environment of the stroma and the lack of protein disulfide isomerase activity. On the other hand, VHHs produced in the chloroplast of the green algae *Chlamydomonas reinhardtii* are competent in binding botulinum neurotoxin and have been shown to accumulate to 5% TSP suggesting that there may be key plant-specific physiological factors that limit production in plant chloroplasts versus *C. reinhardtii* chloroplasts (Barrera et al. 2015). Indeed, the protein disulfide isomerase-like RB60 is partitioned between stroma and thylakoids in *C. reinhardtii* chloroplasts and has been suggested to potentially interface bidirectionally (Treibitsh et al. 2001). Conversely, LTO1, that similarly catalyzes disulfide bond formation, is embedded in thylakoid lumen membrane of plants and is known to be preferentially oriented toward the thylakoid interior (Karamoko et al. 2013). It may thus also be possible that aside from spontaneous disulfide formation that the low levels of VHH-Fc when stromal-targeted and Tat-targeted may be due to trace disulfide isomerase activity at this partition. Therefore, this suggests that the stability, and accumulation thereof, of the VHH-Fc may be a function of its redox potential as it relates to the reactivity of its cysteine residues' thiol groups and/or the availability of disulfide isomerase activity (Figure 20).

I also introduced a *de novo* disulfide pair G196C/R219C into the VHH-Fc targeted with the Sec signal and found a significant yield improvement. Compared to the ten-fold yield improvement observed in Chapter 3 when targeted to the ER, the yield improvement is substantially less. It's unclear why this is but may possibly be due to the availability of relevant chaperones across the two compartments. The lumen is known to contain a unique chaperonin cpn60/cpn10 system distinct from the stromal chaperonin system or the HSP family in the ER (Schlicher and Soll 1996). Alternatively, the difference in yield improvement may be due to different activities of LTO1 in the lumen versus the protein disulfide isomerase (PDI)-mediated folding reactions in the ER.

Structural considerations for thylakoid antibodies

Although the thylakoid lumen is capable of disulfide formation, it lacks the machinery for glycosylation. Given that VHHs are not natively glycosylated by their host camelids, and are also competent when produced in *E. coli*, I hypothesized that the VHH would retain functionality when folded in the thylakoid (Herrmann et al. 2009). Additionally, VHHs have been shown to be effective in neutralizing a broad array of other enteric pathogens (King et al. 2018; Schmidt et al. 2016; Shkoporov et al. 2015; Vega et al. 2013). Accordingly, the binding and neutralizing assays suggest that efficacy is retained despite the lack of glycosylation. Although the VHH alone may be sufficient for neutralization, yields are usually low and attaching the Fc has been shown to improve accumulation (Virdi et al. 2019). The Fc also enables improved avidity via its ability to multimerize thereby mediating agglutination. In enteromucosal conditions, neutralization of a pathogen's ability to colonize epithelial cells is predominantly by steric hindrance via

agglutination (Li et al. 2020). Although the bovine IgA Fc is natively glycosylated, a recent study that characterized glycosylation on a plant-made ER- and apoplast-targeted Fc fusion protein demonstrated that preventing glycan attachment did not prevent the Fc from correctly folding (Xiong et al. 2019). Therefore, the thylakoid may be a suitable compartment for folding and accumulating VHH-Fc fusion proteins despite the lack of glycosylation.

Overall, this study provides a proof of concept that targeting to the thylakoid lumen via the Sec pathway allows for accumulation of a functional VHH-Fc fusion and may thus be a strategic way of producing these therapeutics while accruing the benefits of plastidial encapsulation.

5.0 GENERAL DISCUSSION AND CONCLUSION

Overall, this thesis has described the development of a plant-based anti-EHEC antibody and has explored two main strategies that are conceptually novel research avenues for improving its yield. With regards to the research objectives, I have 1) demonstrated binding and neutralization competency of a synthetic antibody against four of the seven most prevalent strains of EHEC, 2) identified and characterized a combination of six rationally designed mutations that enable a twenty-fold improved yield without sacrificing functional efficacy, and 3) enabled targeting of the antibody to the thylakoid lumen for folding and assessed its relative accumulation and efficacy.

5.1 Significance of a VHH-sIgA with cross serotype protection

Chapter 2 describes the initial design of a plant-produced synthetic antibody with secretory IgA functionality against four EHEC strains, O26:H11, O111:Hnm, O145:Hnm and O157:H7. Collectively, O26, O111, O145 and O157 account for nearly 72% of all EHEC incidences at a rate of approximately 1,158 cases per 100,000 individuals across the US (CDC, 2017). The finding that VHH10-sIgA offers cross-serotype protection is significant because the vast majority of previously developed therapeutics against EHEC have focused on O157 only, despite the clinical relevance of the “Big Six” strains. Because of its multivalency, the antibody may be useful if developed as a diagnostic reagent for detecting O26, O111, O145, and O157 in food, the environment, colonized animals, or in infected individuals. In particular, a cross-serotype diagnostic could potentially be of great value for identifying super shedders which are considered to be important hubs in a cattle population for maintaining the penetrance of EHEC infections. If these animals could be

identified prior to harvest or mixing with other animals, these could be targeted for intervention to reduce the likelihood of product contamination or cross-transmission across herds. Currently, there are no EHEC diagnostics available on the market that can detect both O157 and non-O157 strains.

The antibody market is still dominated by mammalian and microbial expression systems, with the former being preferred for complex glycosylated assemblies and the latter being appealing for high production of simpler proteins at low cost. Although plant systems have progressively made ground on industry acceptance as an alternative platform for larger scale production, there is still significant resistance to market entry. The vast majority of studies involving the plant-based production of antibodies have not been able to transition into commercial development and instead have been stuck at the same early-stage objectives explored in this thesis, namely the verification of expression, optimization of production and the completion of initial functionality assays (Schillberg et al. 2019). Overcoming the financial and organizational hurdles to translate a proof of concept to a product can be difficult, especially when market potential and intellectual property rights are unclear. Industry support favors antibody business models with a long and proven track record with key considerations being high quality, high yield and low overall costs. Although scalability and the low cost of plant cultivation are appealing drivers for reduced manufacturing costs, their benefits are reliant on high enough yields to make large scale production worth the investment. Therefore, there is value in developing yield improvement strategies, especially ones that can be incorporated at the protein design level without scaling expenses.

5.2 Significance of a rationally designed IgA Fc

Chapter 3 documents the development of an engineered Fc in which I demonstrated that by making seven particular amino acid substitutions to a native IgA Fc scaffold, yield of a subsequent VHH-Fc fusion can be improved twenty-fold without sacrificing efficacy. Based on a stability estimating algorithm across globular proteins, about 70% of amino acid mutations are destabilizing and ~20% are significantly destabilizing (Tokuriki et al. 2007). Within the scope of all possible amino acid variations of the Fc protein being 1.15×10^{48} , the chance of isolating a stabilized Fc by random chance can be considered negligible. Therefore, techniques to identify stabilizing mutations are required.

In chapter 3, I developed (5+1)Fc by rational design using a screen comprised of three strategies and found a combination of supercharging and *de novo* disulfides improved accumulation when fused to an anti-EHEC VHH by twenty-fold. Of the three strategies, supercharging seemed to have the highest success rate with five out of six candidates showing improved yield. In another related project, I have performed a similar screen with supercharging candidates on a subunit vaccine against EHEC and found two supercharging mutants to improve yield by about 20% (unpublished). This suggests that supercharging as a design strategy can be extended to other immunotherapeutics against EHEC.

Although the chapter was structured around neutralizing EHEC as a proof of concept, the original intent of engineering the Fc was for it to be a universal scaffold to which various types of binders could be attached and be afforded improved stability. Future studies will test if the yield-improving effect can be extended to other VHHs. Although rationally designed Fc chains have been patented for other isotypes of antibodies with

similar strategies, there are currently no patents or published data for a rationally-designed IgA Fc.

One emerging biotech trend is a focus on conceptually novel antibody-based synthetics including Fc-fusion proteins for improved stability or immunogenicity (Ning et al. 2019). These synthetics bypass a traditional focus on full size native antibody production which have had a variety of technical hurdles with folding, assembly and homogenous production. Currently, a majority of FDA approved mAbs in the clinical pipeline are full-size IgG1 antibodies of about 150 kDa (Kaplun et al. 2020). Therefore, much work in the past decade has focused on scaffold development on which a small scale binder (~15kDa), such as a VHH, can be fused or conjugated. The resulting therapeutic is of much smaller size and usually of higher stability than its full-size counterpart. Of these, Fc scaffolds have been one of the most promising scaffolds for therapeutics (Liu et al. 2017; Verdino et al. 2018). The appeal of the Fc as a scaffold is afforded by its structural modularity allowing independent folding of its fusion partner while still providing structural stability, improved avidity and enabling alternative effector functions (Park et al. 2016). Therefore, Fc(5+1) may potentially be of value as a universal IgA type scaffold for enteric immunotherapeutics.

5.3 Significance of a thylakoid targeting system for antibody production

Chapter 4 explored the viability of targeting a VHH-Fc for oxidative folding in the chloroplast thylakoid in a molecular farming context. Accumulation was compared for a transiently targeted VHH-Fc when imported into the chloroplast thylakoid compartment via either Sec or Tat pathways or to the chloroplast stroma in *N. benthamiana* leaf cells.

The VHH-Fc targeted to either the thylakoid or stromal compartments retained functionality in binding and neutralizing adherence of *E. coli* O157:H7 to epithelial cells in *in vitro* assays. This study is notable because it suggests that oxidative folding environment of the thylakoid lumen is conducive for antibody folding. Compared to stromal, cytoplasm and Tat-imported VHH-Fc, the Sec-targeted VHH-Fc showed superior accumulation, but 33% less than that of the ER-targeted protein. Although yield was not improved when Sec-targeted, it may nonetheless be a strategic alternative to the ER in cases where production in the ER is suspected to be limited by ER-specific misfolding and proteolysis, or in developing aglycosylated antibodies, and especially if transplastomic expression produces higher yields and avoids *Agrobacterium* growth and labor-intensive agroinfiltration.

Antibodies expressed in or targeted to the chloroplast are not glycosylated. For serum localized antibodies, glycosylation is known to be required for antibody-dependent, cell-mediated cytotoxicity and for significantly extending the half-life in circulation (Cymer et al. 2018). However, in enteromucosal conditions, neutralization is not mediated by effector functions but rather predominately by steric hindrance via agglutination of the pathogens (Li et al. 2020). VHHs do not require glycosylation for folding nor binding and have been shown to be effective in neutralizing a broad array of enteric pathogens (King et al. 2018; Schmidt et al. 2016; Shkoporov et al. 2015; Vega et al. 2013). Also, glycosylation serves no purpose if the antibody is to be used as a diagnostic or research reagent. Therefore, while glycosylation is an important general consideration for antibody production, depending on the end-use, glycosylation may not be required. Indeed, because

there are still concerns about the safety and efficacy of plant-specific glycans, particularly with regards to potential immunogenicity, producing an aglycosylated product that is more homogenous may be more acceptable by regulatory agencies.

In general, subcellular localization strongly influences the final yield of a recombinant antibody because each compartment has a unique environment (pH, salts, redox state etc.) that differentially selects for the native fold amongst other possible intermediates in a conformational landscape (Streatfield 2007). Additionally, targeting the antibody for encapsulation can reduce its exposure to relevant proteases, and also to protect the host cell against the potentially harmful effects of accumulating misfolded aggregates (Benchabane et al., 2008; Conley et al., 2009; Joensuu et al., 2010; Torrent et al., 2009). The negative impact of endogenous proteases on the yields of plant recombinant proteins targeted to the secretory pathway in *N. benthamiana* is well documented (Goulet et al. 2012; Jutras et al. 2015; Jutras et al. 2018a; Jutras et al. 2019; Jutras et al. 2016; Jutras et al. 2018b; Robert et al. 2013). Therefore, developing a chloroplast thylakoid targeting system provides an additional tool for screening new antibody candidates for optimal yield.

The robust accumulation of sec-targeted VHH-Fc in the thylakoid suggests the possibility that accumulation would also be robust in transplastomic plants. Transplastomically-expressed recombinant proteins can potentially be of higher yield than when nuclear-expressed because of the high gene copy number, with as many as 3,300 plastome copies being present in mature diploid mesophyll cells (Greiner et al. 2020). Also, chloroplasts retain a prokaryotic expression system, and transcription is not subject to silencing and positional gene interaction effects (Bock 2007; Daniell et al. 2009). Because

expression involves multiple genes arranged in tandem that are all transcribed as a single multicistronic transcript which is then separately cleaved (Bock 2014), this could potentially allow better stoichiometric control of transcription of antibody subunits if the genes were to be stacked in parallel and thus be of value for producing multi-subunit antibodies. Additionally, maternal inheritance of the plastome virtually eliminates the prospect of gene escape to the environment by pollen (Kumar et al. 2004). Therefore, there is value in developing transplastomic plants with antibodies encapsulated in the thylakoid.

5.4 Future work

Stable lines of *N. tabacum* transplastomically expressing the anti-intimin VHH in the chloroplast stroma have been developed (Reza Saberianfar, Nicolas Boisset, personal communication). I have performed *in vitro* neutralization assays with VHHs extracted from these lines and found that the pattern of cross-serotype neutralization against the seven EHEC strains is retained (not shown). I speculate that this neutralization is mediated by competitive inhibition for intimin's docking site with its translocated receptor Tir rather than agglutination of the bacteria via the Fc. A similar mechanism has been demonstrated for a VHH that prevents the enteric colonization of *Listeria monocytogenes* (King et al. 2018). This suggests that blocking of the Tir-intimin interaction may be sufficient for preventing colonisation, at least in an *in vitro* model where saturation of intimin sites is possible. However, for *in vivo* oral administration, the presence of the Fc would be required to agglutinate and eliminate the bacteria by peristalsis. For this, I have synthesized a VHH-(5+1)Fc fusion with a Sec transit peptide which is now ready to be cloned into a chloroplast transformation vector (Kolotilin et al. 2013). Another student will continue the work in

assessing accumulation and testing functionality. In chapter 4, I showed that the yield improving effect of a *de novo* disulfide identified in chapter 3 was retained when transiently targeting the VHH-Fc to the thylakoid with a Sec transit signal. Therefore, I expect that the yield improving effect of the (5+1)Fc will also be retained with transplastomic expression and sec targeting. We are also currently working on organizing animal trials with the VHH-Fc fusions to determine if they can neutralize EHEC *in vivo* as well as its utility for fast acting prevention and intervention.

5.5 Concluding remarks

Overall, this thesis has functionally characterized an anti-EHEC antibody and explored two design strategies that add to the toolbox for understanding the factors that influence antibody yield. These two strategies rely on standard methods and can therefore be applied to other similar antibodies. The engineered Fc may be of value as a general IgA type scaffold that allows not only improved yield, but also the option to dimerize for improved avidity or form a secretory complex for improved proteolytic resistance. The thylakoid targeting of antibodies may allow a better understanding of host factors and cellular processes involved in oxidative folding and degradation and thus may potentially carve out a niche mostly exclusive to a plant platform. As antibody yield progressively becomes less of a hurdle toward commercialization, the unique features of a plant platform, such as prospects for oral delivery and cross-over with agricultural systems, may better define its niche. For the treatment of enteric diseases, such as EHEC, in animals, it is particularly well-suited because of the increased safety and convenience for the producer by orally administering transformed plant tissue. However, this mode of administration

may require a much larger dose for efficacy since much of the antibody formulation may be degraded during its movement through the animal's gut prior to reaching the site of colonization (Rybicki 2010). Therefore, going forward, key research targets for developing plant-based anti-EHEC antibodies should arguably be: 1) better yield to accommodate the gram quantities needed per dose per animal 2) improved protein stability to enable longer shelf-life and for maintaining efficacy in feed formulations 3) designs or formulations geared toward bioavailability and avidity to improve the effective neutralizing power per individual peptide. Additionally, the use of VHH-Fc antibodies as diagnostics could potentially be an easily incorporated method of identifying supershedders in an infected herd. This technology is still in its early development stage with most immediate major milestone requirements before commercialization being proof of efficacy *in vivo* and across environments. Yet, I am optimistic that the contributions demonstrated in this thesis will be of value toward improving antibody yields and ultimately toward the control of food safety.

6.0 REFERENCES

- Almagro Armenteros JJ, Salvatore M, Emanuelsson O, Winther O, von Heijne G, Elofsson A, Nielsen H (2019) Detecting sequence signals in targeting peptides using deep learning. *Life Science Alliance* 2 pii: e201900429
- Almon E, Khoury T, Drori A, Gingis-Velitski S, Alon S, Chertkoff R, Mushkat M, Shaaltiel Y, Ilan Y (2017) An oral administration of a recombinant anti-TNF fusion protein is biologically active in the gut promoting regulatory T cells: Results of a phase I clinical trial using a novel oral anti-TNF alpha-based therapy. *Journal of Immunological Methods* 446:21-29
- Almquist KC, McLean MD, Niu Y, Byrne G, Olea-Popelka FC, Murrant C, Barclay J, Hall JC (2006) Expression of an anti-botulinum toxin A neutralizing single-chain Fv recombinant antibody in transgenic tobacco. *Vaccine* 24:2079-2086
- Baines D, Masson L, McAllister T (2008) Escherichia coli O157:H7-secreted cytotoxins are toxic to enterocytes and increase Escherichia coli O157 : H7 colonization of jejunum and descending colon in cattle. *Canadian Journal of Animal Science* 88:41-50
- Bakema JE, Ganzevles SH, Fluitsma DM, Schilham MW, Beelen RH, Valerius T, Lohse S, Glennie MJ, Medema JP, van Egmond M (2011) Targeting Fc α RI on polymorphonuclear cells induces tumor cell killing through autophagy. *Journal of Immunology* 187:726-732
- Bakema JE, van Egmond M (2011) Immunoglobulin A: A next generation of therapeutic antibodies? *mAbs* 3:352-361
- Bally J, Paget E, Droux M, Job C, Job D, Dubald M (2008) Both the stroma and thylakoid lumen of tobacco chloroplasts are competent for the formation of disulphide bonds in recombinant proteins. *Plant Biotechnology Journal* 6:46-61
- Barrera DJ, Rosenberg JN, Chiu JG, Chang YN, Debatis M, Ngoi SM, Chang JT, Shoemaker CB, Oyler GA, Mayfield SP (2015) Algal chloroplast produced camelid VHH antitoxins are capable of neutralizing botulinum neurotoxin. *Plant Biotechnology Journal*. 13:117-24.
- Bastian A, Kratzin H, Eckart K, Hilschmann N (1992) Intra- and interchain disulfide bridges of the human J chain in secretory immunoglobulin A. *Biological Chemistry* 373:1255-1263
- Bell BP, Goldoft M, Griffin PM, Davis MA, Gordon DC, Tarr PI, Bartleson CA, Lewis JH, Barrett TJ, Wells JG (1994) A multistate outbreak of escherichia coli O157: H7—associated bloody diarrhea and hemolytic uremic syndrome from hamburgers: the Washington experience. *JAMA* 272:1349-1353
- Benchabane M, Goulet C, Rivard D, Faye L, Gomord V, Michaud D (2008) Preventing unintended proteolysis in plant protein biofactories. *Plant Biotechnology Journal* 6:633-648
- Bennett LD, Yang Q, Berquist BR, Giddens JP, Ren Z, Kommineni V, Murray RP, White EL, Holtz BR, Wang LX, Marcel S (2018) Implementation of Glycan Remodeling to Plant-Made Therapeutic Antibodies. *International Journal of Molecular Sciences* 19

- Berry ED, Wells JE, Bono JL, Woodbury BL, Kalchayanand N, Norman KN, Suslow TV, López-Velasco G, Millner PD (2015) Effect of proximity to a cattle feedlot on *Escherichia coli* O157: H7 contamination of leafy greens and evaluation of the potential for airborne transmission. *Applied and Environmental Microbiology* 81:1101-1110
- Besser TE, Hancock DD, Pritchett LC, McRae EM, Rice DH, Tarr PI (1997) Duration of detection of fecal excretion of *Escherichia coli* O157: H7 in cattle. *Journal of Infectious Diseases* 175:726-729
- Best A, La Ragione RM, Sayers AR, Woodward MJ (2005) Role for flagella but not intimin in the persistent infection of the gastrointestinal tissues of specific-pathogen-free chicks by Shiga toxin-negative *Escherichia coli* O157: H7. *Infection and immunity* 73:1836-1846
- Beutin L, Geier D, Steinrueck H, Zimmermann S, Scheutz F (1993a) Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. *Journal of Clinical Microbiology* 31:2483-2488
- Beutin L, Geier D, Steinrück H, Zimmermann S, Scheutz F (1993b) Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. *Journal of Clinical Microbiology* 31:2483-2488
- Bock R (2007) Plastid biotechnology: prospects for herbicide and insect resistance, metabolic engineering and molecular farming. *Current Opinion in Biotechnology* 18:100-106
- Bock R (2014) Genetic engineering of the chloroplast: novel tools and new applications. *Current Opinion in Biotechnology* 26:7-13
- Bonner A, Perrier C, Corthesy B, Perkins SJ (2007) Solution structure of human secretory component and implications for biological function. *Journal of Biological Chemistry* 282:16969-16980
- Boyd B, Lingwood C (1989) Verotoxin receptor glycolipid in human renal tissue. *Nephron* 51:207-210
- Brigham CJ, Sinskey AJ, Song Y, Matsumoto Ki, Yamada M, Gohda A, Taguchi S (2011) Engineered *Corynebacterium glutamicum* as an endotoxin-free platform strain for lactate-based polyester production. *Applied Microbiology and Biotechnology* 93:1917-1925
- Brodzik R, Glogowska M, Bandurska K, Okulicz M, Deka D, Ko K, van der Linden J, Leusen JH, Pogrebnyak N, Golovkin M, Steplewski Z, Koprowski H (2006) Plant-derived anti-Lewis Y mAb exhibits biological activities for efficient immunotherapy against human cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* 103:8804-8809
- Buchanan A, Clementel V, Woods R, Harn N, Bowen MA, Mo W, Popovic B, Bishop SM, Dall'Acqua W, Minter R, Jermutus L, Bedian V (2013) Engineering a therapeutic IgG molecule to address cysteinylolation, aggregation and enhance thermal stability and expression. *mAbs* 5:255-262

- Burkinshaw BJ, Deng W, Lameignère E, Wasney GA, Zhu H, Worrall LJ, Finlay BB, Strynadka NC (2015) Structural analysis of a specialized type III secretion system peptidoglycan-cleaving enzyme. *Journal of Biological Chemistry* 290:10406-10417
- Büttner D (2012) Protein export according to schedule: architecture, assembly, and regulation of type III secretion systems from plant-and animal-pathogenic bacteria. *Microbiology and Molecular Biology Reviews* 76:262-310
- Capodicasa C, Chiani P, Bromuro C, De Bernardis F, Catellani M, Palma AS, Liu Y, Feizi T, Cassone A, Benvenuto E, Torosantucci A (2011) Plant production of anti-beta-glucan antibodies for immunotherapy of fungal infections in humans. *Plant Biotechnology Journal* 9:776-787
- Carballo-Amador MA, McKenzie EA, Dickson AJ, Warwicker J (2019) Surface patches on recombinant erythropoietin predict protein solubility: engineering proteins to minimise aggregation. *BMC Biotechnology* 19:26
- Castilho A, Strasser R, Stadlmann J, Grass J, Jez J, Gattinger P, Kunert R, Quendler H, Pabst M, Leonard R (2010) In planta protein sialylation through overexpression of the respective mammalian pathway. *Journal of Biological Chemistry* 285:15923-15930
- CDC (2011) Vital signs: incidence and trends of infection with pathogens transmitted commonly through food-foodborne diseases active surveillance network, 10 US sites, 1996-2010. *Morbidity and Mortality Weekly Report* 60:749
- CDC (2017) Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet 2015 Surveillance Report (Final Data). Atlanta, Georgia: U.S. Department of Health and Human Services. Retrieved from <https://www.cdc.gov/foodnet/reports/annual-reports-2015.html>
- Chan HT, Daniell H (2015) Plant-made oral vaccines against human infectious diseases—are we there yet? *Plant Biotechnology Journal* 13:1056-1070
- Chandler PG, Broendum SS, Riley BT, Spence MA, Jackson CJ, McGowan S, Buckle AM. Strategies for Increasing Protein Stability (2020) *InProtein Nanotechnology* 163-181
- Chapman AM, McNaughton BR (2016) Scratching the surface: resurfacing proteins to endow new properties and function. *Cell Chemical Biology* 23:543-553
- Charatan F (1999) New York outbreak of *E coli* poisoning affects 1000 and kills two. *British Medical Journal* 319:873
- Choi YS, Moon JH, Kim TG, Lee JY (2016) Potent In Vitro and In Vivo Activity of Plantibody Specific for *Porphyromonas gingivalis* FimA. *Clinical and Vaccine Immunology* : CVI 23:346-352
- Chong Y, Fitzhenry R, Heuschkel R, Torrente F, Frankel G, Phillips AD (2007) Human intestinal tissue tropism in *Escherichia coli* O157: H7—initial colonization of terminal ileum and Peyer's patches and minimal colonic adhesion ex vivo. *Microbiology* 153:794-802
- Cleary TG (2004) The role of Shiga-toxin-producing *Escherichia coli* in hemorrhagic colitis and hemolytic uremic syndrome. *Seminars in Pediatric Infectious Diseases* 15:260-265

- Corthesy B (2003) Recombinant secretory immunoglobulin A in passive immunotherapy: linking immunology and biotechnology. *Current Pharmaceutical Biotechnology* 4:51-67
- Corthesy B (2010) Role of secretory immunoglobulin A and secretory component in the protection of mucosal surfaces. *Future Microbiology* 5:817-829
- Corthesy B (2013) Role of secretory IgA in infection and maintenance of homeostasis. *Autoimmunity Reviews* 12:661-665
- Courtois F, Schneider CP, Agrawal NJ, Trout BL (2015) Rational Design of Biobetters with Enhanced Stability. *Pharmaceutical Sciences* 104:2433-2440
- Cymer F, Beck H, Rohde A, Reusch D (2018) Therapeutic monoclonal antibody N-glycosylation - Structure, function and therapeutic potential. *Biologicals* 52:1-11
- Czajkowsky DM, Hu J, Shao Z, Pleass RJ (2012) Fc-fusion proteins: new developments and future perspectives. *EMBO Molecular Medicine* 4:1015-1028
- D'Aoust MA, Lavoie PO, Belles-Isles J, Bechtold N, Martel M, Vezina LP (2009) Transient expression of antibodies in plants using syringe agroinfiltration. *Methods in Molecular Biology* 483:41-50
- Daniell H, Lee SB, Panchal T, Wiebe PO (2001) Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. *Journal of Molecular Biology* 311:1001-1009
- Daniell H, Singh ND, Mason H, Streatfield SJ (2009) Plant-made vaccine antigens and biopharmaceuticals. *Trends in Plant Science* 14:669-679
- De Buck S, Nolf J, De Meyer T, Viridi V, De Wilde K, Van Lerberge E, Van Droogenbroeck B, Depicker A (2013) Fusion of an Fc chain to a VHH boosts the accumulation levels in Arabidopsis seeds. *Journal of Plant Biotechnology* 11:1006-1016
- De Greve H, Viridi V, Bakshi S, Depicker A (2020) Simplified monomeric VHH-Fc antibodies provide new opportunities for passive immunization. *Current Opinion in Biotechnology* 61:96-101
- De Marchis F, Pompa A, Mannucci R, Morosinotto T, Bellucci M (2011) A plant secretory signal peptide targets plastome-encoded recombinant proteins to the thylakoid membrane. *Plant Molecular Biology* 76:427-441
- De Meyer T, Muyltermans S, Depicker A (2014) Nanobody-based products as research and diagnostic tools. *Trends in Biotechnology* 32:263-270
- De Muynck B, Navarre C, Nizet Y, Stadlmann J, Boutry M (2009) Different subcellular localization and glycosylation for a functional antibody expressed in *Nicotiana tabacum* plants and suspension cells. *Transgenic Research* 18:467-482
- De Wilde K, De Buck S, Vanneste K, Depicker A (2013) Recombinant antibody production in Arabidopsis seeds triggers an unfolded protein response. *Plant Physiology* 161:1021-1033
- Delahay RM, Knutton S, Shaw RK, Hartland EL, Pallen MJ, Frankel G (1999) The Coiled-coil Domain of EspA Is Essential for the Assembly of the Type III Secretion Translocon on the Surface of Enteropathogenic *Escherichia coli*. *Journal of Biological Chemistry* 274:35969-35974

- Dill KA, Ozkan SB, Shell MS, Weikl TR (2008) The protein folding problem. *Annual Review of Biophysics* 37:289-316
- Dimitrov DS (2009) Engineered CH2 domains (nanoantibodies). *mAbs* 1:26-28
- Donnenberg MS, Tzipori S, McKee ML, O'Brien AD, Alroy J, Kaper JB (1993) The role of the *eae* gene of enterohemorrhagic *Escherichia coli* in intimate attachment in vitro and in a porcine model. *Journal of Clinical Investigation* 92:1418
- Craig DB, Dombkowski AA (2013) Disulfide by Design 2.0: a web-based tool for disulfide engineering in proteins. *BMC bioinformatics* 14:346
- Doyle M, Archer J, Kaspar C, Weiss R (2006) FRI Briefing: Human illness caused by *E. coli* O157: H7 from food and non-food sources. Food Research Institute Briefings. Retrieved from https://fri.wisc.edu/files/Briefs_File/FRIBrief_EcoliO157H7humanillness.pdf
- Dudgeon K, Rouet R, Kokmeijer I, Schofield P, Stolp J, Langley D, Stock D, Christ D (2012) General strategy for the generation of human antibody variable domains with increased aggregation resistance. *Proceedings of the National Academy of Sciences of the United States of America* 109:10879-10884
- Dziva F, van Diemen PM, Stevens MP, Smith AJ, Wallis TS (2004) Identification of *Escherichia coli* O157: H7 genes influencing colonization of the bovine gastrointestinal tract using signature-tagged mutagenesis. *Journal of Microbiology* 150:3631-3645
- Ecker DM, Jones SD, Levine HL (2015) The therapeutic monoclonal antibody market. *mAbs* 7:9-14
- Emanuelsson O, Nielsen H, von Heijne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Science* 8:978-984
- Endo Y, Tsurugi K, Lambert JM (1988) The site of action of six different ribosome-inactivating proteins from plants on eukaryotic ribosomes: the RNA N-glycosidase activity of the proteins. *Biochemical and Biophysical Research Communications* 150:1032-1036
- Fernandez DE (2018) Two paths diverged in the stroma: targeting to dual SEC translocase systems in chloroplasts. *Photosynthesis Research* 138:277-287
- Fivaz M, van der Goot FG (1999) The tip of a molecular syringe. *Trends in Microbiology* 7:341-343
- Frenzel A, Hust M, Schirrmann T (2013) Expression of recombinant antibodies. *Frontiers in Immunology* 4:217
- Fukushima H, Hashizume T, Morita Y, Tanaka J, Azuma K, Mizumoto Y, Kaneno M, Matsu-Ura MO, Kitani KK (1999) Clinical experiences in Sakai City Hospital during the massive outbreak of enterohemorrhagic *Escherichia coli* O157 infections in Sakai City, 1996. *Journal of Pediatrics International* 41:213-217
- Garmendia J, Phillips AD, Carlier MF, Chong Y, Schüller S, Marches O, Dahan S, Oswald E, Shaw RK, Knutton S (2004) TccP is an enterohaemorrhagic *Escherichia coli* O157: H7 type III effector protein that couples Tir to the actin-cytoskeleton. *Cellular Microbiology* 6:1167-1183

- Gasdaska JR, Sherwood S, Regan JT, Dickey LF (2012) An afucosylated anti-CD20 monoclonal antibody with greater antibody-dependent cellular cytotoxicity and B-cell depletion and lower complement-dependent cytotoxicity than rituximab. *Molecular Immunology* 50:134-141
- Gelova Z, Ten Hoopen P, Novak O, Motyka V, Pernisova M, Dabravolski S, Didi V, Tillack I, Oklestkova J, Strnad M, Hause B, Harustiakova D, Conrad U, Janda L, Hejatko J (2018) Antibody-mediated modulation of cytokinins in tobacco: organ-specific changes in cytokinin homeostasis. *Journal of Experimental Botany* 69:441-454
- Gill A, Gill CO (2010) Non-O157 verotoxigenic *Escherichia coli* and beef: a Canadian perspective. *Canadian Journal of Veterinary Research* 74:161-169
- Girard LS, Fabis MJ, Bastin M, Courtois D, Pétiard V, Koprowski H, communication (2006) Expression of a human anti-rabies virus monoclonal antibody in tobacco cell culture. *Biochemistry and Biophysics Research Community* 345:602-607
- Gleba YY, Giritch A (2011) Plant viral vectors for protein expression. *Recent Advances in Plant Virology* 387-412
- Gomes M, Alvarez MA, Quellis LR, Becher ML, Castro JMA, Gameiro J, Caporrino MC, Moura-da-Silva AM, de Oliveira Santos M (2019) Expression of an scFv antibody fragment in *Nicotiana benthamiana* and in vitro assessment of its neutralizing potential against the snake venom metalloproteinase BaP1 from *Bothrops asper*. *Toxicon* 160:38-46
- Gomord V, Fitchette AC, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, Michaud D, Faye LJ (2010) Plant-specific glycosylation patterns in the context of therapeutic protein production. *Plant Biotechnology Journal* 8:564-587
- Gould LH, Demma L, Jones TF, Hurd S, Vugia DJ, Smith K, Shiferaw B, Segler S, Palmer A, Zansky S (2009) Hemolytic uremic syndrome and death in persons with *Escherichia coli* O157: H7 infection, foodborne diseases active surveillance network sites, 2000–2006. *Clinical Infectious Diseases* 49:1480-1485
- Greiner S, Golczyk H, Malinova I, Pellizzer T, Bock R, Börner T, Herrmann RG (2020) Chloroplast nucleoids are highly dynamic in ploidy, number, and structure during leaf development. *The Plant Journal* 102: 730-46
- Griffin P (1995) *Escherichia coli* O157: H7 and other enterohemorrhagic *Escherichia coli*. In: Blaser M, Smith P, Ravdin J, Greenberg H and Guerrant R (eds) *Infections of the Gastrointestinal Tract*. Raven Press Ltd, New York, pp739-761
- Griffin PM, Olmstead LC, Petras RE (1990) *Escherichia coli* O157: H7-associated colitis. *Journal of Gastroenterology* 99:142-149
- Griffin PM, Tauxe RV (1991) The epidemiology of infections caused by *Escherichia coli* O157: H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiologic Reviews* 13:60-98
- Grohs BM, Niu Y, Veldhuis LJ, Trabelsi S, Garabagi F, Hassell JA, McLean MD, Hall JC (2010) Plant-produced trastuzumab inhibits the growth of HER2 positive cancer cells. *Journal of Agricultural and Food Chemistry* 58:10056-10063
- Gruenheid S, Sekirov I, Thomas NA, Deng W, O'donnell P, Goode D, Li Y, Frey EA, Brown NF, Metalnikov P (2004) Identification and characterization of NleA, a non-

- LEE-encoded type III translocated virulence factor of enterohaemorrhagic *Escherichia coli* O157: H7. *Molecular Microbiology* 51:1233-1249
- Guerler A, Govindarajoo B, Zhang Y. Mapping monomeric threading to protein-protein structure prediction (2013) *Journal of Chemical Information and Modeling*. 53:717-25
- Gupta SK, Shukla PJCrim (2017) Microbial platform technology for recombinant antibody fragment production: a review. *Critical Reviews in Microbiology* 43:31-42
- Hale CR, Scallan E, Cronquist AB, Dunn J, Smith K, Robinson T, Lathrop S, Tobin-D'Angelo M, Clogher P (2012) Estimates of enteric illness attributable to contact with animals and their environments in the United States. *Clinical Infectious Diseases* 54:S472-S479
- Harmsen MM, De Haard HJ (2007) Properties, production, and applications of camelid single-domain antibody fragments. *Applied Microbiology and Biotechnology* 77:13-22
- Henry KA, Kandalaf H, Lowden MJ, Rossotti MA, van Faassen H, Hussack G, Durocher Y, Kim DY, Tanha J (2017) A disulfide-stabilized human VL single-domain antibody library is a source of soluble and highly thermostable binders. *Molecular Immunology* 90:190-196
- Hernandez-Velazquez A, Lopez-Quesada A, Ceballo-Camara Y, Cabrera-Herrera G, Tiel-Gonzalez K, Mirabal-Ortega L, Perez-Martinez M, Perez-Castillo R, Rosabal-Ayan Y, Ramos-Gonzalez O, Enriquez-Obregon G, Depicker A, Pujol-Ferrer M (2015) Tobacco seeds as efficient production platform for a biologically active anti-HBsAg monoclonal antibody. *Transgenic Research* 24:897-909
- Herrmann JM, Kauff F, Neuhaus HE (2009) Thiol oxidation in bacteria, mitochondria and chloroplasts: common principles but three unrelated machineries? *Biochimica et Biophysica Acta* 1793:71-77
- Heuvelink A, Van Heerwaarden C, Zwartkruis-Nahuis J, Van Oosterom R, Edink K, Van Duynhoven Y, De Boer E (2002) *Escherichia coli* O157 infection associated with a petting zoo. *Epidemiology & Infection* 129:295-302
- Hiatt A, Cafferkey R, Bowdish K (1989) Production of antibodies in transgenic plants. *Nature* 342:76-78
- Hrudey S, Payment P, Huck P, Gillham R, Hrudey E (2003) A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. *Water Science and Technology* 47:7-14
- Huang Y, Zhang L (2017) An In Vitro Single-Primer Site-Directed Mutagenesis Method for Use in Biotechnology. *Methods in Molecular Biology* 1498:375-383
- Huang Z, Phoolcharoen W, Lai H, Piensook K, Cardineau G, Zeitlin L, Whaley KJ, Arntzen CJ, Mason HS, Chen Q (2010) High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. *Bioengineering* 106:9-17
- Hull AK, Yusibov V, Mett V (2005) Inducible expression in plants by virus-mediated transgene activation. *Transgenic Research* 14:407-416
- Ilan Y, Gingis-Velitski S, Ben Ya'aco A, Shabbat Y, Zolotarov L, Almon E, Shaaltiel Y (2017) A plant cell-expressed recombinant anti-TNF fusion protein is biologically

- active in the gut and alleviates immune-mediated hepatitis and colitis. *Journal of Immunobiology* 222:544-551
- Inobe T, Matouschek A (2014) Paradigms of protein degradation by the proteasome. *Current Opinion in Structural Biology* 24:156-164
- Ismaili A, Philpott DJ, Dytoc MT, Sherman PM (1995) Signal transduction responses following adhesion of verocytotoxin-producing *Escherichia coli*. *Infection and Immunity* 63:3316-3326
- Jacewicz MS, Acheson D, Mobassaleh M, Donohue-Rolfe A, Balasubramanian K, Keusch GT (1995) Maturation regulation of globotriaosylceramide, the Shiga-like toxin 1 receptor, in cultured human gut epithelial cells. *Journal of Clinical Investigation* 96:1328
- Jarvis KG, Giron JA, Jerse AE, McDaniel TK, Donnenberg MS, Kaper JB (1995) Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. *Proceedings of the National Academy of Sciences of the United States of America* 92:7996-8000
- Jarvis KG, Kaper JB (1996) Secretion of extracellular proteins by enterohemorrhagic *Escherichia coli* via a putative type III secretion system. *Infection and Immunity* 64:4826-4829
- Jefferis R (2009) Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. *Trends in Pharmacological Sciences* 30:356-362
- Jiang X, Morgan J, Doyle MP (2002) Fate of *Escherichia coli* O157: H7 in manure-amended soil. *Journal of Applied and Environmental Microbiology* 68:2605-2609
- Jobling SA, Jarman C, Teh MM, Holmberg N, Blake C, Verhoeven ME (2003) Immunomodulation of enzyme function in plants by single-domain antibody fragments. *Nature Biotechnology* 21:77-80
- Johnson MP, Wientjes E (2019) The relevance of dynamic thylakoid organisation to photosynthetic regulation. *Biochimica et Biophysica Acta* 4: 148039
- Juarez P, Presa S, Espi J, Pineda B, Anton MT, Moreno V, Buesa J, Granell A, Orzaez D (2012) Neutralizing antibodies against rotavirus produced in transgenically labelled purple tomatoes. *Journal of Plant Biotechnology* 10:341-352
- Kallolimath S, Gruber C, Steinkellner H, Castilho A (2018) Promoter Choice Impacts the Efficiency of Plant Glyco-Engineering. *Journal of Biotechnology* 13:1700380
- Kallolimath S, Steinkellner H (2015) Glycosylation of plant produced human antibodies. *Journal of Human Antibodies* 23:45-48
- Kandel G, Donohue-Rolfe A, Donowitz M, Keusch G (1989) Pathogenesis of *Shigella* diarrhea. XVI. Selective targeting of Shiga toxin to villus cells of rabbit jejunum explains the effect of the toxin on intestinal electrolyte transport. *Journal of Clinical Investigation* 84:1509
- Kaplon H, Muralidharan M, Schneider Z, Reichert JM (2020) Antibodies to watch in 2020. *mAbs* 12:1703531
- Karamoko M, Gabilly ST, Hamel PP (2013) Operation of trans-thylakoid thiol-metabolizing pathways in photosynthesis. *Frontiers Plant Science* 4:476

- Karmali M, Petric M, Lim C, Fleming P, Steele B (1983) Escherichia coli cytotoxin, haemolytic-uraemic syndrome, and haemorrhagic colitis. *The Lancet* 322:1299-1300
- Karmali MA (2004) Prospects for preventing serious systemic toxemic complications of Shiga toxin-producing Escherichia coli infections using Shiga toxin receptor analogues. *Journal of Infectious Diseases* 189:355-359
- Kenny B, DeVinney R, Stein M, Reinscheid DJ, Frey EA, Finlay BB (1997) Enteropathogenic E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* 91:511-520
- Kenny B, Finlay BB (1995) Protein secretion by enteropathogenic Escherichia coli is essential for transducing signals to epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America* 92:7991-7995
- Kieselbach T, Schroder WP (2003) The proteome of the chloroplast lumen of higher plants. *Journal of Photosynthesis Research* 78:249-264
- Kim J, Thanabalasuriar A, Chaworth-Musters T, Fromme JC, Frey EA, Lario PI, Metalnikov P, Rizg K, Thomas NA, Lee SF (2007) The bacterial virulence factor NleA inhibits cellular protein secretion by disrupting mammalian COPII function. *Cell Host & Microbe* 2:160-171
- King MT, Huh I, Shenai A, Brooks TM, Brooks CL (2018) Structural basis of VHH-mediated neutralization of the food-borne pathogen *Listeria monocytogenes*. *Journal of Biological Chemistry* 293:13626-13635
- Kirchhoff H (2019) Chloroplast ultrastructure in plants. *New Phytologist* 223:565-574
- Knutton S, Baldwin T, Williams P, McNeish A (1989) Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic Escherichia coli. *Infection and Immunity* 57:1290-1298
- Ko K, Tekoah Y, Rudd PM, Harvey DJ, Dwek RA, Spitsin S, Hanlon CA, Rupprecht C, Dietzschold B, Golovkin M, Koprowski H (2003) Function and glycosylation of plant-derived antiviral monoclonal antibody. *Proceedings of the National Academy of Sciences of the United States of America* 100:8013-8018
- Kolotilin I, Kaldis A, Pereira EO, Laberge S, Menassa R (2013) Optimization of transplastomic production of hemicellulases in tobacco: effects of expression cassette configuration and tobacco cultivar used as production platform on recombinant protein yields. *Biotechnology for Biofuels* 6:65
- Krugmann S, Pleass RJ, Atkin JD, Woof JM (1997) Structural requirements for assembly of dimeric IgA probed by site-directed mutagenesis of J chain and a cysteine residue of the alpha-chain CH2 domain. *Journal of Immunology* 159:244-249
- Kubis SE, Lilley KS, Jarvis P (2008) Isolation and preparation of chloroplasts from *Arabidopsis thaliana* plants. *Methods in Molecular Biology* 425: 171-86
- Kumar S, Dhingra A, Daniell H (2004) Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. *Plant Molecular Biology* 56:203-216
- Kumar N, Arthur CP, Ciferri C, Matsumoto ML (2020) Structure of the secretory immunoglobulin A core. *Science* 367:1008-14

- La Ragione R, Ahmed NM, Best A, Clifford D, Weyer U, Cooley W, Johnson L, Pearson G, Woodward MJ (2005) Colonization of 8-week-old conventionally reared goats by *Escherichia coli* O157: H7 after oral inoculation. *Journal of Medical Microbiology* 54:485-492
- La Ragione R, Best A, Clifford D, Weyer U, Johnson L, Marshall R, Marshall J, Cooley W, Farrelly S, Pearson G (2006) Influence of colostrum deprivation and concurrent *Cryptosporidium parvum* infection on the colonization and persistence of *Escherichia coli* O157: H7 in young lambs. *Journal of Medical Microbiology* 55:819-828
- Lagasse HA, Alexaki A, Simhadri VL, Katagiri NH, Jankowski W, Sauna ZE, Kimchi-Sarfaty C (2017) Recent advances in (therapeutic protein) drug development. *F1000Research* 6:113
- Lai H, He J, Hurtado J, Stahnke J, Fuchs A, Mehlhop E, Gorlatov S, Loos A, Diamond MS, Chen Q (2014) Structural and functional characterization of an anti-West Nile virus monoclonal antibody and its single-chain variant produced in glycoengineered plants. *Plant Biotechnology Journal* 12:1098-1107
- Lai LC, Wainwright LA, Stone KD, Donnenberg MS (1997) A third secreted protein that is encoded by the enteropathogenic *Escherichia coli* pathogenicity island is required for transduction of signals and for attaching and effacing activities in host cells. *Infection and Immunity* 65:2211-2217
- Lawrence MS, Phillips KJ, Liu DR (2007) Supercharging proteins can impart unusual resilience. *Journal of the American Chemical Society* 129:10110-10112
- Lee CC, Perchiacca JM, Tessier PM (2013) Toward aggregation-resistant antibodies by design. *Trends in Biotechnology* 31:612-620
- Lee DW, Lee J, Hwang IJ (2017) Sorting of nuclear-encoded chloroplast membrane proteins. *Current Opinion in Plant Biology* 40:1-7
- LeJeune JT, Besser TE, Hancock DD (2001) Cattle Water Troughs as Reservoirs of *Escherichia coli* O157. *Applied and Environmental Microbiology* 67:3053-3057
- Leuenberger P, Gansch S, Kahraman A, Cappelletti V, Boersema PJ, von Mering C, Claassen M, Picotti P (2017) Cell-wide analysis of protein thermal unfolding reveals determinants of thermostability. *Science* 355
- Li MZ, Elledge SJ (2007) Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. *Nature Methods* 4:251-256
- Li Y, Jin L, Chen T (2020) The Effects of Secretory IgA in the Mucosal Immune System. *BioMed Research International* DOI:10.1155/2020/2032057
- Liang X, Peng L, Li K, Peterson T, Katzen F (2012) A method for multi-site-directed mutagenesis based on homologous recombination. *Analytical Biochemistry* 427:99-101
- Lim JY, Li J, Sheng H, Besser TE, Potter K, Hovde CJ (2007) *Escherichia coli* O157: H7 colonization at the rectoanal junction of long-duration culture-positive cattle. *Applied and Environmental Microbiology* 73:1380-1382
- Liu H, Saxena A, Sidhu SS, Wu D (2017) Fc engineering for developing therapeutic bispecific antibodies and novel scaffolds. *Frontiers in Immunology* 8:38

- Lohse S, Derer S, Beyer T, Klausz K, Peipp M, Leusen JH, van de Winkel JG, Dechant M, Valerius T (2011) Recombinant dimeric IgA antibodies against the epidermal growth factor receptor mediate effective tumor cell killing. *Journal of Immunology* 186:3770-3778
- Louise CB, Tran MC, O'Brig TG (1997) Sensitization of human umbilical vein endothelial cells to Shiga toxin: involvement of protein kinase C and NF-kappaB. *Infection and Immunity* 65:3337-3344
- Lu RM, Hwang YC, Liu JJ, Lee CC, Tsai HZ, Li HJ, Wu HC (2020) Development of therapeutic antibodies for the treatment of diseases. *Journal of Biomedical Science* 27:1
- Ludwig DL, Witte L, Hicklin DJ, Prewett M, Bassi R, Burtrum D, Pereira DS, Jimenez X, Fox F, Saxena B (2004) Conservation of receptor antagonist anti-tumor activity by epidermal growth factor receptor antibody expressed in transgenic corn seed. *Human Antibodies* 13:81-90
- Ma JKC, Hikmat BY, Wycoff K, Vine ND, Chargelegue D, Yu L, Hein MB, Lehner T (1998) Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. *Nature Medicine* 4:601-606
- Ma JKC, Drossard J, Lewis D, Altmann F, Boyle J, Christou P, Cole T, Dale P, van Dolleweerd CJ, Isitt V (2015) Regulatory approval and a first-in-human phase I clinical trial of a monoclonal antibody produced in transgenic tobacco plants. *Plant Biotechnology Journal* 13:1106-1120
- Magee AM, Coyne S, Murphy D, Horvath EM, Medgyesy P, Kavanagh TA (2004) T7 RNA polymerase-directed expression of an antibody fragment transgene in plastids causes a semi-lethal pale-green seedling phenotype. *Transgenic Research* 13:325-337
- Majowicz SE, Scallan E, Jones-Bitton A, Sargeant JM, Stapleton J, Angulo FJ, Yeung DH, Kirk MD (2014a) Global incidence of human Shiga toxin-producing *Escherichia coli* infections and deaths: a systematic review and knowledge synthesis. *Foodborne Pathogens and Disease* 11:447-455
- Majowicz SE, Scallan E, Jones-Bitton A, Sargeant JM, Stapleton J, Angulo FJ, Yeung DH, Kirk MD (2014b) Global incidence of human Shiga toxin-producing *Escherichia coli* infections and deaths: a systematic review and knowledge synthesis. *Foodborne Pathogens and Disease* 11:447-455
- Makvandi-Nejad S, McLean MD, Hiramata T, Almquist KC, MacKenzie CR, Hall JC (2005) Transgenic tobacco plants expressing a dimeric single-chain variable fragment (scfv) antibody against *Salmonella enterica* serotype Paratyphi B. *Transgenic Research* 14:785-792
- Marusic C, Novelli F, Salzano AM, Scaloni A, Benvenuto E, Pioli C, Donini M (2016) Production of an active anti-CD20-hIL-2 immunocytokine in *Nicotiana benthamiana*. *Plant Biotechnology Journal* 14:240-251
- Mathusa EC, Chen Y, Enache E, Hontz L (2010) Non-O157 Shiga toxin-producing *Escherichia coli* in foods. *Journal of Food Protection* 73:1721-1736
- Matthews L, Reeve R, Gally DL, Low JC, Woolhouse ME, McAteer SP, Locking ME, Chase-Topping ME, Haydon DT, Allison LJ (2013) Predicting the public health

- benefit of vaccinating cattle against *Escherichia coli* O157. *Proceedings of the National Academy of Sciences of the United States of America* 110:16265-16270
- Mayfield SP, Franklin SE, Lerner RA (2003) Expression and assembly of a fully active antibody in algae. *Proceedings of the National Academy of Sciences of the United States of America* 100:438-442
- McCormick A, Reddy S, Reindl S, Cameron T, Czerwinski D, Vojdani F, Hanley K, Garger S, White E, Novak JJ (2008) Plant-produced idiotype vaccines for the treatment of non-Hodgkin's lymphoma: safety and immunogenicity in a phase I clinical study. *Proceedings of the National Academy of Sciences of the United States of America* 105:10131-10136
- Menassa R, Ahmad A, Joensuu JJ. Transient expression using agroinfiltration and its applications in molecular farming. In A. Wang and S. Ma (eds.), *Molecular farming in plants: recent advances and future prospects* pp 183-198. Springer, Berlin, Heidelberg, New York
- Montenegro MA, Bulte M, Trunpf T, Aleksic S, Reuter G, Bulling E, Helmuth R (1990a) Detection and characterization of fecal verotoxin-producing *Escherichia coli* from healthy cattle. *Journal of Clinical Microbiology* 28:1417-1421
- Montenegro MA, Bulte M, Trunpf T, Aleksic S, Reuter G, Bulling E, Helmuth R (1990b) Detection and characterization of fecal verotoxin-producing *Escherichia coli* from healthy cattle. *Journal of Clinical Microbiology* 28:1417-1421
- Montero-Morales L, Maresch D, Crescioli S, Castilho A, Ilieva KM, Mele S, Karagiannis SN, Altmann F, Steinkellner H (2019) In Planta Glycan Engineering and Functional Activities of IgE Antibodies. *Frontiers in Bioengineering and Technology* 7:242
- Moon H, Whipp S, Argenzio R, Levine M, Giannella R (1983) Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. *Infection and Immunity* 41:1340-1351
- Muyldermans S, Baral T, Retamozzo VC, De Baetselier P, De Genst E, Kinne J, Leonhardt H, Magez S, Nguyen V, Revets H (2009) Camelid immunoglobulins and nanobody technology. *Virology and Immunopathology* 128:178-183
- Nakanishi K, Narimatsu S, Ichikawa S, Tobisawa Y, Kurohane K, Niwa Y, Kobayashi H, Imai Y (2013) Production of hybrid-IgG/IgA plantibodies with neutralizing activity against Shiga toxin 1. *PloS One* 8:e80712
- Nandi S, Kwong AT, Holtz BR, Erwin RL, Marcel S, McDonald KA (2016) Techno-economic analysis of a transient plant-based platform for monoclonal antibody production. *mAbs* 8:1456-1466
- Nart P, Naylor SW, Huntley JF, McKendrick IJ, Gally DL, Low JC (2008) Responses of cattle to gastrointestinal colonization by *Escherichia coli* O157:H7. *Infection and Immunity* 76:5366-5372
- Naylor SW, Low JC, Besser TE, Mahajan A, Gunn GJ, Pearce MC, McKendrick IJ, Smith DG, Gally DL (2003) Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157: H7 in the bovine host. *Infection and Immunity* 71:1505-1512
- Ning L, He B, Zhou P, Derda R, Huang J (2019) Molecular design of peptide-Fc fusion drugs. *Current Drug Metabolism* 20:203-208

- Olsson MH, Sondergaard CR, Rostkowski M, Jensen JH (2011) PROPKA3: Consistent Treatment of Internal and Surface Residues in Empirical pKa Predictions. *Chemical Theory and Computation* 7:525-537
- Omisakin F, MacRae M, Ogden I, Strachan N (2003) Concentration and prevalence of *Escherichia coli* O157 in cattle feces at slaughter. *Applied and Environmental Microbiology* 69:2444-2447
- Onda Y (2013) Oxidative protein-folding systems in plant cells. *International Journal of Cell Biology*: 585431
- Pai C, Kelly J, Meyers G (1986) Experimental infection of infant rabbits with verotoxin-producing *Escherichia coli*. *Infection and Immunity* 51:16-23
- Palaci J, Viridi V, Depicker A (2019) Transformation strategies for stable expression of complex hetero-multimeric proteins like secretory immunoglobulin A in plants. *Plant Biotechnology Journal* 17:1760-1769
- Palmer T, Stansfeld P (2020) Targeting of proteins to the twin-arginine translocation pathway. *Molecular Microbiology* 113: 861-71
- Park HI, Yoon HW, Jung ST (2016) The highly evolvable antibody Fc domain. *Trends in Biotechnology* 34:895-908
- Pereira EO, Kolotilin I, Conley AJ, Menassa R (2014) Production and characterization of in planta transiently produced polygalacturanase from *Aspergillus niger* and its fusions with hydrophobin or ELP tags. *BMC Biotechnology* 14:59
- Perna NT, Mayhew GF, Pósfai G, Elliott S, Donnenberg MS, Kaper JB, Blattner FR (1998) Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157: H7. *Infection and Immunity* 66:3810-3817
- Phillips A, Navabpour S, Hicks S, Dougan G, Wallis T, Frankel G (2000) Enterohaemorrhagic *Escherichia coli* O157: H7 target Peyer's patches in humans and cause attaching/effacing lesions in both human and bovine intestine. *Gut* 47:377-381
- Pogue GP, Vojdani F, Palmer KE, Hiatt E, Hume S, Phelps J, Long L, Bohorova N, Kim D, Pauly M (2010) Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant-based transient expression systems. *Plant Biotechnology Journal* 8:638-654
- Pottosin I, Shabala S (2016) Transport across chloroplast membranes: optimizing photosynthesis for adverse environmental conditions. *Molecular Plant* 9:356-370
- Pruimboom-Brees IM, Morgan TW, Ackermann MR, Nystrom ED, Samuel JE, Cornick NA, Moon HW (2000) Cattle lack vascular receptors for *Escherichia coli* O157: H7 Shiga toxins. *Proceedings of the National Academy of Sciences of the United States of America* 97:10325-10329
- Public Health Agency of Canada (2015) Foodnet Canada Short Report 2015.
- Qiu X, Wong G, Audet J, Bello A, Fernando L, Alimonti JB, Fausther-Bovendo H, Wei H, Aviles J, Hiatt E (2014) Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. *Nature* 514:47-53
- Rademacher T, Sack M, Arcalis E, Stadlmann J, Balzer S, Altmann F, Quendler H, Stiegler G, Kunert R, Fischer R, Stoger E (2008) Recombinant antibody 2G12 produced in

- maize endosperm efficiently neutralizes HIV-1 and contains predominantly single-GlcNAc N-glycans. *Plant Biotechnology Journal* 6:189-201
- Rangel JM, Sparling PH, Crowe C, Griffin PM, Swerdlow DL (2005) Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. *Emerging Infectious Diseases* 11:603-609
- Reusch D, Tejada ML (2015) Fc glycans of therapeutic antibodies as critical quality attributes. *Glycobiology* 25:1325-1334
- Ries F, Herkt C, Willmund F (2020) Co-Translational Protein Folding and Sorting in Chloroplasts. *Plants* 9:214
- Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, Hebert RJ, Olcott ES, Johnson LM, Hargrett NT (1983) Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *New England Journal of Medicine* 308:681-685
- Robert S, Khalf M, Goulet MC, D'Aoust MA, Sainsbury F, Michaud D (2013) Protection of recombinant mammalian antibodies from development-dependent proteolysis in leaves of *Nicotiana benthamiana*. *PloS One* 8:e70203
- Robinson C, Matos CF, Beck D, Ren C, Lawrence J, Vasisht N, Mendel S (2011) Transport and proofreading of proteins by the twin-arginine translocation (Tat) system in bacteria. *Biochimica et Biophysica Acta* 1808:876-884
- Rodríguez M, Ramírez NI, Ayala M, Freyre F, Pérez L, Triguero A, Mateo C, Selman-Housein G, Gavilondo JV, Pujol M (2005) Transient expression in tobacco leaves of an aglycosylated recombinant antibody against the epidermal growth factor receptor. *Biotechnology and Bioengineering* 89:188-194
- Rybicki EP (2010) Plant-made vaccines for humans and animals. *Plant Biotechnology Journal* 8:620-637
- Sabalza M, Christou P, Capell T (2014) Recombinant plant-derived pharmaceutical proteins: current technical and economic bottlenecks. *Biotechnology Letters* 36:2367-2379
- Saberianfar R, Chin-Fatt A, Scott A, Henry KA, Topp E, Menassa R (2019) Plant-Produced Chimeric VHH-sIgA Against Enterohemorrhagic *E. coli* Intimin Shows Cross-Serotype Inhibition of Bacterial Adhesion to Epithelial Cells. *Frontiers in Plant Science* 10:270
- Sack M, Hofbauer A, Fischer R, Stoger E (2015) The increasing value of plant-made proteins. *Current Opinion in Biotechnology* 32:163-170
- Sack M, Paetz A, Kunert R, Bomble M, Hesse F, Stiegler G, Fischer R, Katinger H, Stoeger E, Rademacher TJ (2007) Functional analysis of the broadly neutralizing human anti-HIV-1 antibody 2F5 produced in transgenic BY-2 suspension cultures. *The FASEB Journal* 21:1655-1664
- Safarnejad MR, Fischer R, Commandeur U (2008) Generation and characterization of functional recombinant antibody fragments against tomato yellow leaf curl virus replication-associated protein. *Communications in Agricultural and Applied Biological Sciences* 73:311-321
- Sainsbury F, Lomonosoff GP (2008) Extremely high-level and rapid transient protein production in plants without the use of viral replication. *Plant Physiology* 148:1212-1218

- Sainsbury F, Sack M, Stadlmann J, Quendler H, Fischer R, Lomonossoff GP (2010) Rapid transient production in plants by replicating and non-replicating vectors yields high quality functional anti-HIV antibody. *PLoS One* 5: e13976
- Sainsbury F, Thuenemann EC, Lomonossoff GP (2009) pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnology Journal* 7:682-693
- Sanderson MW, Sargeant JM, Shi X, Nagaraja T, Zurek L, Alam M (2006) Longitudinal emergence and distribution of *Escherichia coli* O157 genotypes in a beef feedlot. *Applied and Environmental Microbiology* 72:7614-7619
- Sargeant J, Amezcua M, Rajic A, Waddell L (2007) Pre-harvest interventions to reduce the shedding of *E. coli* O157 in the faeces of weaned domestic ruminants: a systematic review. *Zoonoses and Public Health* 54:260-277
- Schillberg S, Raven N, Spiegel H, Rasche S, Buntru M (2019) Critical Analysis of the Commercial Potential of Plants for the Production of Recombinant Proteins. *Frontiers in Plant Science* 10:720
- Schlischer T, Soll J (1996) Molecular chaperones are present in the thylakoid lumen of pea chloroplasts. *FEBS Letters* 5:302-4
- Schmidt DJ, Beamer G, Tremblay JM, Steele JA, Kim HB, Wang Y, Debatis M, Sun X, Kashentseva EA, Dmitriev IP (2016) A tetraspecific VHH-based neutralizing antibody modifies disease outcome in three animal models of *Clostridium difficile* infection. *Clinical Vaccine Immunology* 23:774-784
- Schrodinger LLC (2010) The PyMOL molecular graphics system. Version, 1(5), 0.
- Schubert M, Petersson UA, Haas BJ, Funk C, Schroder WP, Kieselbach T (2002) Proteome map of the chloroplast lumen of *Arabidopsis thaliana*. *Journal of Biological Chemistry* 277:8354-8365
- Schymkowitz J, Borg J, Stricher F, Nys R, Rousseau F, Serrano L (2005) The FoldX web server: an online force field. *Nucleic Acids Research*. 33:W382-8.
- Shaaltiel Y, Gingis-Velitski S, Tzaban S, Fiks N, Tekoah Y, Aviezer D (2015) Plant-based oral delivery of beta-glucocerebrosidase as an enzyme replacement therapy for Gaucher's disease. *Plant Biotechnology Journal* 13:1033-1040
- Shkoporov A, Khokhlova E, Savochkin K, Kafarskaia L, Efimov B (2015) Production of biologically active scFv and VHH antibody fragments in *Bifidobacterium longum*. *FEMS Microbiology Letters* 362: fmv083
- Silhavy D, Molnar A, Lucioli A, Szittyá G, Hornyik C, Tavazza M, Burgyan J (2002) A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. *The EMBO Journal* 21:3070-3080
- Slutsker L, Ries AA, Greene KD, Wells JG, Hutwagner L, Griffin PM (1997) *Escherichia coli* O157: H7 diarrhea in the United States: clinical and epidemiologic features. *Annals of Internal Medicine* 126:505-513
- Smith DR, Moxley RA, Klopfenstein TJ, Erickson GE (2009a) A randomized longitudinal trial to test the effect of regional vaccination within a cattle feedyard on *Escherichia coli* O157: H7 rectal colonization, fecal shedding, and hide contamination. *Foodborne Pathogens and Disease* 6:885-892

- Smith DR, Moxley RA, Peterson RE, Klopfenstein TJ, Erickson GE, Bretschneider G, Berberov EM, Clowser S (2009b) A two-dose regimen of a vaccine against type III secreted proteins reduced *Escherichia coli* O157: H7 colonization of the terminal rectum in beef cattle in commercial feedlots. *Foodborne Pathogens and Disease* 6:155-161
- Snedeker K, Campbell M, Sargeant J (2012) A systematic review of vaccinations to reduce the shedding of *Escherichia coli* O157 in the faeces of domestic ruminants. *Zoonoses and Public Health* 59:126-138
- Stanford K, Stephens TP, McAllister TA (2011) Use of model super-shedders to define the role of pen floor and hide contamination in the transmission of *Escherichia coli* O157:H7. *Journal of Animal Science* 89:237-244
- Staub JM, Garcia B, Graves J, Hajdukiewicz PT, Hunter P, Nehra N, Paradkar V, Schlittler M, Carroll JA, Spatola L, Ward D, Ye G, Russell DA (2000) High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nature Biotechnology* 18:333-338
- Stephens TP, McAllister TA, Stanford K (2009) Perineal swabs reveal effect of super shedders on the transmission of *Escherichia coli* O157:H7 in commercial feedlots. *Journal of Animal Science* 87:4151-4160
- Stevens LH, Stoop GM, Elbers IJ, Molthoff JW, Bakker HA, Lommen A, Bosch D, Jordi W (2000) Effect of climate conditions and plant developmental stage on the stability of antibodies expressed in transgenic tobacco. *Journal of Plant Physiology* 124:173-182
- Strachan NJ, Doyle MP, Kasuga F, Rotariu O, Ogden ID (2005) Dose response modelling of *Escherichia coli* O157 incorporating data from foodborne and environmental outbreaks. *International Journal of Food Microbiology* 103:35-47
- Strasser R (2016) Plant protein glycosylation. *Journal of Glycobiology* 26:926-939
- Strasser R, Castilho A, Stadlmann J, Kunert R, Quendler H, Gattinger P, Jez J, Rademacher T, Altmann F, Mach L (2009) Improved virus neutralization by plant-produced anti-HIV antibodies with a homogeneous β 1, 4-galactosylated N-glycan profile. *Journal of Biological Chemistry* 284:20479-20485
- Streatfield SJ. Approaches to achieve high-level heterologous protein production in plants (2007) *Plant Biotechnology Journal* 5:2-15
- Tissot G, Canard H, Nadai M, Martone A, Botterman J, Dubald M (2008) Translocation of aprotinin, a therapeutic protease inhibitor, into the thylakoid lumen of genetically engineered tobacco chloroplasts. *Plant Biotechnology Journal* 6:309-320
- Tobe T, Beatson SA, Taniguchi H, Abe H, Bailey CM, Fivian A, Younis R, Matthews S, Marches O, Frankel G (2006) An extensive repertoire of type III secretion effectors in *Escherichia coli* O157 and the role of lambdoid phages in their dissemination. *Proceedings of the National Academy of Sciences of the United States of America* 103:14941-14946
- Tokuhara D, Álvarez B, Mejima M, Hiroiwa T, Takahashi Y, Kurokawa S, Kuroda M, Oyama M, Kozuka-Hata H, Nochi T (2013) Rice-based oral antibody fragment prophylaxis and therapy against rotavirus infection. *Journal of Clinical Investigation* 123:3829-3838

- Tokuriki N, Stricher F, Schymkowitz J, Serrano L, Tawfik DS (2007) The stability effects of protein mutations appear to be universally distributed. *Journal of Molecular Biology* 369:1318-1332
- Topp E, Irwin R, McAllister T, Lessard M, Joensuu JJ, Kolotilin I, Conrad U, Stöger E, Mor T, Warzecha H. et al. (2016) The case for plant-made veterinary immunotherapeutics. *Biotechnology Advances* 34:597-604
- Trebitsh T, Meiri E, Ostersetzer O, Adam Z, Danon A (2001) The protein disulfide isomerase-like RB60 is partitioned between stroma and thylakoids in *Chlamydomonas reinhardtii* chloroplasts. *Journal of Biological Chemistry* 276:4564-4569
- Tuttle J, Gomez T, Doyle M, Wells J, Zhao T, Tauxe R, Griffin P (1999) Lessons from a large outbreak of *Escherichia coli* O157: H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiology and Infection* 122:185
- Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. *Trends in Biotechnology* 21:570-578
- Tzipori S, Gibson R, Montanaro J (1989) Nature and distribution of mucosal lesions associated with enteropathogenic and enterohemorrhagic *Escherichia coli* in piglets and the role of plasmid-mediated factors. *Infection and Immunity* 57:1142-1150
- Tzipori S, Gunzer F, Donnenberg MS, de Montigny L, Kaper JB, Donohue-Rolfe A (1995) The role of the *eaeA* gene in diarrhea and neurological complications in a gnotobiotic piglet model of enterohemorrhagic *Escherichia coli* infection. *Infection and Immunity* 63:3621-3627
- Unicef (2010) Diarrhoea: why children are still dying and what can be done. Final Diarrhoea Report - October 2009
- Vanmarsenille C, Elseviers J, Yvanoff C, Hassanzadeh-Ghassabeh G, Garcia Rodriguez G, Martens E, Depicker A, Martel A, Haesebrouck F, Pasmans F, Hernalsteens JP, De Greve H (2018) In planta expression of nanobody-based designer chicken antibodies targeting *Campylobacter*. *PloS One* 13:e0204222
- Vasilev N, Smales CM, Schillberg S, Fischer R, Schiermeyer A (2016) Developments in the production of mucosal antibodies in plants. *Biotechnology Advances* 34:77-87
- Vega CG, Bok M, Vlasova AN, Chattha KS, Gómez-Sebastián S, Nuñez C, Alvarado C, Lasa R, Escribano JM, Garaicoechea LL (2013) Recombinant monovalent llama-derived antibody fragments (VHH) to rotavirus VP6 protect neonatal gnotobiotic piglets against human rotavirus-induced diarrhea. *PLoS Pathogens* 9: e1003334
- Verch T, Yusibov V, Koprowski H (1998) Expression and assembly of a full-length monoclonal antibody in plants using a plant virus vector. *Immunological Methods* 220:69-75
- Verdino P, Atwell S, Demarest SJ (2018) Emerging trends in bispecific antibody and scaffold protein therapeutics. *Current Opinion in Chemical Engineering* 19:107-123

- Vial PA, Robins-Browne R, Lior H, Prado V, Kaper JB, Nataro JP, Maneval D, Levine MM (1988) Characterization of enteroadherent-aggregative *Escherichia coli*, a putative agent of diarrheal disease. *Infectious Diseases* 158:70-79
- Villani ME, Morgun B, Brunetti P, Marusic C, Lombardi R, Pisoni I, Bacci C, Desiderio A, Benvenuto E, Donini M (2009) Plant pharming of a full-sized, tumour-targeting antibody using different expression strategies. *Plant Biotechnology Journal* 7:59-72
- Vincke C, Muyldermans S (2012) Introduction to heavy chain antibodies and derived Nanobodies. *Methods Molecular Biology* 911:15-26
- Virdi V, Coddens A, De Buck S, Millet S, Goddeeris BM, Cox E, De Greve H, Depicker A (2013) Orally fed seeds producing designer IgAs protect weaned piglets against enterotoxigenic *Escherichia coli* infection. *Proceedings of the National Academy of Sciences of the United States of America* 110:11809-11814
- Virdi V, Palaci J, Laukens B, Ryckaert S, Cox E, Vanderbeke E, Depicker A, Callewaert N (2019) Yeast-secreted, dried and food-admixed monomeric IgA prevents gastrointestinal infection in a piglet model. *Nature Biotechnology* 37:527-530
- Wagner PL, Waldor MK (2002) Bacteriophage control of bacterial virulence. *Infection and Immunity* 70:3985-3993
- Walsh G (2006) Biopharmaceutical benchmarks 2006. *Nature Biotechnology* 24:769-776
- Walther DM, Kasturi P, Zheng M, Pinkert S, Vecchi G, Ciryam P, Morimoto RI, Dobson CM, Vendruscolo M, Mann M, Hartl FU (2015) Widespread Proteome Remodeling and Aggregation in Aging *C. elegans*. *Cell* 161:919-932
- Warawa J, Finlay BB, Kenny B (1999) Type III secretion-dependent hemolytic activity of enteropathogenic *Escherichia coli*. *Journal of Infection and Immunity* 67:5538-5540
- Weintraub JA, Hilton JF, White JM, Hoover CI, Wycoff KL, Yu L, Larrick JW, Featherstone JD (2005) Clinical trial of a plant-derived antibody on recolonization of mutans streptococci. *Caries Research* 39:241-250
- Wendel AM, Johnson DH, Sharapov U, Grant J, Archer JR, Monson T, Koschmann C, Davis JP (2009) Multistate outbreak of *Escherichia coli* O157: H7 infection associated with consumption of packaged spinach, August–September 2006: the Wisconsin investigation. *Clinical Infectious Diseases* 48:1079-1086
- Wesolowski J, Alzogaray V, Reyelt J, Unger M, Juarez K, Urrutia M, Cauherff A, Danquah W, Rissiek B, Scheuplein F, Schwarz N, Adriouch S, Boyer O, Seman M, Licea A, Serreze DV, Goldbaum FA, Haag F, Koch-Nolte F (2009) Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. *Medical Microbiology and Immunology* 198:157-174
- Wheeler T, Kalchayanand N, Bosilevac JM (2014) Pre-and post-harvest interventions to reduce pathogen contamination in the US beef industry. *Meat Science* 98:372-382
- Williams MS, Withee JL, Ebel ED, Bauer Jr NE, Schlosser WD, Disney WT, Smith DR, Moxley RA (2010) Determining relationships between the seasonal occurrence of *Escherichia coli* O157: H7 in live cattle, ground beef, and humans. *Foodborne Pathogens and Disease* 7:1247-1254

- Wong AR, Pearson JS, Bright MD, Munera D, Robinson KS, Lee SF, Frankel G, Hartland EL (2011) Enteropathogenic and enterohaemorrhagic *Escherichia coli*: even more subversive elements. *Molecular Microbiology* 80:1420-1438
- Wong MS, Wu S, Causey TB, Bennett GN, San K-Y (2008) Reduction of acetate accumulation in *Escherichia coli* cultures for increased recombinant protein production. *Medical Engineering* 10:97-108
- Woodward MJ, Best A, Sprigings KA, Pearson GR, Skuse AM, Wales A, Hayes CM, Roe JM, Low JC, La Ragione RM (2003) Non-toxicogenic *Escherichia coli* O157: H7 strain NCTC12900 causes attaching-effacing lesions and *eae*-dependent persistence in weaned sheep. *International Journal of Medical Microbiology* 293:299-308
- Woof J, Russell M (2011) Structure and function relationships in IgA. *Mucosal Immunology* 4:590-597
- Wu S, Zhang Y (2008) MUSTER: Improving protein sequence profile-profile alignments by using multiple sources of structure information. *Proteins* 72:547-556
- Wurm FM (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. *Nature Biotechnology* 22:1393-1398
- Wycoff KL (2005) Secretory IgA antibodies from plants. *Current Pharmaceutical Design* 1:2429-37
- Xiong Y, Karuppanan K, Bernardi A, Li Q, Kommineni V, Dandekar AM, Lebrilla CB, Faller R, McDonald KA, Nandi S (2019) Effects of N-Glycosylation on the Structure, Function, and Stability of a Plant-Made Fc-Fusion Anthrax Decoy Protein. *Frontiers in Plant Science* 10:768
- Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y (2015) The I-TASSER Suite: protein structure and function prediction. *Nature Methods* 12:7-8
- Yano A, Maeda F, Takekoshi M (2004) Transgenic tobacco cells producing the human monoclonal antibody to hepatitis B virus surface antigen. *Medical Virology* 73:208-215
- Zabetakis D, Olson MA, Anderson GP, Legler PM, Goldman ER (2014) Evaluation of disulfide bond position to enhance the thermal stability of a highly stable single domain antibody. *PLoS One* 9:e115405
- Zeitlin L, Bohorov O, Bohorova N, Hiatt A, Kim D, Pauly M, Velasco J, Whaley K, Barnard D, Bates J (2013) Prophylactic and therapeutic testing of *Nicotiana*-derived RSV-neutralizing human monoclonal antibodies in the cotton rat model. *mAbs* 5:263-269
- Zeitlin L, Olmsted SS, Moench TR, Co MS, Martinell BJ, Paradkar VM, Russell DR, Queen C, Cone RA, Whaley KJ (1998) A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. *Nature Biotechnology* 16:1361-1364
- Zhang Y (2008) I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics* 9:40

7.0 APPENDICES

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8.0 CURRICULUM VITAE

Adam Chin-Fatt

Protein Engineer

Summary

- Project interests: Biobetter development, vaccine & antibody engineering, drug discovery
- Main skillset: Rational design, protein modelling, protein production, cell culture, synthetic biology, microscopy, functional genomics, biophysical characterization
- 12 years of lab experience in molecular biology

Education

Ph.D. candidate, Biology, Western University (2015-current)

M.Sc., Plant Science, University of Manitoba (2011-2014)

Honors B.Sc., Genetics and Biotechnology, University of Toronto (2005-2010)

Laboratory Experience

Production of a plant-based synthetic secretory immunoglobulin A against Enterohemorrhagic *E. coli* (EHEC). Menassa Lab, University of Western Ontario. (2015-current)

Rational design of a bovine IgA Fc for improved antibody yield against EHEC. Menassa Lab, University of Western Ontario. (2015-current)

Chaperone coexpression for improving yield of a chloroplast targeted subunit vaccine against EHEC. Menassa Lab, University of Western Ontario. Ph.D. thesis (2015-current)

Production and rational design of a chloroplast targeted subunit vaccine against EHEC; Menassa Lab, University of Western Ontario. (2015-current)

Linkage mapping and quantitative trait loci identification of cyanogenic glucosides in flax; Cloutier Lab, University of Manitoba. (2011-2014)

Development of a near infrared spectroscopic method for cyanogenic glucoside quantification in flaxseed; Cloutier Lab, University of Manitoba. (2011-2014)

Characterization of an EMS-induced calcium channel mutant's defense response in plant-pathogen interactions; Yoshioka Lab, University of Toronto. (2010)

Publications

Saberianfar R, Chin-Fatt A, Scott A, Henry KA, Topp E, Menassa R (2019) Plant-Produced Chimeric VHH-sIgA Against Enterohemorrhagic *E. coli* Intimin Shows Cross-Serotype Inhibition of Bacterial Adhesion to Epithelial Cells. *Frontiers in Plant Science* 10:270

- MacDonald J, Miletic S, Gaidry T, Chin-Fatt A, Menassa R (2017) Co-expression with the Type 3 Secretion Chaperone CesT from Enterohemorrhagic *E. coli* Increases Accumulation of Recombinant Tir in Plant Chloroplasts. *Frontiers in plant science* 8:283
- Chin-Fatt A, Topp E, Menassa R (2018) The Benefit of a Plant-Based Cattle Vaccine for Reducing Enterohemorrhagic *Escherichia Coli* Shedding and Improving Food Safety. In: MacDonald J (ed) *Prospects of Plant-Based Vaccines in Veterinary Medicine*. Springer International Publishing, Cham, pp 285-310
- Oncoming: A rationally designed bovine IgA Fc scaffold for enhanced in planta accumulation of a VHH-Fc fusion that binds enterohemorrhagic *E. coli*.
- Oncoming: A VHH-Fc fusion targeted to the thylakoid lumen binds and neutralizes Enterohemorrhagic *E. coli* O157:H7.
- Oncoming: Identification of QTL associated with the accumulation of linustatin and neolinustatin in the mature flax seed.

Awards

Travel awards

- George H. Duff travel award. By The Canadian Society of Plant Biologists, For conference in Kingston, Ontario. July 2016
- Graduate travel award. By Biology Graduate Education Committee, Western University. For conference in Gent, Belgium. June 2016.
- Travel bursary. International Society for Plant Molecular Farming. Conference For conference in Gent, Belgium. June 2016.
- Protein Science Travel Award. By John Wiley & Sons Inc. For conference in Boston, Massachusetts. July 2018.
- Travel Award. By Canadian Association of Plant Biotechnology. For conference in Guelph, Ontario. July 2019
- Best presentation. By Canadian Association of Plant Biotechnology. At Plant Biotech 2016 Conference. Kingston, Ontario. July 2016
- Best presentation. By Society of Biology Graduate Students. At Biology Graduate Research Forum, Western University. London, Ontario. October 2019
- Best poster. By Canadian Association of Plant Biotechnology. At Plant Canada conference. Guelph, Ontario. July 2019