A Novel Lactic Acid Bacteria (LAB)-based Vaccine Candidate for Human Norovirus

Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

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The Ohio State University
2018

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Abstract

Human noroviruses (HuNoVs) are responsible for more than 95% of the non-bacterial acute gastroenteritis epidemics in the world. The CDC estimates that every year 21 million individuals suffer from HuNoV-induced gastroenteritis in the U.S. Currently, there is no FDA-approved vaccine for HuNoVs. Development of an effective vaccine has been seriously hampered by the lack of an efficient cell culture system for HuNoVs and a suitable small animal model. The goal of this study is to develop lactic acid bacteria (LAB) as a vector to deliver HuNoV antigen. To do this, a LAB bacteria strain (*Lactococcus lactis*) carrying VP1 gene of a HuNoV GII.4 virus (LAB-VP1) was constructed. It was found that HuNoV VP1 protein was highly expressed by LAB vector. Subsequently, a novel microencapsulation technology was developed to enhance the stability of LABs in low and high pH environments. To test whether LAB-based HuNoV vaccine is immunogenic, 4-day-old gnotobiotic piglets were orally inoculated with various doses of LAB-VP1 either with or without microencapsulation. It was found that LABs were persistent in the small intestine of piglets and shed in pig feces for at least 25 days post inoculation. Live LABs or LAB DNA were found in mesenteric lymph nodes and spleen tissue in LAB-VP1 inoculated groups. HuNoV-specific IgG and IgA were detectable in serum and feces at day 13 post-inoculation, respectively, and further increased at late time points. After challenge with HuNoV GII.4 strain, a large amount of HuNoV antigens were observed in the duodenum, jejunum, and ileum sections of the intestine in the LAB control group. In contrast, significantly less or no HuNoV antigens were detected in the LAB-VP1 immunized groups. Collectively, these

results demonstrate that LAB-based HuNoV vaccine induces protective immunity in gnotobiotic piglets.

Acknowledgments

I would like to thank you those who helped mentor me through graduate school. I would like to give my gratitude to my advisor Dr. Jianrong Li, for the guidance and support he provided throughout my graduate school. Additionally, the Li lab members including Dr. Niraj Makadiya, Dr. Mijia Lu, Miaoge Xue, Anzhong Li, Dr. Xueya Liang, Dr. Yang Yu, Yilong Wang and Juan Zhang provided mentorship through my research and I would like to express my gratitude. I would like to thank Dr. Lucia Rosas for the guidance with the gnotobiotic piglets as she provided exceptional advice. I would like to thank Dr. Andrew Niehaus for performing the surgery for the gnotobiotic piglets and Dr. Ryan Jennings for help with the necropsy of the piglets. I would like to thank Dr. Rachael Rayner, Lauren Doolittle and Dr. Wessel Dirksen for the helpful knowledge and support during my research. My committee including Dr. Melvin Pascall and Dr. Prosper Boyaka have provided their time and wisdom to help with my thesis and I would like to thank them. Additionally, I would like to thank my family and friends for their love and support through the process of graduate school.

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Abbreviations

CDC – Centers for Disease Control and Prevention

CFU – Colony forming unit

CPE- cytopathic effect

ELISA – Enzyme-linked immunosorbant assay

FCV – Feline calicivirus

FDA – Food and Drug Administration

FUT2- fucosyltransferase 2

GI – Gastrointestinal

HBGA – Histo-blood group antigen

IgA- immunoglobulin A

IgG- immunoglobulin G

IN- intranasal

NICE- Nisin Controlled Expression

IM- intramuscular

LAB- lactic acid bacteria

LN- lymph nodes

NDV- Newcastle Disease virus

HuNoV – Human norovirus

MNV- Murine Norovirus

MP- microparticles

NIAID – National institute of Allergy and Infectious Diseases

NoV – Norovirus

ORF – Open reading frame

RdRp – RNA-dependent RNA polymerase

RNA – Ribonucleic acid

SDS-PAGE – Sodium dodecyl sulfate- polyacrylamide gel electrophoresis

IFA- Immunofluorescence assay

UTR- untranslated region

VEE- Venezuelan equine encephalitis

VLP – Virus-like particle

VP1- viral protein 1

VP2- viral protein 2

VRP- VEE replicon particles

VSV- Vesicular stomatitis virus

WHO- World Health Organization

CHAPTER 1

LITERATURE REVIEW

1.1. Human norovirus and foodborne illnesses

Foodborne illnesses are defined as infections of the gastrointestinal tract caused by digestion of food contaminated with pathogenic viruses, bacteria, parasites, fungi, prions, or chemicals (Kirk et al., 2015). Currently, there are more than 250 different foodborne diseases and new diseases are continuing to be found. The typical symptoms of gastroenteritis caused by foodborne diseases include nausea, vomiting, stomach aches, diarrhea, and malaise (CDC, 2018). The populations most at risk for foodborne disease include elderly, young children, immunocompromised, and pregnant people (Havelaar et al., 2016). HuNoV causes acute gastroenteritis symptoms including nausea, abdominal cramps, vomiting, diarrhea, low grade fever, myalgia and malaise (Atmar et al., 2008).

The World Health Organization (WHO) listed HuNoV as the leading cause of global foodborne illnesses with 658 million global cases per year. In addition, HuNoV is the leading cause of death among foodborne diseases (Kirk et al., 2015). HuNoV accounts for 58% of foodborne gastroenteritis (Scallan et al., 2011). Diseases causing gastroenteritis/diarrhea are one of the leading causes of death in developing countries with HuNoV as a major contributor

(S. M. Ahmed et al., 2014). The majority of deaths from diarrhea diseases occur in developing countries (Patel et al., 2008).

The outbreaks of HuNoV occur in areas with high densities of people, such as schools, restaurants, nursing homes, and cruise ships. There is a significantly lower risk of transmission through healthcare facilities, while transmission through food and water contamination has a significantly higher risk of causing outbreaks (Matthews et al., 2012). The primary spread of HuNoV is through person-to-person contact via fecal-oral routes (Hall, Glass, & Parashar, 2016). Direct contact with fecal matter or aerosolized vomitus can cause infection in addition to contaminated food, water and fomite transmission (contaminated surfaces). HuNoV is highly stable in the environment to allow for increased risk of contamination (Debbink, Lindesmith, & Baric, 2014). Another factor that increases the risk of infection is the low infectious dose, which can be as low as under 10 particles (Estes, Prasad, & Atmar, 2006).

1.2. Virology of human norovirus

Human norovirus, previously called Norwalk-like virus, was discovered in 1968 in Norwalk, OH. The students and teachers at Bronson Elementary School developed acute gastroenteritis and the Norwalk agent was found as the pathogen. Virus particles ranging from 27-32nm in size were observed by the immune electron microscopy technique (Kapikian et al., 1972). HuNoV has also been referred to as the small round structured viruses, winter vomit

bug, and the stomach flu. Since the discovery of HuNoV, it is now recognized as the most prominent non-bacterial cause of gastroenteritis (Glass, Parashar, & Estes, 2009). In hospitals, HuNoV is the leading cause of gastroenteritis and the leading cause of death from acute gastroenteritis (Hall et al., 2016). It causes on average 21 million cases in the U.S and makes up 18% of all gastroenteritis cases in the world (S. M. Ahmed et al., 2014; Glass et al., 2009; Pringle et al., 2015; Scallan et al., 2011; Vinjé, 2015). The average incubation period for HuNoV is 1-2 days then gastroenteritis symptoms typically last for 12-60 hours (R. M. Lee et al., 2013). Common symptoms include diarrhea which occurs in 87% of HuNoV infected individuals, abdominal pain in 51% and vomiting occurs in 74%. The duration of gastroenteritis symptoms is longer in children and can last for 5-6 days (Rockx et al., 2002).

The HuNoV capsid protein has distinct cup shaped depressions that lead to the family name *Caliciviridae*, derived from the Greek word calyx meaning cup (Glass et al., 2009). HuNoV is a member of the *Norovirus* genus within the *Caliciviridae* family. *Sapovirus, Vesivirus, Lagovirus* , and *Recovirus* are the other four genera included in the *Caliciviridae* family. HuNoV are nonenveloped with icosahedral shaped capsid and have a single-stranded positive-sense RNA genome that is approximately 7.7kb in length (Karst, 2010).

HuNoV is highly diverse, both genetically and antigenically. HuNoV can be classified into 7 genogroups (GI to VII), and each genogroup can further be divided into different genotypes. To date, at least 40 different genotypes have been identified. Genogroup GI and GII have 9 and 22 genotypes, respectively. Noroviruses can infect humans, cattle, swine, and mice. The GI, GII

and GIV genogroups infect humans and 90% of infections include GII. The GII strains including GII.11, GII.18, and GII.19 infect swine, while the 16 other genotypes infect humans (Debbink et al., 2014; Q.-H. Wang et al., 2005). The GIII genogroup infects cattle and GV infects mice (Karst, 2010; Mattison et al., 2007). Dogs can be a carrier for HuNoV and antibodies have been detected in dogs for GII.4, GIV.1, GIV.2, and GVI.2 strains (Di Martino et al., 2017). The genotype GII.4 is the most common human genotype including 70% of infections (Debbink et al., 2014). GII.4 has six subclusters including the Camberwell cluster that was circulating from 1987 to 1995, the Grimsby cluster circulating from 1995 to 2002, the Farmington Hills cluster circulating from 2002 to 2004, the Hunter cluster circulating from 2004 to 2006, and the Sakai cluster circulating 2004-2006. Then, there is the Minerva cluster for isolates found after 2006. The GII.4 genotype evolved in the 1980s making this strain more recent than others (Donaldson, Lindesmith, Lobue, & Baric, 2008).

These mutation rates allow for there to be new strains of HuNoV every year and GI has a mean evolutionary rate of 1.26×10^{-3} substitutions/site/year however, the rates of the different GI genotypes are highly variable. The evolutionary rate of the GI and GII capsid genes are both similar and quite rapid (Kobayashi et al., 2015). The different genogroups of HuNoV have high genetic divergence leading to the variety of hosts HuNoV is able to infect. There is about a 20-40% nucleotide difference between the genotypes whereas within a genogroup there is over a 40% difference between different genogroups of HuNoV (Bok et al., 2009). The homology of different subclusters within a genotype is usually high (around 2% nucleotide difference) . There are new outbreaks from a newly evolved strain every 1-2 years, which is a similar pattern

to influenza virus (L. C. Lindesmith et al., 2008). Outbreaks of GI strains are commonly found in contaminated water while GII outbreaks are commonly in healthcare facilities, food and other environmental sources (Scallan et al., 2011). The GII genogroup is highly specific to infecting humans due to natural selection. The GII.4 strain has been the most prevalent worldwide (Kobayashi et al., 2016; Siebenga et al., 2009). HuNoV strains are given nomenclature based on the strains genogroup/genotype, host species, country, year, city of the first outbreak and the assigned serial number such as norovirus GII/Hu/FR/2004/GII.12/Paris25 (NIPHE, 2013).

The increase in HuNoV reported cases since the 2000s is related with the rise of the GII.4 variant. There are mutations as a result of selective pressure on the virus as a result of the host's immune system to bring out multiple variants of GII.4. The surface proteins of HuNoV are evolving at a higher rate due to the pressure of the immune system. The mutations are located in the carbohydrate-binding domains and drifts in the P2 subdomain receptor binding regions (L. C. Lindesmith et al., 2008). The evolutionary mutations the virus acquires build up over time but then the strains revert back to the old strains structures. The GII.3 was the predominant variant before 1991 then in 1995-6 the GII.4 variant started to increase. There are several variants of GII.4 strains in the 2000s that cause epidemics repeatedly. This is due to the immune system's lack of ability to protect people from further infections (Siebenga et al., 2010). The HBGA binding sites for the HuNoV have remained unchanged since the discovery in 1970s (Bok et al., 2009).

1.3. Epidemiology of human norovirus

HuNoV affects people of all ages and infects 6.5-10% of the U.S. population (Scallan et al., 2011). Among those infected, 10% will seek medical attention and HuNoV causes 797 deaths per year in the U.S. (Hall et al., 2016; Rockx et al., 2002). In addition, the diagnostic tests for HuNoV were not widely available until 1998 and 35% of healthcare professionals do not take samples when gastroenteritis symptoms are present (Hall et al., 2016; T. F. Jones & Yackley, 2018). The lack of people seeking healthcare and lack of samples tested cause HuNoV to be underreported. When fecal samples are tested for HuNoV, then the results can be complicated as healthy people can shed HuNoV and shedding is sometimes delayed (Belliot, Lopman, Ambert-Balay, & Pothier, 2014; Rockx et al., 2002). The shedding of HuNoV is highly variable and can last from 8-60 days (Teunis et al., 2008). The data on HuNoV frequency is often underreported due to the lack of people seeking healthcare for a disease that has short duration and usually mild symptoms. The GII.4 genotype causes more severe acute gastroenteritis as there are a higher amount of hospitalizations and deaths annually from it (Desai et al., 2012). The health care needed for HuNoV infections and the societal costs combined cost the U.S. $2 billion per year and $60.3 billion globally per year (Bartsch, Lopman, Ozawa, Hall, & Lee, 2016; Belliot et al., 2014). The economic burden is the largest part of the cost, as the majority do not seek healthcare with gastroenteritis. The economic burden shared among low and high income countries suggests that HuNoV is a global concern that needs a preventative.

The spread of HuNoV is similar to influenza virus as new variants of GII.4 spread globally in a rapid manner. New epidemic strains evolve every 1-2 years after herd immunity has occurred to the previous epidemic strain. HuNoV has epochal evolution, which includes periods of stasis in between the epidemic strains. Currently, strains are evolving faster rate than a decade ago that had new epidemics every 2-3 yrs. The new stains evolving are different due to their antibody recognition sites causing different binding patterns (Donaldson et al., 2008).

The first GII.4 strain to spread globally was the 1996 strain and also became the first GII.4 epidemic that was most prevalent in Europe and Hong Kong. Following the 1996 epidemic there was a new strain in the winter of 2002/2003 that caused an epidemic in Asia. The 2006a variant caused an epidemic in Europe, North America, and Oceania. Interestingly, this 2002/2003 variant barely spread to other continents, while the 2006a variant barely spread to Asia. Oceania appears to be ahead of most countries for epidemics, based on the 2006 and 2004 epidemic strains, that were first observed there (Siebenga et al., 2009). There were other epidemic strains including one in 2002 that covered the United States, Canada, and Europe (Lopman et al., 2004). The 2004 and 2006b strains were completely global as they affected areas all over the world. There were some variants that affected fewer countries such as 2001 Japan affected Japan, the Netherlands, Chile and Malawi. Then, the 2003 Asia variant affected China, Japan, New Zealand and the UK.

Outbreaks of HuNoV have a seasonal connection with a significantly higher risk during the winter months as represented in Fig. 1. The trends of people congregating staying indoors

during colder weather can lead to a higher risk of transmission during the winter. Then, hospitals have a higher amount of patients in the winter months creating a larger chance of person-to-person contact for a higher chance of transmission (Matthews et al., 2012). The peaks for HuNoV incidences are in December and January each year and last from November to February (Gastañaduy, Hall, Curns, Parashar, & Lopman, 2013).

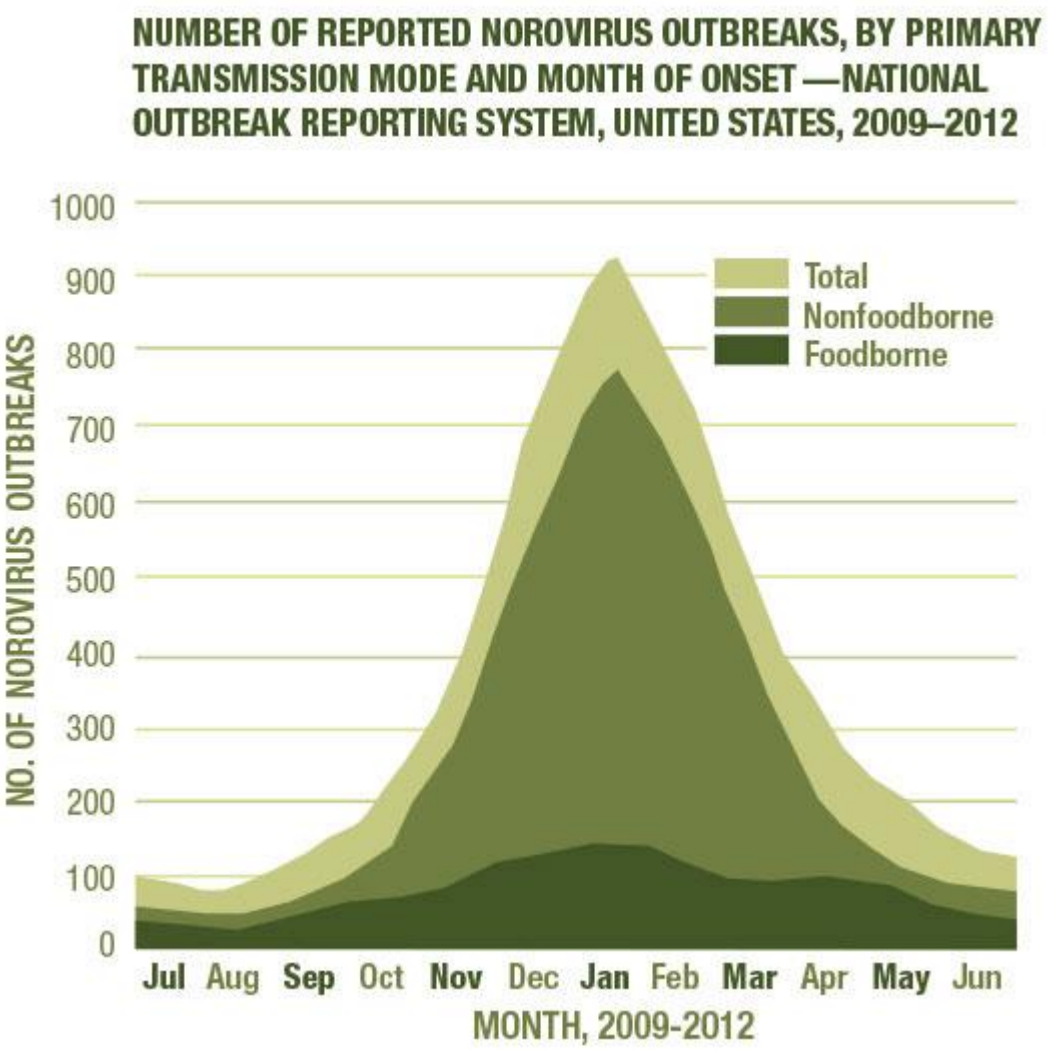


Figure 1. Norovirus outbreaks 2009-2012 (CDC, 2018)

HuNoV infections are more prevalent in children, elderly and immunocompromised people. The highest cost per person is among the elderly (>55 yrs) but most of the costs are from infected children (Bartsch et al., 2016). HuNoV affects the elderly population differently by the symptoms lasting longer. The symptoms that linger after HuNoV such as dehydration, anorexia, lethargy and vertigo can last for up to 19 days after infection in elderly people. HuNoV sheds for longer periods of time in elderly than younger people. It can shed in stools for up to 15 days in elderly with the median being 8.6 days. The care for elderly people with HuNoV needs to extend long after the symptoms have ceased to ensure they reach full health and prevent further spread (Goller, Dimitriadis, Tan, Kelly, & Marshall, 2004). The elderly population of the U.S. makes up 90% of the 797 annuals deaths from HuNoV (Hall et al., 2016). The high amount of deaths being from the elderly population is due to outbreaks taking place in nursing homes and hospitals. Their weaker immune system and the dehydration symptom cause a more severe disease in the elderly population from HuNoV.

Children have a higher prevalence of HuNoV as HuNoV is found in 25% of children with acute gastroenteritis (Payne et al., 2013). Children are one of the main transmitters of HuNoV to people of all ages as they come in contact with others more often and are in the process of learning good hygiene (Simmons, Gambhir, Leon, & Lopman, 2013). Before children are 5 years old 1 in 6 will go to outpatient for HuNoV and 1 in 278 will be hospitalized from HuNoV. The healthcare burden for children under 5 for HuNoV is over $273 million per year. Children from 6 to 18 months of age have the highest rate of seeking healthcare for HuNoV (Payne et al., 2013). In developing countries norovirus causes over 200,000 deaths per year in children under

5 years old (Patel et al., 2008). In children under 1 year, HuNoV sheds for longer periods of time compared to older children/adults infected and on average 1-3 infections will have already occurred (Blazevic, Malm, & Vesikari, 2015; Rockx et al., 2002).

People with immunosuppressive diseases and oncological disorders often have persistent HuNoV infection. There have been several cases with immunodeficient patients shedding HuNoV for >9 months. HuNoV is the most prevalent gastroenteritis cause in immunodeficient children (Frange et al., 2012). In a study done by Frange et al. (2012), 45.8% of the immunodeficient children with acute gastroenteritis had HuNoV. Then, all of the major histocompatibility complex class II expression deficiency children in the study were positive for HuNoV shedding (Frange et al., 2012).

The relationship between symptoms and virus excretion is complicated with virus shedding occurring after diarrhea has ceased and it can occurs in 7% of individuals who exhibit no symptoms (S. M. Ahmed et al., 2014; Goller et al., 2004). The HuNoV amount is not significantly different in loose or formed stool samples indicating that symptoms did not signify the amount of HuNoV being shed (Goller et al., 2004). Shedding of HuNoV in feces can last for more than 22 days in 26% of infections (Rockx et al., 2002). The prolonged shedding leads to further spread of HuNoV especially with asymptomatic people.

Human norovirus is highly stable in the environment, food and water causing higher rates of transmission. HuNoV GI.I can remain infectious in groundwater for over 61 days then

remains detectable in groundwater for over 3 years (Seitz et al., 2011). The temperature and pH of the groundwater in different areas affect the longevity of its survival (Moreno-Espinosa, Farkas, & Jiang, 2004). The HuNoV has the ability to aggregate helping the virus to stay stable for long periods of time in the environment such as groundwater.

1.4. Molecular biology of human norovirus

1.4.1. Genome structure

The HuNoV genome consists of positive-sense single-stranded RNA with 3 open reading frames (ORF), including structural proteins and nonstructural proteins as depicted in Fig. 2. The murine norovirus (MNV) has a fourth ORF, an alternative ORF within ORF2, coding for virulence factor 1 (VF1). VF1 protein delays the upregulation of the innate immune system in the mitochondria, including interferon-β (McFadden et al., 2011). The 5' end of HuNoV genome is covalently linked to the VPg protein and is polyadenylated at the 3' end. The untranslated regions (UTR) are relatively short on norovirus, with the 3' UTR consisting of only 48 nucleotides (Thorne & Goodfellow, 2014). The UTRs include highly conserved secondary RNA structures and are present at the 5' end, 3' end, and throughout the genome (Simmons et al., 2013).

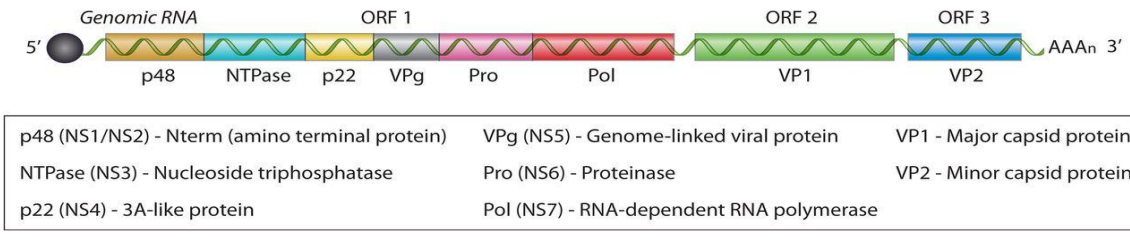


Figure 2. Genome of human norovirus (Robilotti, Deresinski, & Pinsky, 2015)

The ORF1 encodes six proteins, which originate as a polyprotein that is co-translationally and post-translationally cleaved via the virally encoded protease (Blakeney, Cahill, & Reilly, 2003; Donaldson et al., 2008). These six proteins include p48 (NS1/2/N-term), nucleoside triphosphatase (NTPase/NS3/2C-like/p40/p41), p20 (p22/NS4/3A-like), virus protein genome linked (VPg/NS5), protease (NS6/Pro/3C-like), and the RNA-dependent RNA polymerase (Pol/3Dpol/RdRp/NS7) (Blakeney et al., 2003; Donaldson et al., 2008; Sosnovtsev et al., 2006). The ORF2 encodes for the structural protein named viral protein 1 (VP1), which is the major capsid protein and the antigen for antibodies to bind. The ORF3 is translated into viral protein 2 (VP2), the minor capsid protein, which is located on the inside of VP1, which stabilizes the virus structure.

The non-structural proteins play important roles in the virus replication cycle and their functions are listed in Table 1. The protein VPg is 16 kDa and used as a primer in transcription and translation (Donaldson et al., 2008). VPg was observed binding to initiation factors such as eIF3, which is bound to ribosomal subunit 40s thus allowing for transcription/replication initiation to occur (Daughenbaugh, Fraser, Hershey, & Hardy, 2003; Donaldson et al., 2008). Additionally, VPg is involved in cell-to-cell movement for long distance (Hardy, 2005). The NTPase protein is about 40 kDa and has been shown to bind and hydrolyze NTPs needed for transcription and replication but does not act as a helicase (Donaldson et al., 2008; Hardy, 2005). The protease (Pro) protein is a 19 kDa protein, which cleaves the polyprotein in *trans* at 5 sites (Sosnovtsev et al., 2006). Also, Pro can inhibit host protein synthesis by inhibiting the host's translation pathway. This is inhibited via cleaving proteins that bind to cellular poly-A

such as translation initiation factors (Blakeney et al., 2003; Donaldson et al., 2008). The protein p48 is a 48 kDa protein, which includes a transmembrane domain and plays a role in intracellular protein trafficking and formation of viral replication complex. The p20 protein is named after its 20 kDa mass and has an unknown function.

Table 1. List of HuNoV proteins and their functions

MNV	HuNoV	Function
NS1/2	p48 (N-term)	Replication complex formation†, contributes to persistence in MNV infections
NS3	NTPase (2C-like)	RNA helicase†/NTPase
NS4	p22 (3A-like)	Replication complex formation†
NS5	VPg	Genome-linked protein involved in translation and replication
NS6	Pro (3C-like)	Protease
NS7	Pol/3Dpol	RdRp
VP1	VP1	Major capsid protein
VP2	VP2	Minor capsid protein
VF1	No equivalent	Virulence factor

The RNA-dependent RNA polymerase (pol) is approximately 57 kDa and cleaved from the C-terminal section of the polyprotein. The pol protein is responsible for replication of viral genome and subgenome. The fingers, palm and thumb domains of HuNoV pol are comparable to other polymerases while the C terminal domain varies from other virus pol (Hardy, 2005). The Pol is from the Gly–Asp–Asp (GDD) polymerase family, a common feature among positive-sense RNA viruses including HuNoV. For replication the pol uses VPg for a primer and binds to the poly A region of the RNA to initiate replication by first synthesizing full-length negative-sense RNA genome, which in turn serves as a template to synthesize the positive-sense RNA genome to be packaged in the capsid VP1 and VP2 (Donaldson et al., 2008).

1.4.2. Major capsid protein VP1

Viral protein 1 (VP1) is the major capsid protein which includes 2 principal domains called shell (S) domain and protruding (P) domain with a hinge region connecting them as represented in Fig. 3. The shell domain provides the icosahedral shell structure while the protruding domain gives the protruding portion from the shell via dimers. The protruding domain is made from 2 subdomains P1 and P2 (Prasad et al., 1999). The P1 subdomain has residues at 230 to 285 and 406 to 516 and has a mix of α-helix and β-sheets in the structure including two twisted antiparallel β-sheets and a single α-helix. The P2 gene is in the middle of the P1 gene and includes residues 286-405. The P2 protein folds into a six-stranded antiparallel β-barrel. The protrusion domain is stabilized by hydrogen bonds between P1 and P2 in addition to hydrophobic interactions (Bu et al., 2008).

The P2 subdomain is highly variable and recognizes the histo-blood group antigen (HBGA) receptors for the virus to bind to on the host cells (Glass et al., 2009). The P2 subdomain also interacts with neutralizing antibodies generated by the immune response. As the population becomes immune to a particular strain, the HuNoV P2 subdomain has to evolve via antigenic drift and there are a variety of HBGA to be used as antigens (L. C. Lindesmith et al., 2008).

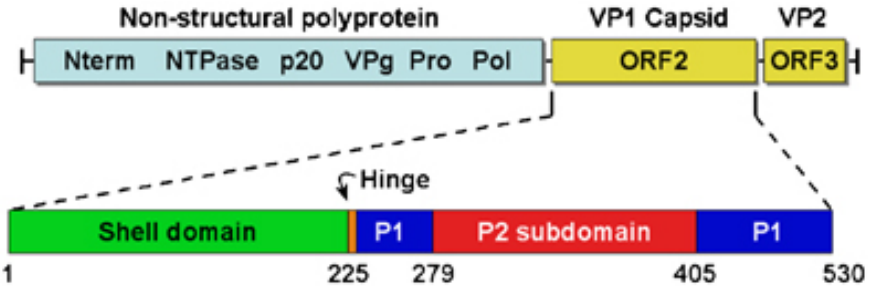


Figure 3. The P and S domains of the major capsid protein (VP1) (Donaldson et al., 2008)

The icosahedral structure of T=3 includes subunits A, B and C with a total of 180 capsid proteins arranged in 90 dimers and formed into 30 hexagonal facets as shown in Fig. 4 (Bally Marta et al., 2012; Donaldson et al., 2008). The A/B dimer from the S domain forms a bent configuration that protrudes from the surface of the capsid. While the C/C dimer has a flat conformation to make the capsid shell and gives the cup appearance (Glass et al., 2009).

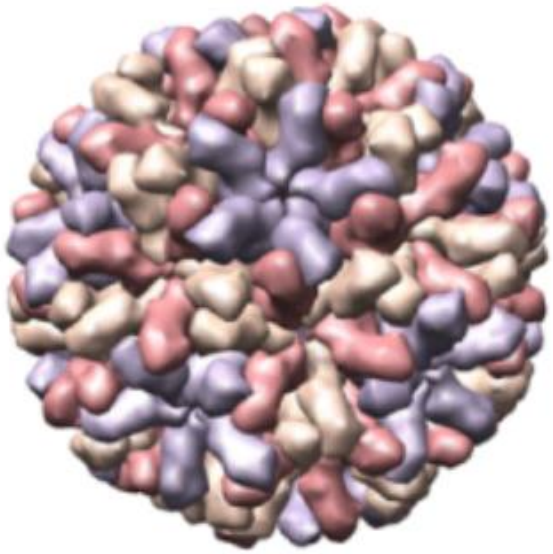


Figure 4. Capsid assembly representing the different dimers necessary for assembly. Red: A monomer, blue: B monomer, yellow: C monomer (Donaldson et al., 2008)

As the VP1 gene is the outer capsid protein, it is the most variable gene and can be genotyped to create a HuNoV phylogeny tree. The variability of VP1 stems from the need to evade the immune response, stability improvements and change tropisms allowing the virus to adapt. The surface exposed section (protruding domain 2) of the gene has the highest variability to help the virus evolve to new epidemic causing strains over time. The herd immunity that develops after a new strain causes mutated viruses to be more successful in replicating in hosts (Donaldson et al., 2008; L. C. Lindesmith et al., 2008).

1.4.3. Minor capsid protein VP2 of human norovirus

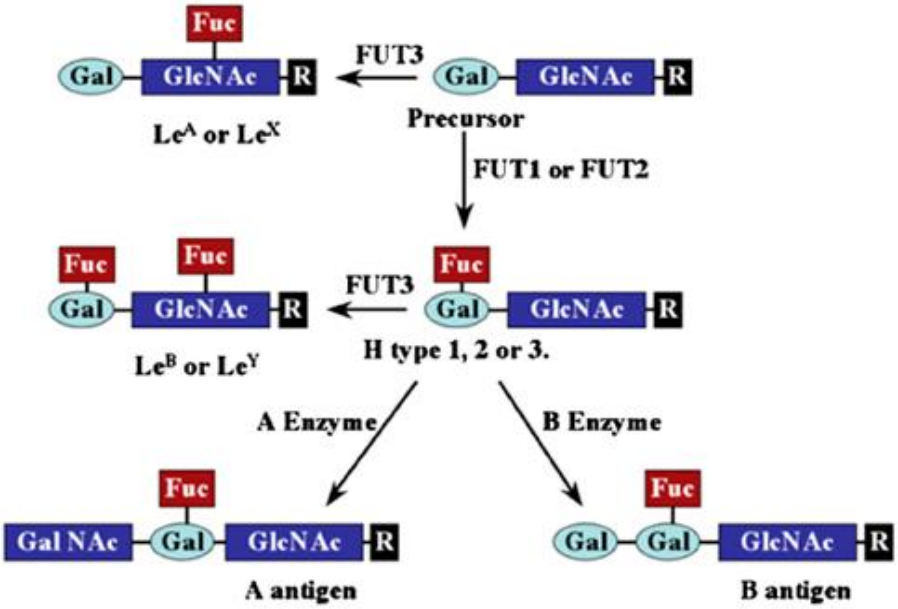
Viral protein 2 (VP2) from ORF3 is 20 kDa. This protein functions as a minor structural protein by interacting with the shell domain of VP1 on the internal side to enhance VP1 stability in forming the capsid protein (Le Pendu, Ruvoën-Clouet, Kindberg, & Svensson, 2006). In

addition to enhancing stability, VP2 helps with the encapsulation of genomic RNA and the process of assembling the capsid (Vongpunsawad, Prasad, & Estes, 2013).

1.5. Receptors and host susceptibility of human norovirus

The receptor for HuNoV to bind to the intestinal epithelium is ABH histo-blood group antigen glycans (HBGA) on glycoproteins and glycosphingolipids. HBGA are monosaccharides added onto the glycosphingolipids and glycoprotein chains (HBGA disaccharide precursors) by fucosyltransferase 2 (FUT2), creating a trisaccharide as shown in Fig. 5 (Bally Marta et al., 2012; Donaldson et al., 2008; Ravn & Dabelsteen, 2000). The FUT1 is found in red blood cells and has the same enzymatic activity as FUT2, which is found on mucosal linings. Then, FUT3 modifies the precursor (Galβ1-3GlcNAc for type 1 pathway) or the FUT1/2 products to create Lewis type HBGAs and Lewis types can include secretors or non-secretors as shown in Fig. 5 (Marionneau et al., 2001). The lewis A (LeA or LeX) non-secretor occurs when FUT3 adds fucose residues in α-1,3 or α-1,4 linkages to the precursor. For the H type, the FUT2 (for FUT1) adds a monosaccharide to the precursor in an α-1,2 linkage. Then, FUT3 can modify the H type trisaccharide to create the Lewis B (LeB or LeY) secretor tetrasaccharide. In addition, A enzyme

can modify H type by adding a N-acetylgalactosamine via α-1,3 linkage to the trisaccharide to



create A type 1 or B enzyme can add a galactose to the trisaccharide in an α-1,3 linkage to create B type 1 (Donaldson et al., 2008).

Figure 5. HBGA pathway including enzymatic modification to FUT1, FUT1 and FUT3 genes (Donaldson et al., 2008)

HBGAs are present on red blood cells, gastrointestinal tract epithelium, genitourinary tract epithelium, peripheral nervous system, thymus epithelium, and respiratory tract epithelium. In addition, HBGAs are found in bodily secretions such as saliva. Other microorganisms use HBGA as receptors and the host's susceptibility to them depends on if the host possesses a secretor gene (FUT2 gene). For example, *Escherichia coli* binds to non-

secretors who possess galactosylgloboside, which is only exposed without FUT2 adding trisaccharides to HBGAs (Marionneau et al., 2001).

The VLP of HuNoV has been observed to bind specifically to α 1,2-fucosylated glycans and it can recognize the H type 1 and Le^b epitopes on glycosphingolipids. Then, there is a second binding to sialylated structures such as SLe^x based on studies using VLPs (Rydell, Svensson, Larson, Johannes, & Römer, 2013). In addition to binding the α- fucose, there is a β-GalNAc region on the A-trisaccharide that binds to Ser377, Asp327, His329, and Ser380 residues on the P2 domain for the GI.1 strain and GII.4 strain. The binding patterns observed for A and H antigens are similar suggesting the binding sites are the same for both antigen types

(Bu et al., 2008). Only a few strains of HuNoV bind to Lewis and B antigens as most strains bind to H and A antigens. Surprisingly, only one or a few alterations to amino acids in VP1 protein giving rise to a new strain capable of using different HBGAs as receptors. The Farmington Hills 2002 strain uses LeY and H3 as a receptor, however after evolving to 2002a the receptors changed to LeA, LeX and A antigens (Donaldson et al., 2008). This strain variation occurred from two amino acid changes including a substitution in P1 with a Pro changing to a Ser at 226 and another substitution at P2 with Ala changing to Thr at 395 (L. C. Lindesmith et al., 2008).

This is the first strain to be able to bind to non-secretor HBGAs giving the opportunity for this strain to infect a population that does not have prior immunity. Understanding the evolution pattern of HuNoV receptor binding can influence vaccine development (Donaldson et al., 2008).

Table 2. The different HBGAs used as receptors for different strains of HuNoV (Donaldson et al., 2008)

VLP	GG	Year	Le^A	H1	Le^B	A	B	H3	Le^X	Le^Y
Norwalk	I.1	1968		X	X	X		X		
West Chester	I.1	2001						X		
SoV	I.2	1999	X					X		
DSV	I.3	1999	X			X				
Chiba	I.4	2000	X							
HV	II.1	1971				X				
Weisbaden	II.1	2001								
SMV	II.2	1976						X		
Buds	II.2	2002								
Ina	II.2	2002								
TV	II.3	1999				X		X		
GII.4.1987	II.4	1987						X		X
GII.4.1987.D393G	II.4	2007 *				X	X	X		
GII.4.1997	II.4	1997				X	X	X		X
GII.4.2002a	II.4	2002		X		X			X	
GII.4.2002	II.4	2004						X		X
GII.4.2004	II.4	2004								
GII.4.2005	II.4	2005								
GII.4.2006	II.4	2006				X	X	X		
M7	II.4	1999								
MNV	V	2004								

People who are non-secretors do not have HBGAs therefore, most strains of HuNoV are unable to bind to the intestinal epithelium HBGAs as represented in Table 2. However, strains such as GI.3 from an outbreak in 2007 in Jönköping, Sweden are able to bind to HBGA regardless of secretor status (Nordgren, Kindberg, Lindgren, Matussek, & Svensson, 2010). People who are non-secretors do not possess the gene for FUT2, therefore there are no α 1,2-fucosylated glycans added to glycosphingolipids and glycoproteins for HuNoV to bind to (Ravn & Dabelsteen, 2000; Rydell et al., 2013). Eighty percent of the population are secretors, therefore 20% are resistant without the FUT2 gene. The different genogroups have different bonds and interactions with the HBGA in the GI tract but all HuNoV bind to HBGAs. There are two groups of receptor binding for HuNoV including one group that binds A/B type and/or H type antigens then the other group binds Lewis and/or H antigens (Nordgren et al., 2010). The different blood types have a role in susceptibility to infection, as the HBGA in the blood can give insight to the HBGAs present in the GI tract. People with type O blood are observed to be more susceptible to infection (L. Lindesmith et al., 2003).

On the HuNoV capsid protein, the section of VP1 subdomain P2 that binds to the host receptor is between amino acids 300 and 384 and the attachment is site is predicted to be in this region of P2 as well (White et al., 1996). To stabilize the receptor binding process there is weaker long distance binding between P2 and β-Gal ring of the trisaccharide (Cao et al., 2007).

1.6. Challenges in human norovirus research

For decades, there have been no cell lines found to be able to be infected with HuNoV. Many efforts have been devoted to develop a cell culture system for HuNoV. Firstly, it was found that HuNoV binds at a high efficiency to receptors on human colon carcinoma cell line (D-Caco-2 cells) however, no replication was observed. To help with internalization of HuNoV in D-Caco-2 cells trypsin and pancreatin were used, however no internalization resulted (White et al., 1996). Later, a 3-D cell culture modeling the human intestinal lining (Caco-2 cells) showed a 2 log increase in HuNoV RNA copies in a study done by Straub et al. (2011). HuNoV from both genogroup I and II were tested on the 3-D Caco-2 cells (Straub et al., 2011). However, these cell culture systems results were not reproduced by other researchers (Takanashi et al., 2014).

In Karst et al. study (2014), a B cell culture system was developed for HuNoV (Karst, 2010). Interestingly, HuNoV infection of B cells required the presence of HBGA-expressing enteric bacteria. They found that GII.4 and GII.3 strains had 600 fold increases in RNA genome copies when unfiltered samples were used for infection. However, no RNA genome replication was detected when the stool sample was filtered or ultraviolet treated. This result suggests enteric bacteria promote HuNoV replication in B cell culture. Additionally, there are enteric bacteria present in humans that contain HBGA and they may play an important role in B cell culture infections. One of the enteric bacteria, *Enterobacter cloacae,* expresses H type HBGA

that the GII.4 genotype can bind to and help with infection in B cells. The enteric bacteria helped in the HuNoV infection process in the B cell culture system (T. F. Jones & Yackley, 2018).

In a study done by Ettayebi et al. (2016), multiple HuNoV strains were successfully cultivated in stem cell–derived human enteroids (Ettayebi et al., 2016). The human intestinal enteroid (HIE) monolayer cell culture is made from stem cells in human intestinal crypts and it can model the natural human intestinal epithelium. This culture includes goblet cells, paneth cells, enterocytes, and enteroendocrine that contribute to replicate the environment present in human intestines. Using this culture system, a 1.5-2.5 log increase in RNA copies was observed along with cytopathic effect (CPE) including cell death (Ettayebi et al., 2016). This result was observed for multiple HuNoV genogroups including GII.4 and GII.3. In addition, they found that bile enhanced infection of HuNoV and some strains (such as GI.1, GII.3 and GII.17) strains requires bile to infect the HIE (Ettayebi et al., 2016).

1.7. Virus-like-particles (VLPs) of human norovirus

There is no commonly used cell line that HuNoV infects, as the HIE culture requires primary cells. However, the VLPs possess the same virus-ligand binding abilities as the original virus and allow for the binding of VLPs to cells to be studied (Bally Marta et al., 2012). There are several virus-like particle (VLP) expression systems that have been developed that produce VP1 proteins in a comparable structure to the entire HuNoV capsid structure with VP2 and the RNA genome. The most common way to produce VLPs is using the baculovirus expression system. It has been used since 1983 and has produced high quantities of viral proteins that exhibit virus

like properties (Jarvis, 2009). Another method to produce VLPs is using the Venezuelan equine encephalitis (VEE) replicon system. The HuNoV structural genes (ORF1 & 2) are inserted in VEE genome to replace VEE's structural genes and HuNoV structural genes are translated using 26S promoter (Donaldson et al., 2008). The VLPs are immunoreactive allowing them to be used for immune response and vaccine research. In addition, VLPs are commonly used as the antigen in ELISAs (Jiang, Wang, Graham, & Estes, 1992). The fact that HuNoV VP1 protein is able to self-assemble into empty capsid particles as depicted in Fig. 6, implies that mainly protein-protein interactions play a role in capsid assembly (White et al., 1996). The VLPs have led to many discoveries about HuNoV, such as the VP1 protein has a molecular mass of 58 kDa.

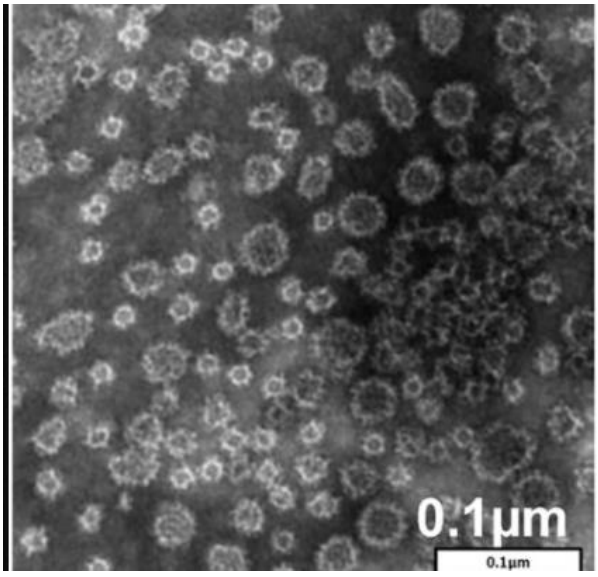


Figure 6. Virus-like particles generated through the baculovirus expression system (Lou et al., 2012)

1.8. Human norovirus vaccine candidates

For the prevention of diseases, vaccination is the most efficient strategy. Since the discovery of HuNoV in Norwalk, OH 1968, a tremendous amount of efforts have been devoted to developing a safe and efficacious HuNoV vaccine. However, it has been a challenge to develop a HuNoV vaccine because of the lack of small animal model and an efficient cell culture system. In addition, the lack of antigenic cross-reactivity between genogroups and the wide diversity between the genogroups have made vaccine development difficult (Bernstein et al., 2015; Richardson, Bargatze, Goodwin, & Mendelman, 2013). Mice have been used in studies to determine the immune response after a vaccine was administered but this model is limited by the inability to challenge mice (Ma & Li, 2011). The animal models used for vaccine experiments including a challenge to measure the level of protection are chimpanzees and gnotobiotic piglets. To overcome strain diversity, vaccines made will need to be multivalent to cover multiple common genotypes to provide a broader immunity to multiple strains (Debbink et al., 2014; Swanstrom, Lindesmith, Donaldson, Yount, & Baric, 2014). The duration of immunity resulting from HuNoV is variable and can last for 8 weeks or up to 8 years based on human challenge clinical studies (Parrino, Schreiber, Trier, Kapikian, & Blacklow, 1977; Simmons et al., 2013). These findings raise concern over the length of time a HuNoV vaccine will remain effective to protect the population against HuNoV. Although currently there are no FDA-approved vaccines, several HuNoV vaccine candidates have been in preclinical trials or human clinical trials.

The availability of a HuNoV vaccine would significantly reduce the economic burden and provide sustainable preventive health benefits to society. The best target populations would be people under 5 or over 65 years of age, as they are the most at risk. The duration of protection, cost of vaccine and age of the vaccinated person need to be taken into consideration (Bartsch, Lopman, Hall, Parashar, & Lee, 2012).

1.8.1. VLP-based subunit vaccine candidates

It is known that the major capsid protein VP1 is the major antigen that is responsible for triggering protective immunity against HuNoV. Thus, most HuNoV vaccine research has been focused on VP1. In 1992, Jiang et al., performed the first HuNoV vaccine study and evaluated the immunogenicity of HuNoV VP1 in mice, rabbits and guinea pigs. They found that that the expression of VP1 formed VLP and bound to antibodies in immunoprecipitation (Jiang et al., 1992). Following the IM injection of VLP in all three animal models, high titers of HuNoV-specific antibody response were detected, including IgG, IgA, and IgM (Jiang et al., 1992). Since then, VP1 protein has been expressed in many systems including *E. coli*., yeast, insect cells, mammalian cells, and potatoes. Mice immunized with VP1 or VLP expressed from these systems generated a variable degree of HuNoV-specific humoral, cellular, and mucosal immunities. Among these systems, baculovirus-insect cell expression system has been shown to be most efficient system to generate VLPs. In order to create a cross-reactive vaccine, Parra et al. (2012) designed bivalent VLPs with GI.1 VP1 and GII.4 VP1 engineered to contain proteins from 3 different strains (Parra et al., 2012). Rabbits were used as the animal model and the

VLPs were administered IM with aluminum hydroxide gel (Al(OH)3) as an adjuvant in 50 µg or 150 µg of doses. Immunoglobulin G (IgG) GMT from sera was higher when given IM with Al(OH)3 than intranasally with chitosan, suggesting IM as the best route for VLP immunizations and Al(OH)3 is a better adjuvant. However, low cross-reactivity was observed following IM immunization and the VLP protection was not studied due to the limitation that rabbits cannot be infected by HuNoV. (Parra et al., 2012).

Gnotobiotic piglets have been used as a challenge model to measure whether VLP vaccines can protect from HuNoV infection. The HuNoV- GII.4 HS66 strain VLPs were given intranasally (IN)/orally with immunostimulating complexes (ISCOM) or mutant *E. coli* LT toxin (mLT) as mucosal adjuvants to generate an immune response in gnotobiotic piglets. For the challenge, the half of the piglets were challenged and the other half was euthanized (Souza, Costantini, Azevedo, & Saif, 2007). There was 100% protection in both vaccination groups against virus shedding. IgM was first observed in both vaccinated groups at PID 10. Following IgM, IgA and IgG was first observed at PID 21 then both increased by day 28. In the VLP + ISCOM group, the antibody secreting cells (ASC) were the highest in the intestinal content suggesting that, a combination of VLP with ISCOM is an effective adjuvant for generating an immune response. In addition, the combination of oral and IN inoculation in multiple doses proves effective at generating an immune response robust enough to protect the piglets from HuNoV shedding (Souza et al., 2007).

The VLP vaccine has gone through phase 1 trials via intramuscular, intranasal and oral routes. A clinical study done by Bernstein et al. (2015) with VLP from GI.1 and GII.4 given IM to human volunteers using one dose of 100μg (Bernstein et al., 2015). They observed decreased disease severity and an increase in total IgG immune response. However, the response did not support the primary endpoint of the study (Bernstein et al., 2015).

For oral administration of the VLP vaccine, it requires a high dose to trigger an immune response. The doses levels used in Tacket et al. (2003) study included 250 μg to 2000μg VLP administered orally with sodium bicarbonate in two separate doses 21 days apart (Carol O Tacket, Sztein, Losonsky, Wasserman, & Estes, 2003). The genogroup/genotype of VLP used was not mentioned in this article, as genotyping was not routine in 2003. The immune response from the vaccine generated high IgG levels in the 250 μg group but the higher dose levels did not generate significantly higher IgG levels compared to the 250 μg dose (Carol O Tacket et al., 2003). However, the IgG titers had a 4 fold increase in a study done with a 250 μg dose compared to a 100 μg dose (Ball, Hardy, Atmar, Conner, & Estes, 1998). The IgG titers from the VLP oral vaccine were lower than IgG titers seroconverted after HuNoV challenges suggesting the need for improvement to the VLP vaccine candidate. This study did not challenge the volunteers therefore, we cannot determine how well orally administered VLP protect against HuNoV infections (Carol O Tacket et al., 2003).

The VLP vaccine candidate was administered IN in a human clinical trial by El-Kamary et al. (2010) (El-Kamary et al., 2010). The GI.1 VLP were administered in two separate doses 21

days apart in this study with two adjuvants including monophosphoryl lipid A and mucoadherent chitosan. There were several dose levels ranging from 5 µg-100µg. The VLP vaccine stimulated an immune response with increased HuNoV specific IgG and IgA and they were further increased with the second dose. This adjuvanted VLP vaccine clinical trial generated higher antibody secreting cells (ASCs) than the non-adjuvanted VLP oral vaccine trial done by Tacket et al. (2003). This clinical trial did not measure this vaccines ability to protect against HuNoV infection (El-Kamary et al., 2010). In another study with VLP administered IN, the volunteers were challenged with HuNoV. The GI.1 VLPs were inoculated IN in a dose of 100 µg with chitosan as an adjuvant. For the immune response, 70% of the vaccine volunteers had an IgA response. Following the HuNoV challenge, only 37% of the vaccinated group were infected, which is a significant difference from the control group with 69% of the group infected. This suggests that IN administration with chitosan adjuvant generates an immune response and significant protection from HuNoV infection (Atmar et al., 2011).

1.8.2. P particle-based subunit vaccine candidate

VP1 protein can be divided into 2 principal domains called shell (S) domain and protruding (P) domain with a hinge region connecting. It was found that expression of P domain of VP1 can form another type particle called P particle. Importantly, the P particle has immunogenic characteristics observed with higher HBGA binding activity when compared to VP1 VLPs. The high stability and immunogenic characteristics of P particles lead to the idea of using the P particle as a subunit vaccine (Tan & Jiang, 2005). In Tan et al. (2011) study evaluated the immune response of GII.4 HuNoV P particles with a rotavirus VP8 insertion,

which were expressed via *E. coli*. The immunogenicity of the P particles was evaluated in a mice model with subcutaneous injections of P particles over several doses in conjunction with Freund's adjuvant in a two-week period. The sera collected from the immunized mice were able to prevent the binding of HuNoV VLPs to the HBGA receptors. This provides evidence towards the P particle vaccines potential to prevent HuNoV disease. In addition, this P particle could stimulate an immune response for rotavirus with high titers of anti-rotavirus neutralizing antibodies (Tan et al., 2011).

1.8.3. Viral vectored vaccine candidates

Viral vector based HuNoV vaccines have been an effective vaccine approach and have the potential to prevent HuNoV infection. The viral vectors that have been constructed to deliver antigens for HuNoV include Venezuelan equine encephalitis (VEE), adenovirus, vesicular stomatitis virus (VSV), and Newcastle Disease virus (NDV) (Guo et al., 2008; Harrington et al., 2002; S.-H. Kim, Chen, Jiang, Green, & Samal, 2014; Ma & Li, 2011).

The first viral vector vaccine for HuNoV VP1 used is the VEE vector in 2002. VEE replicon expression system was used for expressing the HuNoV VP1 antigen. The VEE replicon expression system is commonly used to express heterologous proteins. In this system, VEE structural proteins are replaced with the VP1 gene to reduce the pathogenesis potential of VEE. The VEE replicon particles (VRP) vectored with HuNoV VP1 are able to produce high amounts of HuNoV VLPs. When VRP expressing VP1 was administered to mice subcutaneously, high antibody titers were observed. By day 7 post-vaccination, high HuNoV-specific serum IgM titers

were detected in addition to HuNoV-specific serum IgG. Following the booster on day 21, the serum IgG titers increased by almost 10-fold, suggesting the importance of a vaccination booster. When administered orally, the HuNoV-specific IgG response was lower showing that subcutaneous route is ideal for administering the VRP vectored VP1 vaccine. The HuNoV GI-specific IgG was able to cross-react with a different HuNoV GI strain (NCFL) suggesting the potential for heterotypic immunity. The HuNoV-specific fecal IgA response was significantly higher for the subcutaneous group compared to the oral group. The VRP vectored VP1 induced systemic and mucosal immune responses suggests the potential for its protection from HuNoV infections (Harrington et al., 2002).

Adenovirus has previously been used as a vaccine vector for several pathogens such as HIV and Ebola virus. However, the concern with using adenovirus based vector is the stress response in addition to the respiratory symptoms. In 2008, the adenovirus vectored HuNoV GII.4 VP1 was constructed and administered to mice intranasally to evaluate the immune response. To enhance the immunogenicity of the vaccine, the mice were boosted twice 14 days apart. High titers of HuNoV-specific serum IgG were observed after the first dose then the strong amounts of IgG were observed following the second and third dose of vaccine. For serum IgA, there was a strong HuNoV-specific IgA response after the second dose and continued to increase following the third dose. For mucosal immunity, HuNoV-specific IgG and IgA were detected in the feces following the first dose and continued through the next two doses. These strong mucosal and systemic immune responses show potential for protection from HuNoV in future studies (Guo et al., 2008).

VSV has been used extensively for its ability to express proteins for vectored vaccine and gene therapy purposes. Multiple vaccine candidates have used VSV as a vector including Ebola virus, HIV, Hepatitis B virus , influenza virus, west Nile virus along with many others (Cobleigh, Buonocore, Uprichard, Rose, & Robek, 2010; Guo et al., 2008; S. M. Jones et al., 2007). A VSV-based HuNoV vaccine was constructed and has been tested in mice and gnotobiotic pig models. The recombinant VSV vectored VP1 was attenuated as observed in cell culture, mice, and gnotobiotic piglets. It was shown that VSV-based vaccine triggered significantly higher humoral, cellular, and mucosal immune response than VLP-based vaccine. In addition, gnotobiotic piglets vaccinated with rVSV-based HuNoV vaccine protected from HuNoV replication in intestine (Ma & Li, 2011).

Newcastle disease virus (NDV) causes acute respiratory disease in birds and in some cases has caused flu-like symptoms in humans. There are three different pathotypes of NDV including lentogenic, mesogenic, and velogenic. The least virulent is lentogenic and recombinant lentogenic NDV has been used as a vector for vaccines since 2001. A NDV-based HuNoV vaccine has been generated and was capable of triggering HuNoV-specific immune responses (S.-H. Kim et al., 2014).

1.8.4. Current problems in HuNoV vaccine development

The development of a HuNoV vaccine is the key for future control and prevention of HuNoV. The cost, immunogenicity, and safety all need to be taken into consideration when

developing a vaccine. With a HuNoV vaccine that is cost effective and safe for all ages, then it would economically benefit society (Bartsch et al., 2012). Although VLP and P-based vaccine candidates are highly promising, there are several limitations. First, production of VLP and P particles is time-consuming and expensive. Second, immunization requires high doses of VLP multiple boosters, and mucosal adjuvants such as *V. cholerae* and *E. coli* enterotoxins. Third, immunogenicity of such vaccines is limited as the VLPs are non-replicating immunogens. Although live viral vectored HuNoV vaccine candidates have been shown to induce strong immunity, the safety of these viral vectors is a concern which hampered their practical application in humans. Therefore, exploration of other HuNoV vaccine candidates is urgently needed.

1.9. Animal models for human norovirus

1.9.1. Chimpanzee model

There are no small animals that are able to be infected with HuNoV to be used as a model. Previously, chimpanzees are shown to be susceptible to HuNoV infection but expense needs to be considered. They do not show clinical symptoms of gastroenteritis but the duration of virus shedding in stool samples is similar to humans. The serum antibody responses are also similar to humans allowing us to evaluate the efficacy of the vaccine candidate. The chimpanzees gained immunity to HuNoV and were resistant to reinfection. The chimpanzees vaccinated with GI VLP administered IM were protected from the HuNoV challenge. This shows that chimpanzees are a possible animal model for future vaccine research. In 2015, U.S. Fish

and Wildlife Services classified U.S. chimpanzees as endangered under the Endangered Species Act. Chimpanzee has been banned for use in biomedical research by NIH. However, chimpanzee research is occurring in other countries (Bok et al., 2011).

1.9.2. Gnotobiotic piglet model

Gnotobiotic piglets are germ-free newborn piglets that have served as an animal model in research for 50 years and have led to further knowledge of many pathogens such as Campylobacter pylori, rotavirus, and Escherichia coli (Chattha, Vlasova, Kandasamy, Rajashekara, & Saif, 2013; Krakowka, Morgan, Kraft, & Leunk, 1987; Tzipori et al., 1995). Gnotobiotic piglet is a good model for studying HuNoV due to their susceptibility to HuNoV infection. These piglets share many similarities with humans in gastrointestinal structure, physiology, and immunology. In addition, swine have HBGA phenotypes similar to those of humans, making gnotobiotic piglets an excellent model with which to study HuNoV. Previous studies have shown that gnotobiotic piglets are susceptible to oral infection with several human NoV GII.4 strains, and this animal model has been used for evaluation of the efficacy of vaccine candidates and antiviral therapies against HuNoVs. Oral inoculation of gnotobiotic piglets with HuNoV GII.4 virus developed mild diarrhea, viral shedding in feces, and HuNoV replication in pig intestine (Sonia Cheetham et al., 2006).

1.10. *Lactococcus lactis* and its applications in the food industry

Lactococcus lactis subsp. *cremoris (L. lactis*) is a mesophilic gram-positive bacterium used in the food industry fermented food and dairy products. *L. lactis* is categorized as generally recognized as safe (GRAS) by the FDA and regularly used in the dairy industry. In the dairy industry, *L. lactis* is used for its lactic acid producing characteristic to make fermented dairy products such as cheese (Daly, 1983). The family and order of *L. lactis* are *Streptococcacaeae* and *Lactobacillales*, respectively (Champagne, Piette, & Saint-Gelais, 1995). The environmental survival of *L. lactis* is hindered by its inability to produce spores but has tolerance to acidic environments in addition to resistance to bacteriophages. The ability of *L. lactis* to adapt to acidic environment is due to the upregulation of proteins related to sugar metabolism, amino acid metabolism, and pH homeostasis (Budin-Verneuil Aurélie, Pichereau Vianney, Auffray Yanick, Ehrlich Dusko S., & Maguin Emmanuelle, 2005). Quorum sensing for bacteria to communicate with surrounding bacteria via chemical signal molecules occurs in lactic acid bacteria when antimicrobial peptides are necessary (Kleerebezem Michiel, Quadri Luis E. N., Kuipers Oscar P., & De Vos Willem M., 2003). The antimicrobial peptide nisin is secreted by lactic acid bacteria (LAB) but nisin also acts as a signal molecule for nisK to upregulate nisin production (Kuipers, Beerthuyzen, Ruyter, Luesink, & Vos, 1995).

The optimum growth conditions for *Lactococcus lactis* includes an anaerobic environment with M17 medium supplemented with 1% glucose at 30°C, as it is a facultative

anaerobe. For *L. lactis* subsp. *cremoris* fermentation, the glucose is metabolized to produce lactic acid resulting in a 4.4 pH after about 12 h of growth (Duwat et al., 2001). The doubling time for *L. lactis* is 35-60 min depending on if the growth conditions such as oxygen presence or temperature (Kunji, Slotboom, & Poolman, 2003). The growth of *L. lactis* starts with lag phase, then they start exponentially multiplying with plenty of nutrients in the growth phase. When the resources are limited, an equal amount of growth and death occur in the stationary phase followed by the death phase (Monod, 1949; Zwietering, Jongenburger, Rombouts, & Riet, 1990).

1.11. *Lactococcus lactis* recombinant protein expression system

L. lactis is used extensively for biological engineering including overexpression of genes, metabolic engineering, expression membrane proteins, and protein secretion with anchoring in the cell envelope (Kleerebezem Michiel et al., 2003; Mierau & Kleerebezem, 2005). Wild-type *L. lactis* without the plasmid does not produce extracytoplasmic protease (PrtP). *L. lactis* has been extensively used to express heterologous proteins such as antigens and enzymes. In some cases, these foreign antigens can be secreted into medium. Thus, *L. lactis* has been used as a vector to deliver antigen in vitro and in vivo.

1.12. The NICE system for protein expression

The NICE (Nisin Controlled gene Expression) system is often used to express foreign and proteins via LAB based vector. The synthesis of nisin is encoded and controlled by 11 genes

that occur in some *L. lactis* strains. NICE system uses nisA promoter, nisK histidine kinase sensor and nisR response regulator genes to induce the expression of proteins. The nisA gene encodes for the nisin protein with the promoter at the beginning. The other genes of NICE group play a function in modification, translocation, or processing. The addition of nisin during the log phase induces the production of the exogenous gene with the nisA promoter within the vector plasmid. The NZ9000 strain of *L. lacti*s subsp. *cremoris* was created by inserting the nisK and nisR genes into the pepN gene of the plasmid/nisin free MG1363 strain (Kuipers, de Ruyter, Kleerebezem, & de Vos, 1998; Mierau & Kleerebezem, 2005). The NZ9000 strain has a higher induction efficiency and the most sensitive compared to other stains used in the NICE system (Kuipers et al., 1998).

Nisin is bactericidal and produced by some *L. lactis* strains during growth then the nisin genes are upregulated as a result of quorum sensing (Kleerebezem Michiel et al., 2003). This protein expression system was first discovered in 1995 by Kuipers et al. when nisin transcription was being further analyzed. In Kuiper et al. (1995) study, the insertion of a reporter gene *gusA* after the nisA promoter gene showed the potential for protein expression in *L. lactis* (Kuipers et al., 1995). The protein expression in the NICE expression is induced by the addition of nisin to the medium, which binds to nisK in the PZ9000 cell starting a signal transduction pathway as shown in Fig. 7. Then, the nisK activates nisR by phosphorylation and nisR stimulates transcription at the nisA promoter region. The target gene follows the nisA promoter in the vector/plasmid that was inserted into the NZ9000 strain (Mierau & Kleerebezem, 2005).

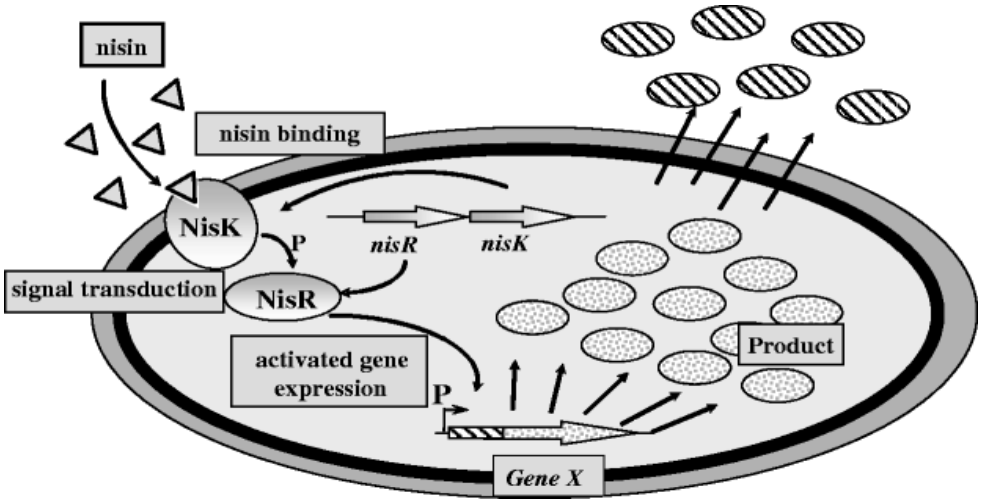


Figure 7. The Nisin signaling pathway to induce gene expression (Mierau & Kleerebezem, 2005)

The NICE is a common system due to its flexibility, low cost, GRAS for food and generates high protein expression. The flexibility is based on the control of the protein expression, which as a linear relationship to the concentration of nisin (Kuipers et al., 1998). The NICE system has been used to study a variety of different proteins including virus proteins. Discoveries about enzymes, pathogenic bacteria, and bacteriophages have been found as a result of the NICE system (Mierau & Kleerebezem, 2005). Over the last decade, *L. lactis* has been used as a vaccine vector for the prevention of many bacteria and virus pathogens.

1.13. Principle of bacteria vectored vaccines

An exciting development in modern vaccinology is the use of bacteria as a vector to deliver animal and human vaccines. The principle of this vaccine strategy is simple. Basically, this is involved in

construction of a plasmid encoding immunogenic genes from other pathogens and transformation of this plasmid into bacterial cells, which results in construction of a bacteria strain carrying this foreign gene. Once this bacteria being delivered into animals or humans, the foreign protein will be expressed *in vivo* which in turn triggers antigen-specific immune responses. An important advantage of bacteria-vectored vaccine is that it can be delivered orally thus enhances mucosal IgA response on the mucosal lining. This is particularly attractive for prevention of pathogens that replicate in mucosal surface. Importantly, the mucosal lining is the first line of defense against mucosal pathogens and strengthening the IgA mucosal immune response is a key for preventing mucosal diseases. The administration of purified antigens can lead to degradation or poor absorption by the GI tract. An advantage of using bacteria as an antigen delivery vector would provide continuous local protein expression to stimulate pathogen-specific mucosal immunity.

Initially, several intracellular pathogenic bacteria have been used for vaccine vectors which include *Salmonella typhimurium, Listeria monocytogenes,* and *Mycobacterium bovis* (Mercenier, Müller-Alouf, & Grangette, 2000). Recently, many attenuated intracellular bacteria have been developed for vaccine delivery. However, these attenuated pathogenic bacteria vaccines may not be suited for children, elderly, or immunocompromised individuals (Mercenier et al., 2000).

1.14. Lactic acid bacteria (LAB)-based vaccines

Alternatively, non-pathogenic lactic acid bacteria (LAB) have been developed as vaccine vectors including *Lactococcus, Streptococcus and Lactobacillus*. LAB are generally not pathogenic therefore, are a safer option for all ages to consume. The ability of LAB to survive in

acidic environments allows for a higher survival rate after passage through the harsh stomach acid. Some strains from the *Lactobacillus* genus are able to colonize the GI and urogenital tract for long-term survival (Mercenier et al., 2000). The GRAS status and nisin characteristics make *Lactococcus lactis* a common tool for genetic engineering and protein expression (Kuipers et al., 1995). These findings opened a new door to use LAB as a vector to develop a safe and efficacious vaccine. Over the last 28 years, LAB has proved to be an excellent vector for many viral and bacterial pathogens.

1.14.1. Examples of LAB-based vaccines for bacterial pathogens

Starting in 1990, there have been many *L. lactis* vectored vaccine candidates for pathogenic bacteria including tetanus toxin fragment C (TTFC) from *Clostridium tetani, Streptococcus mutans,* Enterohemorrhagic *Escherichia coli, Staphylococcus aureus, Yersinia pseudotuberculosis* and *Rhodococcus equi* (Mercenier et al., 2000). The first *L. lactis* vectored vaccine used the surface protein Pac from *Streptococcus mutans*. *S. mutans* creates plaques on teeth and can cause dental decay. In Iwaki et al. (1990) study, the Pac protein was expressed on the cell surface and in the cytoplasm of *L. lactis* (Iwaki et al., 1990). When killed *L. lactis* vectored Pac was intragastrically inoculated in mice, high titers of Pac-specific mucosal IgA and serum IgG were produced following two boosters. However, the mice were not challenged so the protection from *S. mutans* was not measured (Iwaki et al., 1990).

Streptococcus gordonii is a LAB gram-positive bacteria that normally colonizes the mouth of humans. *S. gordonii* has been used as a vaccine vector for several pathogens such as

Streptococcus pyogenes, human papillomavirus, HIV-1, and measles virus (Mercenier et al., 2000). A vector containing the tetanus toxin fragment C (TTFC) was transformed into *S. gordonii* resulting in expression of TTFC on the surface. The toxin released from *Clostridium tetani* is cleaved into a holotoxin then affects the nervous system by preventing nerves from releasing neurotransmitters. However, the C fragment of the tetanus toxin is not toxic and provides immunogenic properties. When TTFC vectored with *S. gordonii* was immunized in mice, high IgG and IgA titers were observed resulting in protection from the *Clostridium tetani* challenge (Medaglini et al., 1997).

In Ahmed et al. (2014) study, *L. lactis* vectored with the EspB protein of *E. coli* serotype O157:H7 was evaluated for its ability to generate an immune response (B. Ahmed, Loos, Vanrompay, & Cox, 2014). Enterohemorrhagic *Escherichia coli* serotype O157:H7 causes diarrhea symptoms and hemorrhagic colitis in addition to hemolytic–uremic syndrome in severe cases. The EspB protein was intracellularly expressed in *L. lactis* and immunized in mice via oral inoculation. Ten days following an intraperitoneal booster, significant fecal IgA and serum IgG responses were observed showing systemic and mucosal immune responses to EspB (B. Ahmed et al., 2014).

L. lactis vectored with the virulence-associated protein A (VapA) from *Rhodococcus equi* has proven to secrete VapA and was tested for its ability to generate an immune response. *Rhodococcus equi* has caused incidences of disease in people who are immunocompromised as well as acute pneumonia in foals. The immune response and protection was measured in mice

with three doses 2 weeks apart. The mice immunized via an intragastric route had higher mucosal IgA antibody titers compared to intranasal inoculation. Both intranasal and intragastric groups had T-helper based immune responses measured by IL-4 and IFN-γ levels. Interestingly, the intranasal inoculated mice had significantly lower amounts of *R. equi* following the challenge (Cauchard et al., 2011).

Staphylococcus aureus can cause gastroenteritis, respiratory disease and toxic shock syndrome as a result of infection. The exotoxin *Staphylococcal* endotoxin B (SEB) is expressed by *Staphylococcus aureus* during infection and used as the antigen expressed by *L. lactis.* Two variants were constructed including expression of SEB in the cytoplasm and SEB secretion into the medium. Mice immunized orally with *L. lactis* vectored SEB were protected from the *S. aureus* challenge proving its protective properties. The cytoplasm expressed SEB provided complete protection while secretions of SEB provided partial protection. The *L. lactis* vectored SEB also induced an immune response starting day 14 including fecal IgA and serum IgG, then increased over 42 days (Asensi et al., 2013).

To create a vaccine candidate for *Yersinia pseudotuberculosis, L. lactis* was constructed to express *Y. pseudotuberculosis* low calcium response V (LcrV). In humans, *Y. pseudotuberculosis* can induce ileitis and mesenteric lymphadenitis disease. To demonstrate the immune response and protection, mice were immunized intranasally or intragastrically with booster twenty days apart then challenged. The intranasally inoculated mice had a high titer of specific mucosal IgA from bronchoalveolar lavage (BAL) fluid and a high specific IgG response

with majority IgG1. The majority (90%) of the intranasally inoculated mice were protected from the *Y. pseudotuberculosis* challenge representing the protection *L. lactis* vectored LcrV provided (Asensi et al., 2013).

1.14.2. Examples of LAB-based vaccines for viral pathogens

There have been several *L. lactis* vectored vaccines for viral pathogens over the last couple decades including influenza virus and human immunodeficiency virus (HIV). Currently, at least ten independent studies demonstrated that LAB-based vaccine is highly promising for influenza virus. The avian influenza virus H5N1 causes respiratory infections in birds in addition to hundreds of human cases. The influenza hemagglutinin (HA1) containing vector was transformed into *L. lactis* resulting in HA1 expression on the surface. Germ-free mice were immunized with HA1 vectored with *L. lactis* via intragastric lavage then boosted over two intervals. The vaccination with cholera toxin B subunit acting as an adjuvant induced the mice to produce significantly more fecal IgA and serum IgG compared to *L. lactis* vectored HA alone. For the challenge, the cholera toxin B adjuvanted group with *L. lactis* vectored HA vaccine were completely protected from H5N1 influenza (Lei et al., 2011). In another study, Wang et al., (2012) generated *Lactococcus lactis* MG1363 expressing avian influenza virus HA1, and oral vaccination of mice with this vaccine candidate induced specific anti-HA(1) IgA antibody in the intestine, specific anti-HA(1) IgG antibody in the serum, and T cell responses. Most importantly, the mice were protected against lethal challenge of the H5N1 virus. In addition, LAB-based

influenza virus neuraminidase (NA) vaccine has been reported. Mice vaccinated orally with L.lactis/pNZ8110-pgsA-NA could elicit significant NA-specific serum IgG and mucosa IgA antibodies, as well as neuraminidase inhibition (NI) titers. Importantly, L.lactis/pNZ8110-pgsA-NA vaccination triggered cross-protection against different influenza virus strains, providing 80% protection against H5N1 and 60% protection against H3N2 and H1N1. Jee et al., (2017) showed that oral immunization of mice with LAB-based influenza virus vaccine (LL-HA1/L/AcmA) elicited mucosal immunity in both the gastrointestinal tract and the respiratory tract, and provided protection against lethal influenza virus challenge. These results highlight the potential application of *L. lactis* as a platform for delivery of influenza virus vaccine.

Human immunodeficiency virus (HIV) has infected 37,000 people on average in the U.S. every year and about 6,000 die from complications (CDC, 2017). Currently, there is no FDA-approved HIV vaccine. Current most of the current HIV vaccines under development use the I.M. route for immunization, which is relatively poor in generating mucosal immune responses. Recently, A LAB-vectored HIV vaccine candidate was developed. Specifically, *L. lactis* expressing the envelope protein of HIV on the surface of the cell was constructed. Oral immunization of mice with LAB-based HIV vaccine induced high levels of HIV-specific serum IgG and fecal IgA antibodies. A strong cell-mediated immune response was detected using immune cells isolated from lymph nodes and the spleen. To determine the protective efficacy, mice were challenged intraperitoneally with an HIV Env-expressing vaccinia virus. The viral loads in vaccinated mice were 350-fold lower than those of control mice. This demonstrates the immune response to the *L. lactis* vectored HIV env has the potential to protect from the HIV infections (Xin et al., 2003).

Most recently, Chamcha et al., (2015) showed that oral vaccination with a probiotic organism, *Lactococcus lactis*, elicited HIV-specific immune responses in the mucosal and systemic compartments of BALB/c mice. A LAB-based HIV vaccine expressing the HIV-1 Gag-p24 on the tip of the T3 pilus of *Streptococcus pyogenes* as a fusion to the Cpa protein (LL-Gag) was generated. After oral immunization of LL-Gag, strong Gag-specific IgG and IgA responses in serum, feces, and vaginal secretions were detected. These results demonstrate that oral immunization with LAB-based vaccine is an excellent vaccine platform to induce strong mucosal humoral and cellular immunity against HIV.

In conclusion, LAB-based vaccine is highly promising for prevention of infectious diseases. However, whether LAB can be used as a vector to deliver HuNoV vaccine has not been explored, which will be the focus of this study.

CHAPTER 2

A Novel Lactic Acid Bacteria (LAB)-based Vaccine Candidate for Human Norovirus

2.1. Abstract

Human noroviruses (HuNoVs) are responsible for more than 95% of the non-bacterial acute gastroenteritis epidemics in the world. The CDC estimates that every year 21 million individuals suffer from HuNoV-induced gastroenteritis in the U.S. Currently, there is no FDA-approved vaccine for HuNoVs. Development of an effective vaccine has been seriously hampered by the lack of an efficient cell culture system for HuNoVs and a suitable small animal model. The goal of this study is to develop lactic acid bacteria (LAB) as a vector to deliver HuNoV antigen. To do this, a LAB bacteria strain (*Lactococcus lactis*) carrying VP1 gene of a HuNoV GII.4 virus (LAB-VP1) was constructed. It was found that HuNoV VP1 protein was highly expressed by LAB vector. Subsequently, a novel microencapsulation technology was developed to enhance the stability of LABs in low and high pH environments. To test whether LAB-based

HuNoV vaccine is immunogenic, 4-day-old gnotobiotic piglets were orally inoculated with various doses of LAB-VP1 either with or without microencapsulation. It was found that LABs were persistent in the small intestine of piglets and shed in pig feces for at least 25 days post inoculation. Live LABs or LAB DNA were found in mesenteric lymph nodes and spleen tissue in LAB-VP1 inoculated groups. HuNoV-specific IgG and IgA were detectable in serum and feces at day 13 post-inoculation, respectively, and further increased at late time points. After challenge with HuNoV GII.4 strain, a large amount of HuNoV antigens were observed in the duodenum, jejunum, and ileum sections of the intestine in the LAB control group. In contrast, significantly less or no HuNoV antigens were detected in the LAB-VP1 immunized groups. Collectively, these results demonstrate that LAB-based HuNoV vaccine induces protective immunity in gnotobiotic piglets.

2.2 Introduction

Human norovirus (HuNoV) is the causative agent of more than 95% of nonbacterial acute gastroenteritis cases worldwide (Estes et al., 2006). HuNoV is responsible for over 60% of foodborne illnesses in the U.S. It is estimated that 21 million people are infected by HuNoV which result in approximately 800 deaths annually in the U.S. HuNoV causes a global economic burden with $60.3 billion in healthcare costs on a yearly basis (Bartsch et al., 2016; Belliot et al., 2014). HuNoV is highly infectious and contagious, and approximately 10 virus particles are sufficient to establish an infection (Caul, 1994; Donaldson et al., 2008). However, research on

HuNoV has been hampered because it cannot be efficiently grown in cell culture and lacks a suitable small animal model. Currently, HuNoV is listed as a "candidate contaminant" for the regulation of drinking water by EPA and is classified as a Category B Priority Pathogen by the National Institute of Allergy and Infectious Diseases (NIAID, 2016). To date, there is no FDA approved vaccine or antiviral drug to combat with HuNoV. Vaccination is the most effective strategy to prevent infectious diseases. A HuNoV vaccine is urgently needed to protect the population, particularly for the most susceptible populations including infants, children, the elderly, and immunocompromised individual (Bartsch et al., 2016).

Since the discovery of HuNoV in 1968, tremendous efforts have been devoted to develop a safe and efficacious HuNoV vaccine. Currently, most HuNoV vaccine studies have been focused on a subunit vaccine using the viral capsid protein (VP1) as the antigen. Expression of the VP1 gene in insect cells can lead to the subsequent self-assembly of VLPs that are structurally and antigenically similar to native virions. It has been shown that VLP-based vaccines triggered a variable level of HuNoV-specific serum antibody response and mucosal immunity in in mice, rabbits, guinea pigs, gnotobiotic piglets, and chimpanzees (Ball et al., 1998; Bok et al., 2011; Jiang et al., 1992; Souza et al., 2007). Furthermore, the VLPs-based vaccine candidate has been tested in human clinical trials. In 1999, Ball et al. performed the first clinical study to demonstrate that human norovirus VLPs were safe and immunogenic (Ball* et al., 1999). Over the last 18 years, there have been multiple phase I and II clinical trials of VLP based vaccines to assess the protection and immune response potential. A recent human clinical trial showed that Norwalk virus–specific IgA antibody was detected in 70% of the vaccine recipients.

After challenge with the Norwalk virus, it was found that vaccination significantly reduced the frequency of Norwalk virus gastroenteritis. Of the placebo participants, 67% developed gastroenteritis whereas only 37% of vaccine recipients developed symptoms (Atmar et al., 2011). Despite the promise raised by these studies, production of VLPs is time-consuming and expensive, and immunization requires high VLP doses, multiple boosters, and mucosal adjuvants such as *V. cholerae* and *E. coli* enterotoxins (Ball et al., 1998). In addition, immunogenicity of such vaccines is limited as the VLPs are non-replicating immunogens.

Since conventional live attenuated vaccine cannot be developed from a virus that cannot be efficiently propagated *in vitro*, several viral vectors have been reported to deliver HuNoV vaccine candidates. These viral vectors include Venezuelan equine encephalitis (VEE), adenovirus, vesicular stomatitis virus (VSV), and Newcastle disease virus (NDV) (Guo et al., 2008; Harrington et al., 2002; S.-H. Kim et al., 2014; Ma & Li, 2011). Mice vaccination studies immunized with these viral vectored vaccine candidates triggered strong HuNoV-specific immunities. (Guo et al., 2008; Harrington et al., 2002; S.-H. Kim et al., 2014; Ma & Li, 2011). Unfortunately, the safety concern of these viral vectors limited their practical application in humans.

A live bacteria delivery system offers enormous potential for the development of new vaccines against infectious diseases. However, this novel strategy has been explored in HuNoV vaccine development. Food grade lactic acid bacteria (LAB) are an excellent platform to fulfill this requirement. Food grade LAB are an attractive delivery system as they are non-pathogenic,

effective in delivering antigens to the mucosa, and are FDA approved GRAS (Generally Recognized As Safe) agents. Several species of *lactobacilli* and *lactococci* are known to be excellent vehicles for delivery of vaccines against a spectrum of infectious agents including HIV, rotavirus, and human papillomavirus, porcine circovirus type 2 (PCV2), *Streptococcus pneumoniae, Clostridium tetani*, *Brucella abortus, Rhodococcus equi, and Staphylococcus aureus* (Asensi et al., 2013; Cauchard et al., 2011; Frankel et al., 1995; Hanniffy, Carter, Hitchin, & Wells, 2007; Miyoshi et al., 2002; Robinson et al., 1997; K. Wang, Huang, Kong, & Zhang, 2008). *Lactococcus lactis* is a gram-positive lactic acid producing bacterium commonly used in the dairy industry. In addition to its high safety profile, oral vaccination of mice with *Lactococcus lactis* vectored vaccine induced a strong systemic immune response and mucosal immune response. This vaccine strategy is particularly attractive for HuNoV, as an ideal HuNoV vaccine must be safe, stable, inexpensive, easy to deliver, and induce robust humoral, mucosal, and cellular immune responses at sites where pathogens interact with the host.

In this study, we have developed a “live” LAB-based HuNoV vaccine candidate. The major capsid gene (VP1) of a GII.4 HuNoV strain was cloned into a LAB expression vector pNZ8150, which were subsequently transformed into *Lactococcus lactis* by electroporation resulting in a LAB bacteria strain expressing VP1 (LAB-VP1). Subsequently, we showed that HuNoV VP1 protein was highly expressed by LAB vector and the expressed VP1 was secreted into media supernatants. To enhance the stability of LAB-VP1, a novel microencapsulation technology was developed to encapsulate the LAB-VP1 into microparticles. Oral vaccination of LAB-VP1 with or without microencapsulation in gnotobiotic piglets triggered HuNoV-specific IgA

and IgG responses and prevented HuNoV infection in pig intestine. Collectively, these results demonstrate that LAB-based HuNoV vaccine is immunogenic in gnotobiotic piglets. Our results also suggest that LAB-based HuNoV vaccine is a promising vaccine candidate for HuNoV.

2.3. Materials and methods

2.3.1. Preparation of human norovirus inoculum

The HuNoV GII.4 strain 766 was originally obtained from stool samples collected from an outbreak of acute gastroenteritis in Ohio. Stool samples were diluted 1:2 in minimal essential medium (MEM; Gibco-Invitrogen, Carlsbad, CA) and further processed by vortexing, centrifugation at 3,500 × *g* for 20 min, and filtration through a 0.8-µm-pore-size filter followed by a 0.2-µm-pore-size filter. The possibility of the presence of other enteric viral pathogens, such as human rotavirus, human sapovirus, and human astrovirus, was excluded by RT-PCR analysis prior to initiation of the study. The amount of RNA copies in the HuNoV strain 766 filtrate was quantified by real-time RT-PCR, and the level of RNA was 2.1×10^8 RNA copies/ml. Viruses were aliquoted and stored at −80°C until used.

2.3.2. Bacterial cultures

The Nisin controlled gene expression (NICE) system strain NZ9000 *Lactococcus lactis subsp. cremoris* containing regulatory genes nisR and nisK integrated into the pepN gene was used as a vector for HuNoV this study. The VP1 gene of HuNoV GII.4 strain 766 was amplified by RT-PCR then cloned into pNZ8150 NICE expression secretion vector (MoBiTec), which contains a chloramphenicol resistance gene using cloning site NaeI resulting in a pNZ8150- GII.4

VP1 vector. This pNZ8150- GII.4 VP1 vector was transformed into *Lactococcus lactis* via electroporation providing lactic acid bacteria (LAB) capable of expressing GII.4 VP1 protein named LAB-VP1. In addition, the empty pNZ8150 vector was transformed into *Lactococcus lactis* to use for control purposes. This control LAB was named LAB empty vector control. For the culturing of LAB-VP1 and LAB empty control, M17 medium and agar containing 1% (wt/vol) glucose and 10µg/ml chloramphenicol were used along with a GasPak anaerobic chamber providing optimal anaerobic growing conditions for the LAB-VP1 to grow overnight at 30^{o}C. For long term storage of LAB-VP1, M17 medium containing 25% glycerol was aliquoted and placed in -80^{o}C.

2.3.3. Protein expression

The expression of HuNoV VP1 protein was detected using Western blot analysis of the supernatant and cell lysate. A single cloning of LAB-VP1 or LAB control was grown in M17 medium overnight then diluted 1/25 in 2 tubes with 10ml of M17 for it to grow for another 4-5 hours until OD600 of 0.4 was reached. One of the 10ml tubes was induced with 2ng/ml Nisin, and the second tube served as control. The LAB-VP1 was incubated for another 5 hours then the supernatant and cells were harvested separately by centrifugation at 4000 rpm for 10 min at room temperature. The cells were resuspended in 300 µl of lysate buffer (50mM Tris-HCl, 2mM EDTA, 10mM NaCl, 0.1% Triton X-100, pH 8) and 10mg/ml lysozyme and incubated at 37C for 3 hours then ultrasonicated 3 times for 40 s. The supernatant was ultracentrifuged at 30,000 rpm for 2 hours and the pellet was resuspended in lysate buffer. The VP1 protein was analyzed by Western blot analysis. The cell lysate and supernatant from Nisin and control

samples were prepared by adding 5 x loading buffer and boiling for 7 min. For SDS-PAGE, the samples were added to a 10% acrylamide gel then ran at 80 V for 30 min then 120 V for 1 h. Then the gel was transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham) via a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). The primary antibody guinea pig anti-HuNoV VP1 antiserum (a generous gift from Dr. Xi Jiang, Cincinnati Children Hospital) was diluted 1:5000 in blocking buffer (5% non-fat milk) followed by horseradish peroxidase-conjugated goat anti-guinea pig IgG secondary antibody (Santa Cruz) at a dilution of 1:10,000. The blot was developed via a SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and exposed to Kodak BioMax MR film (Kodak).

2.3.4. Development of a novel microencapsulation technology to encapsulate LAB into microparticles

In collaboration with Dr. Xiaoming He's laboratory in the Department of Bioengineering at OSU, we have developed a novel microencapsulation technology to encapsulate LAB into microparticles.

2.3.4.1. Preparation of the oil emulsion

A calcium chloride solution was prepared by dissolving 1 g of calcium chloride in 1 mL of deionized water. A mixture, consisting of 5 mL of mineral oil, 93.3 µl of Span 80, and 1 mL of calcium chloride solution, was emulsified using the Branson 450 Digital Sonifier at the amplitude of 20% for 1 min to obtain the oil emulsion.

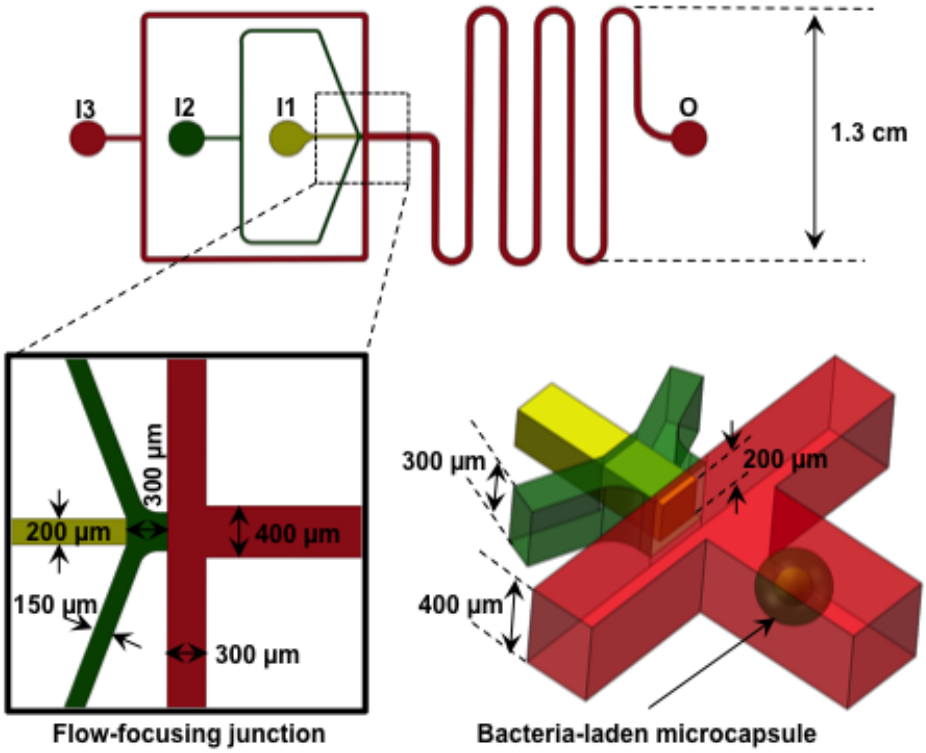


Figure 8. A schematic illustration of the microfluidic device for bacteria encapsulation, and the zoom-in image and 3D image of the flow-focusing junction. The total length of the microchannel between the flow-focusing junction and the exit (O) is ~9.8 cm.

2.3.4.2. Microfluidic encapsulation

As shown in Fig. 8, the bacteria were suspended into a solution of 1% (w/v) high viscosity carboxymethyl cellulose in saline, and introduced into the device via I1 at a flow rate of 100 µl/h. A solution of 2% (w/v) sodium alginate in saline was introduced into the device via I2 at a flow rate of 500 µl/h. The oil emulsion was introduced into the device via I3 at a flow rate of 8 ml/h. The core-shell-structured droplets were generated in the flow-focusing junction, and gelled when moving with the oil emulsion. The bacteria-laden microcapsules were collected from the device via O in a 50 ml centrifuge tube containing 20 ml of saline, and then

centrifuged at 300 rpm (50×g) for 5 min, washed using 20 ml of saline for one time, and suspended in 20 ml of saline in a 50 ml centrifuge tube for further use.

2.3.4.3. Chitosan-alginate-chitosan (C-A-C) coating on microcapsules

Firstly, the bacteria-laden microcapsules were suspended in 5 ml of a solution of 0.4% (w/v) chitosan in saline for 5 min in a 50 ml centrifuge tube to obtain the first layer of chitosan coating. The bacteria-laden microcapsules were then centrifuged at 300 rpm (50×g) for 5 min, washed using 20 ml of saline for one time, and then suspended in 5 mL of a solution of 0.15% (w/v) sodium alginate in saline for 5 min in the 50 ml centrifuge tube to obtain the second layer of alginate coating. Afterwards, the bacteria-laden microcapsules were centrifuged at 300 rpm (50×g) for 5 min, washed using 20 ml of saline for one time, and then suspended in 5 ml of the solution of 0.4% (w/v) chitosan in saline for 5 min in the 50 ml centrifuge tube to obtain the third layer of chitosan coating. Finally, the bacteria-laden microcapsules were centrifuged at 300 rpm (50×g) for 5 min, washed using 20 ml of saline for one time, and suspended in 20 ml of saline in the 50 ml centrifuge tube for further use.

2.3.5. The stability of *L. lactis* in pH differences

The stability of LAB-VP1 was assessed at pH 2, 4, 7, and 8.5 at 37ºC and pH 7 at 4ºC at differing time points. The LAB-VP1 was grown overnight as described above then undiluted, diluted 10 (1:10) and 100 (1:100) times, samples were centrifuged for 1 min at 4,500 rpm at room temperature. For the 10 times concentrated samples, LAB-VP1 was centrifuged 4,000 rpm for 10 min at room temperature. Then saline buffer with different pH was added and the

mixture was aliquoted by adding 0.5 ml per tube and placed at 37ºC. For stability assay at pH 2, the undiluted and 10 x concentrated tubes were removed at 30, 60, 90, and 120 min. The diluted 1:10 and 1:100 tubes (pH 2) were removed at 3, 6, 9, 12, 15, and 18 min. The pH 7 and pH 8.5 tubes were removed at 12, 24, 36, and 48 h. The pH 4 tubes were removed at 4, 8, and 12 h. For stability assay at pH 7 at 4ºC, tubes were removed at 3, 7, 10, 14, 17, 21, 24, and 28 day. The pH reaction was stopped by centrifuging 4,500 rpm for 1 min at room temperature then 1 ml of M17 medium was added and vortexed to resuspend the bacteria. The survival was calculated by a 10-fold serial dilution with saline then plated several dilutions on M17 plates for each time point. After 2 days the colony forming units (cfu) were quantified to determine the original titer at the assigned time points.

2.3.6. The stability of microparticles in different pH

The stability of microencapsulated LAB-VP1 (microparticles) and non-microencapsulated LAB-VP1 was compared at pH 7, 8.5, and 2. The microparticles and LAB-VP1 were placed in pH 7, 8.5, and 2 saline then aliquoted to 0.5 ml per tube and placed at 37ºC. For stability assay at pH 7 and 8.5, each tube was removed at 12, 24, 36, and 48 h. For the stability assay at pH 2, the tubes were removed at 3, 6, 9, 12, and 15 min. To dissolve the microparticles, 1 ml of 75 mM trisodium citrate (TSC) was added and vortexed. Non-microencapsulated LAB-VP1 was used as a control with the same procedure as described previously with 1 ml of TSC to stop the reaction. The survival was quantified by plate counts as described previously.

2.3.7. Delivery of gnotobiotic piglets

The animal protocol used in this study was following the USDA regulations and guidelines of Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Also, the animal protocol was approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee (animal protocol no. 2015A00000118). Two adult artificially inseminated pregnant Landrace sows were purchased from a commercial pork production unit (Shoup Brothers, Smithville, OH) and transported to the Goss Laboratory at The Ohio State University. The sows were on their 113th day of gestation and provided 21 and 19 piglets, respectively. The sows were injected in the spine with lidocaine, ketamine and telazol 10 min prior to the surgery. There were only 25 piglets that survived due to some piglets exhibiting severe hypoglycemia overnight. The piglets were from 2 sows delivered via a closed hysterectomy with a midline abdominal incision then the uterus was delivered through bleach water tunnel into a surgical isolator for the caesarian section. Then the piglets were transferred through a plastic sleeve into an attached rearing isolator in order to attached umbilical clamps, resuscitate and dry them with sterile towels. The rearing isolator was closed and detached then the piglets were separately housed in sterile isolators made of vinyl canopies connected to pentubs with 6 partitions and maintained under positive pressure via 2 AW-40 filters for the entirety of the experiments. The derivation units, 4 isolators and supplies were sterilized via spor-klenz (peracetic acid, hydrogen peroxide, and acetic).

For an external heat source, several heat lamps were stationed around the isolators to keep the temperature at 33-34°C for the first week and reduced over a three week period to 23ºC. The piglets were fed Natrel whole milk three times a day in a weekly increasing volume schedule. On the first day the piglets received vitamin B complex and Iron intramuscularly (IM). Contamination was checked 3 days prior to derivation and 1 day before euthanasia. Sterile swabs (8/isolator) were used to pick up contamination in each isolator then 4 of the swabs were streaked on sheep blood agar plates and 4 of the swabs per isolator were placed in soy broth. The plates were placed in aerobic and anaerobic conditions at 37ºC along with the soy broth. The plates and broth were checked for contamination at 24, 48, and 72h.

2.3.8. Immunogenicity of LAB-VP1 vaccine candidate in gnotobiotic piglets

Briefly, four-day-old newborn gnotobiotic piglets were inoculated orally via oral gavage with 10 ml of LAB-VP1 at three different doses, 10^9 cfu, 10^{10} cfu, and 10^{12} cfu, microparticles containing 10^9 cfu of LAB-VP1, and LAB vector control at 10^{12} cfu in saline. Before inoculation blood, fecal, nasal and vaginal swabs were taken as controls. At day 6, 13, and 20 post inoculation blood, fecal, nasal and vaginal swabs were collected. The blood samples were centrifuged at 10,000 rpm for 2 min to separate the sera which were used for detection of HuNoV-specific IgG by ELISA. The fecal, nasal and vaginal swabs were used for the detection of HuNoV-specific IgA by ELISA. The other half of the fecal swabs was used to enumerate the *L. lactis*. At day 20 post-inoculation, the piglets were challenged with 1.0×10^7 genomic RNA copies of HuNoV GII.4 strain 766 diluted in 5 ml of saline given via oral gavage. The following 4 days fecal samples were collected to quantify the RNA copies of HuNoV. At day 5 post-

challenge, the piglets were euthanized and necropsied. The spleen, mesenteric lymph nodes, intestinal pieces, jejunum content, blood and colon content were taken to quantify *L. lactis* by plating, quantify HuNoV RNA by real-time RT-PCR, detect HuNoV antigen expression by immunofluorescence assay (IFA), and determine the IgA or IgG antibody titers.

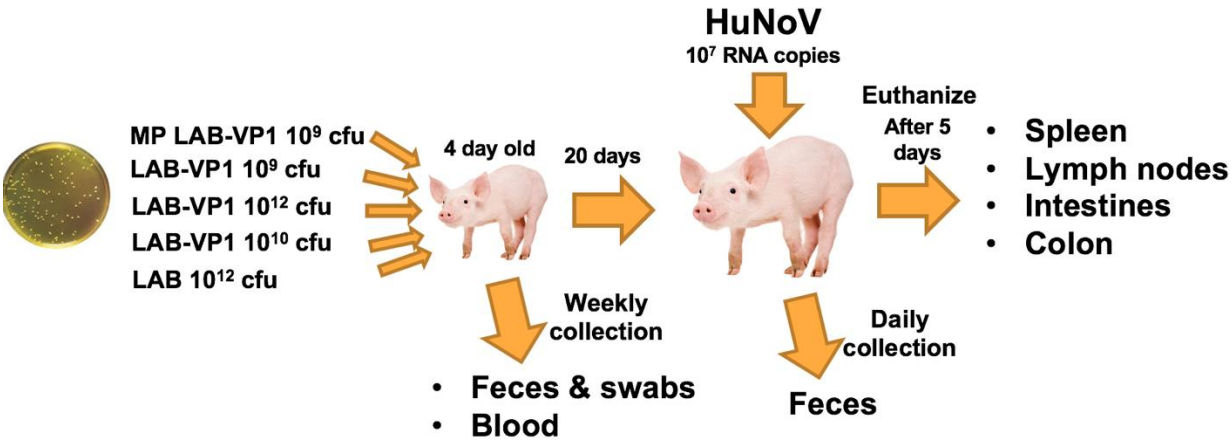


Figure 9. Flow diagram of gnotobiotic piglet experimental design

Table 3. Information about the piglet groups

Group	Dose	Piglets
MP LAB-VP1	10^9 cfu	6
LAB-VP1	10^9 cfu	3
LAB-VP1	10^{12} cfu	6
LAB-VP1	10^{10} cfu	5
LAB	10^{12} cfu	5

Table 4. Samples collected at necropsy and purposes for the samples

Tissues	Purpose
Spleen	Quantify *L. lactis*
Mesenteric Lymph Nodes	Quantify *L. lactis*
Intestinal pieces from all 3 sections	Quantify *L. lactis*
	Immunofluorescence
Jejunum content	Quantify *L. lactis*
Colon content	Quantify *L. lactis*
	Quantify HuNoV

2.3.9. Quantification of LAB titer in pig tissues and LAB shedding in pig feces

The intestinal pieces from duodenum, jejunum and ileum were placed in 2 ml of saline and weighed prior to processing. Each piece was scraped with a scalpel on a petri dish then 10-fold serial diluted in saline and plated on M17 plates as described previously. The colon and jejunum content were weighted then diluted with saline (2x vol/weight) then serially diluted and plated as previously described. The mesenteric lymph nodes and spleen were weighted

first then homogenized with 15 ml dunces in 3 ml saline then serially diluted and plated as described previously. All experiments were done aseptically to avoid any cross-contamination.

2.3.10. Detection of HuNoV RNA by real-time RT-PCR

The piglet fecal samples were eluted in 300 µl of DMEM at a 2:1 (vol/weight) dilution. The fecal samples were vortexed then centrifuged at 10,000 rpm for 15 min at 4ºC. Then the supernatants were collected to extract RNA with the RNeasy Mini kit (Qiagen, Valencia, CA) and eluted in 30 µl of ultrapure water. The HuNoV RNA was detected and quantified by real time RT-PCR using primers annealing to RNA-dependent RNA polymerase (RdRp) gene of HuNoV. The first-strand cDNA was synthesized using SuperScriptase III (Invitrogen) using a RdRp reverse primer (5'- ACCACGCTAGGAGAAAGAAGGTC-3') in addition to 10mM dNTP, 5µl of RNA template, 1 unit of SuperScriptase III, 5x first strand buffer, and 0.1M DTT. To amplify the RdRp gene, a combination of SYBR Green Master Mix (Takara), ROX, forward primer (5'-AGTTGGCATGAATATGAATGAGGA-3') and reverse primer (5' ACCACGCTAGGAGAAAGAAGGTC-3') with 5µl of cDNA template were placed in a StepOne real-time PCR machine (Applied Biosystems, Foster City, CA). For each cycle, a holding stage at 95°C was maintained for 2 min prior to cycling, followed by 40 cycles of 94°C for 15 s for denaturation, 55°C for 30 s for annealing, and 72°C for 15 s for extension. The sample CT values were analyzed with the HuNoV RdRp plasmid of known concentration to calculate the $\log_{10}$ RNA copies/g.

2.3.11. Indirect immunofluorescence assay (IFA) on whole intestinal tissue mounts

Indirect immunofluorescence was performed on whole-mount intestinal tissues. Pieces of duodenum, jejunum, and ileum from inoculated pigs were collected and fixed with 2 ml of 4% paraformaldehyde-0.2% glutaraldehyde in 0.1 M potassium phosphate buffer (PPB) (pH 7.4) for 2 h at RT. The fixed samples were washed four times with PPB, and quenched with 1ml of PPB containing 50 mM glycine and stored at 4ºC. After quenching overnight at 4ºC, sections of the tissues were cut and permeabilized with 0.3ml of 0.1% Triton X-100 in PBS for 1 h then washed 3 times with PBS. Then the tissues were blocked with 0.5 ml PBS containing 2% bovine serum albumin and 5% goat serum for 1.5 h at RT. The tissues were incubated with guinea pig anti-HuNoV at 1:5,000 dilution overnight at 4°C in 0.3 ml of incubation buffer containing 10 mM potassium phosphate buffer (PPB) [pH 7.4], 150 mM NaCl, 10 mM sodium azide, and 0.2% bovine serum albumin. After washing with PBS six times, the tissues were incubated with the secondary antibody goat anti-guinea pig IgG (Invitrogen; A11075) labeled with AlexaFluor488 [Ex (nm) 499, Em (nm) 519], which produces green color at a dilution of 1:800 in 0.3 ml of incubation buffer. The tissues were then stained with the nuclear stain SYTOX orange (Invitrogen; S11368) [Ex (nm) 547, Em (nm) 570] diluted 1:1000 in PBS for 15 min on a shaker, giving a red color. Then the actin was stained with AlexaFluor633-labeled 149 phalloidin (Invitrogen; A222884) [Ex (nm) 632, Em (nm) 648] diluted 1:20 for 45min on a shaker, producing a blue color. Samples were examined using a laser scanning confocal microscope (Olympus FV-1000, Germany) at the Microscopy Facility at The Ohio State University.

2.3.12. Serum IgG ELISA

The serum samples collected at post inoculation day (PID) 6, 13, 20 and post challenge day (PCD) 5 were used to detect HuNoV-specific IgG. Ninety-six-well plates were coated with 50 µl of highly purified HuNoV VP1 protruding domain particles (P particles) at 20 µg/ml concentration in 50 mM NaCO3 buffer (pH 9.6) at 4°C overnight. The plates were blocked to prevent nonspecific proteins binding via 0.2ml per well of 1% (weight/vol) bovine serum albumin (BSA) in PBS-Tween (0.05%) for 2h at 37ºC. Serum samples were 2-fold-serially diluted with 0.5% BSA in PBST and 50µl per well of each dilution was added to P particle-coated wells. After incubation at 37ºC for 2 h, the plates were washed three times with PBST, followed by incubation with 100 µl of rabbit anti-swine IgG horseradish peroxidase (HRP) conjugated secondary antibody (Sigma) at a dilution of 1:500 for 1 h at room temperature (RT). Plates were washed three times with PBST then developed with 100µl of 3'3',5'5'-tetramethylbenzidine (TMB). Then the reaction was stopped with 100µl of 2M sulfuric acid and the optical density (OD) at 450 nm was determined using an enzyme-linked immunosorbent assay (ELISA) plate reader. End point titer values were determined as the reciprocal of the highest dilution that had an absorbance value greater than background level from the LAB empty vector control.

2.3.13. Fecal IgA ELISA

For the fecal samples, HuNoV-specific IgA was determined in PID 6, 13, 20 and PCD5 samples via plates coated with P-particles. Fecal pellets were diluted 2:1 (vol/weight) in PBS containing 0.1% 150 Tween and a Complete EDTA-free protease inhibitor cocktail tablet (Roche). Samples were vortexed twice for 30 s with 5 min on ice in between then centrifuged at 10,000 × g for 10 min. The supernatant was removed to a new tube then centrifuged again. Ninety-six-well plates were coated with 50 µl of highly purified HuNoV P particles at 15 µg/ml concentration in 50 mM NaCO3 buffer (pH 9.6) at 4°C overnight. For blocking, the plates were incubated for 2 h at 37°C with 0.2 ml of 1% (weight/vol) BSA in PBST. The samples were 2-fold serially diluted with 0.5% BSA in PBST and 50 µl per well of each dilution was added to P-particle coated plates. The plates were incubated for 2h at 37ºC then washed 3 times. The secondary antibody goat anti-swine IgA HRP conjugated was added at 1:1,000 diluted in 0.5% BSA in PBST incubated 1 h at RT. Plates were washed three times with PBST and developed with 100µl of 3’3’,5’5’-tetramethylbenzidine (TMB) then the reaction was stopped with 100µl of 2M sulfuric acid. The optical density (OD) at 450 nm was determined using an ELISA plate reader. End point titer values were determined as the reciprocal of the highest dilution that had an absorbance value greater than background level from the LAB empty vector control.

2.3.14. Quantification of LAB DNA in spleen and lymph nodes by real-time PCR

Total DNA was extracted from sections of the spleen and mesenteric lymph nodes collected at necropsy to determine the amount of LAB DNA. The spleen and lymph nodes were weighed then processed according to the directions in the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). The DNA was eluted in 30 µl of water. The LAB DNA was quantified by real-time qPCR using primers and a probe designed by Applied Biosystems and the Taqman Fast Universal PCR Master Mix. The real time qPCR procedure was done as described above. The original amount of LAB vector DNA was calculated based on a standard curve using the CT values generated.

2.3.15. Detection of HuNoV VP1 gene in spleen and lymph nodes by PCR

To determine whether VP1 gene presents in the spleen and lymph nodes, the DNA was subjected to traditional PCR using the Platinum Blue PCR SuperMix (invitrogen) using two primers annealing to VP1 gene, forward primer (5'-ATGAAGATGGCGTCGAATGAC3') and reverse (5'-TTATAATACACGTCTGCGCCC-3'). For the PCR, a holding stage at 95°C was maintained for 1 min prior to cycling. Then for denaturation, 40 cycles at 94°C for 20 s, 58°C for 20 s for annealing, and 72°C for 2 min for extension were done on the samples. The PCR product was run on gel electrophoresis to visualize the presence of HuNoV VP1 gene.

2.3.16. Statistical Analysis

All values are expressed as the means ± standard deviations. Statistical analysis by two-tailed student's t-test was performed. A *P* value of <0.05 was considered statistically significant.

2.4. Results

2.4.1. HuNoV VP1 protein is highly expressed by LAB vector

We have constructed recombinant lactic acid bacteria (LAB) expressing HuNoV VP1. The VP1 gene of HuNoV GII.4 strain 766 were amplified by RT-PCR and cloned into the LAB expression vector pNZ8150, which were subsequently transformed into *Lactococcus lactis* by electroporation resulting in LAB bacteria strains designated LAB-VP1. Subsequently, the expression of VP1 protein by the LAB vector was detected by Western blot. Briefly, LAB-VP1 were grown to mid-log phase (O.D. = 0.4) in M17 broth. The cell pellets and supernatants were collected. Proteins from supernatants were precipitated using trichloroacetic acid (TCA) and pelleted by ultracentrifugation. The total protein from both the supernatant and cell pellet was analyzed by SDS/PAGE. As shown in Fig.10A, a 55 kDa protein, consistent with the size of HuNoV VP1, was found in bacterial cell lysates (Fig. 10A, lane 4) and supernatants (Fig. 10A, lane 2). To further confirm this, Western blots were performed using anti-HuNoV VP1 polyclonal antibody. As shown in Fig. 10B, VP1 protein was found in both the supernatant (Fig.

10B, lane 2) and cell pellet (Fig. 10B, lane 4) from LAB-VP1, but not control LAB (Fig. 10B, lane 3). Collectively, these data confirm that: (i) HuNoV-VP1 protein is expressed by the LAB vector and, (ii) Expressed VP1 protein is secreted into media supernatants.

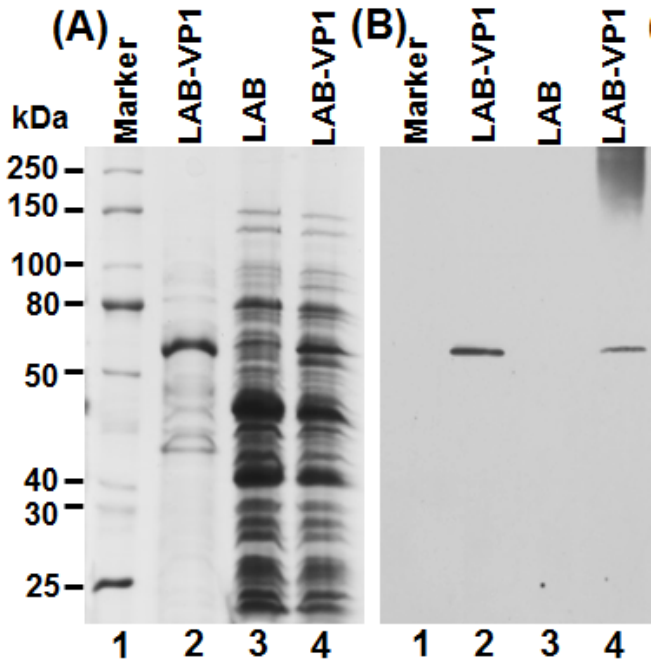


Figure 10. Expression of HuNoV VP1 protein by LAB vector

2.4.2. Stability of LAB-VP1 in neutral pH saline solutions

The stability of LAB-VP1 in acidic and basic solutions was assessed to provide insight to the survival in the digestive system. We first examined the stability of LAB-VP1 in neutral pH (pH 7) saline. Briefly, 10 times concentrated ($10^{10.5}$ cfu/ml), undiluted ($10^{9.5}$ cfu/ml), diluted 1:10 ($10^{8.5}$ cfu/ml), diluted 1:100 ($10^{7.5}$ cfu/ml) LAB-VP1 were resuspended in pH 7 saline and incubated at 37°C for 12, 24, 36 and 48 h. The reaction was neutralized with M17 medium, and

the bacterial titer was determined. As shown in Fig. 11B, a 3.7 log reduction in undiluted LAB-VP1 titer was observed after 12 h incubation at neutral pH and a 6.0-6.8 log reduction was observed after 24-48 h incubation. Interestingly, when LAB-VP1 was concentrated 10 times, the stability was significantly enhanced at 12 and 24 h ($P<0.05$). In contrast, LAB-VP1 survival was significantly reduced when LAB-VP1 was diluted 10 and 100 times. All bacteria were inactivated after 36 h incubation at these two dilutions. These results suggested that survival of LAB-VP1 at neutral pH is concentration-dependent.

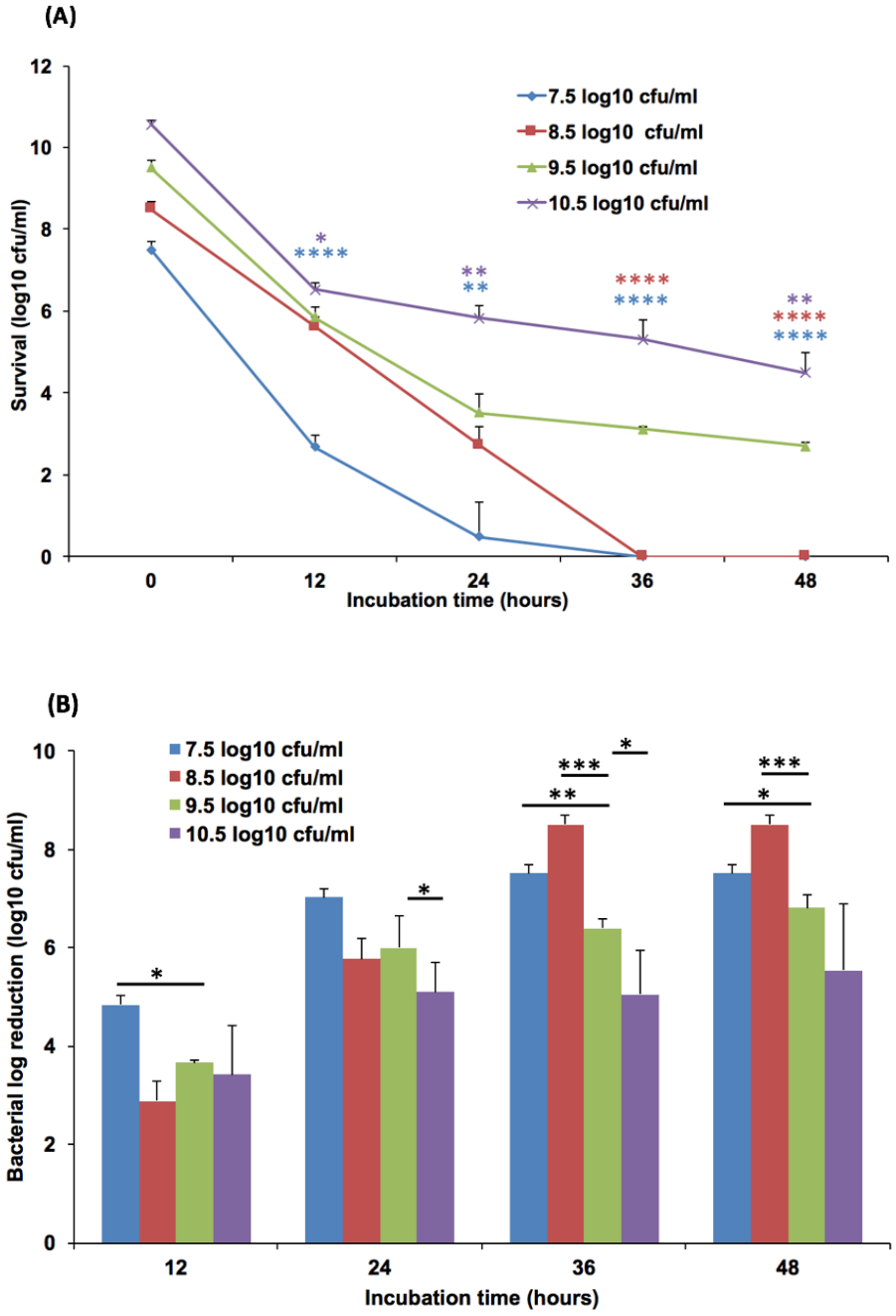


Figure 11. The effects of bacteria concentration and pH 7 on survival of LAB-

(A) The survival of LAB-VP1 at pH 7 solution. The concentrated 10x, undiluted, diluted 1:100 LAB-VP1 were incubated in pH 7 saline solution. The tubes of each concentration were removed at 12, 24, 36, and 48 h, and bacterial survival was determined by plate counts. (B) The log reduction of LAB-VP1 at pH 7 solution.

2.4.3. Stability of LAB-VP1 in pH 8.5 saline solutions

We next measured the stability of LAB-VP1 at pH 8.5, mimicking the pH environment in the duodenum. Briefly, LAB-VP1 of 10 times concentrated, undiluted, diluted 1:10, diluted 1:100 were placed in pH 8.5 saline (adjusted with NaOH) and samples were harvested after 12, 24, 36, and 48h incubation. The reaction was neutralized with M17 medium, and the bacterial titer was determined. For undiluted group, approximately 3.4, 4.2, 4.9, and 5.6 log bacteria reductions were observed after 12, 24, 36, and 48 h incubation. Similarly, survival of LAB-VP1 was increased when it was concentrated 10 times. However, survival was decreased when it was diluted 10 and 100 times. Therefore, these results demonstrated that LAB-VP1 was more stable at pH 8.5 compared to pH 7.0 (compare log reductions in Fig. 12B and 13B). In addition, these results suggested that the concentration of LAB-VP1 affected the survival of bacteria at pH of 8.5.

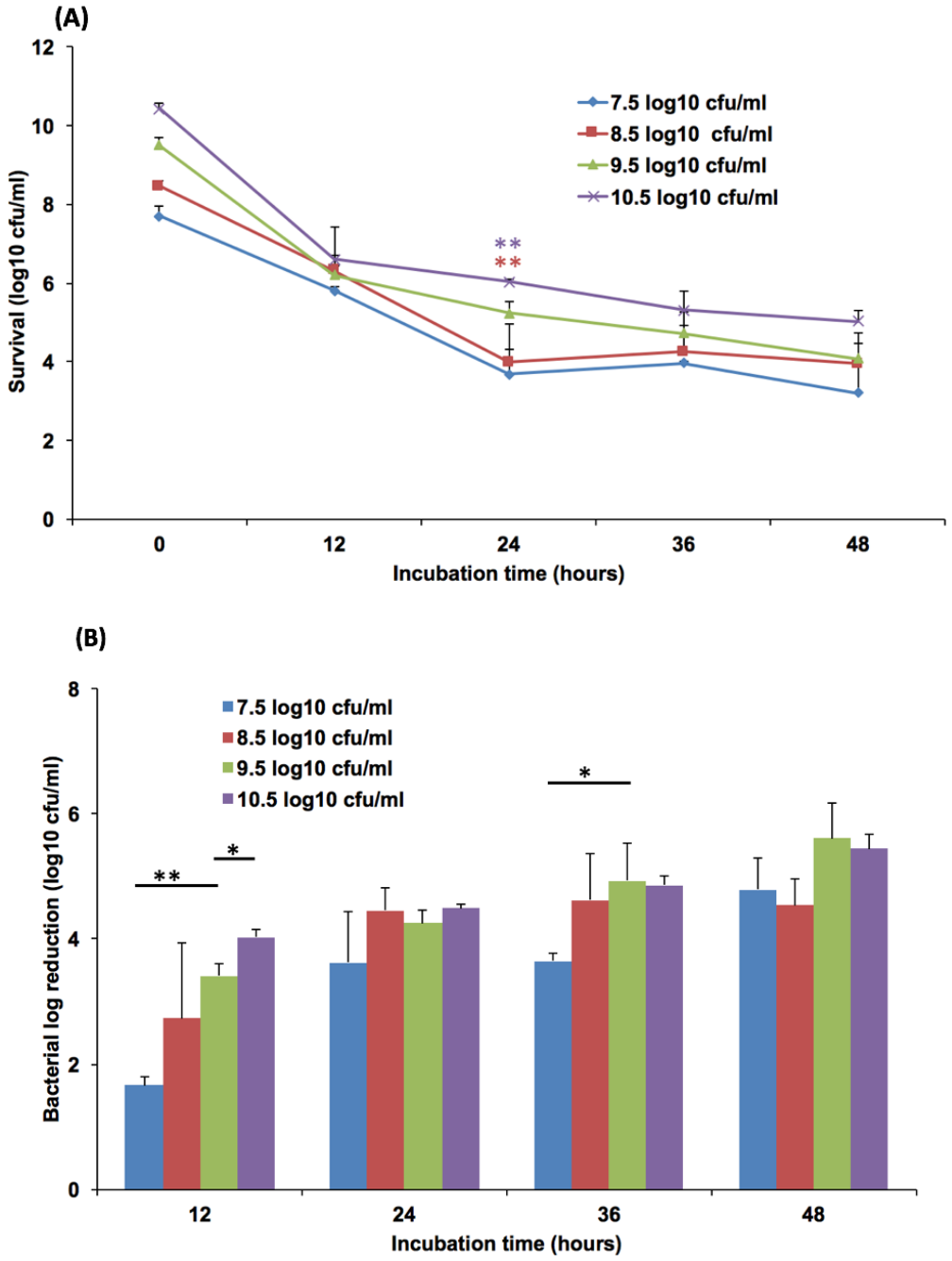


Figure 12. The effects of bacteria concentration and pH 8.5 on survival of LAB-VP1

(A) The survival of LAB-VP1 at pH 8.5 solution. The LAB-VP1 concentrated 10x, undiluted, diluted 1:10, and diluted 1:100 were placed in pH 8.5 saline solution. The tubes of each concentration were removed at 12, 24, 36, and 48 h, and bacterial survival was determined by plate counting. (B) The log reduction of LAB-VP1 at pH 8.5 solution.

2.4.4. Stability of LAB-VP1 in pH 4 saline solutions

We next determined the survival of LAB-VP1 at pH of 4.0. Since LAB-VP1 was significantly less stable at pH 4.0 in the pilot experiment (not shown), we determined the survival of 10 x concentrated, undiluted, diluted 1:10, diluted 1:100 LAB-VP1 in pH 4 saline with a shorter incubation time, harvesting samples at 2, 4, 6, 8, and 12 h. The reaction was neutralized with M17 medium, and the bacterial titer was determined. For undiluted group, as shown in Fig. 13A, 0.32, 3.0, 3.6, 3.8, and 4.8 log bacterial reductions were found after 2, 4, 6, 8, and 12h incubation, respectively. Similar to the previous observation in other pH conditions, bacterial survival was enhanced when they were concentrated 10 times, whereas the survival was reduced when they were diluted 10 and 100 times.

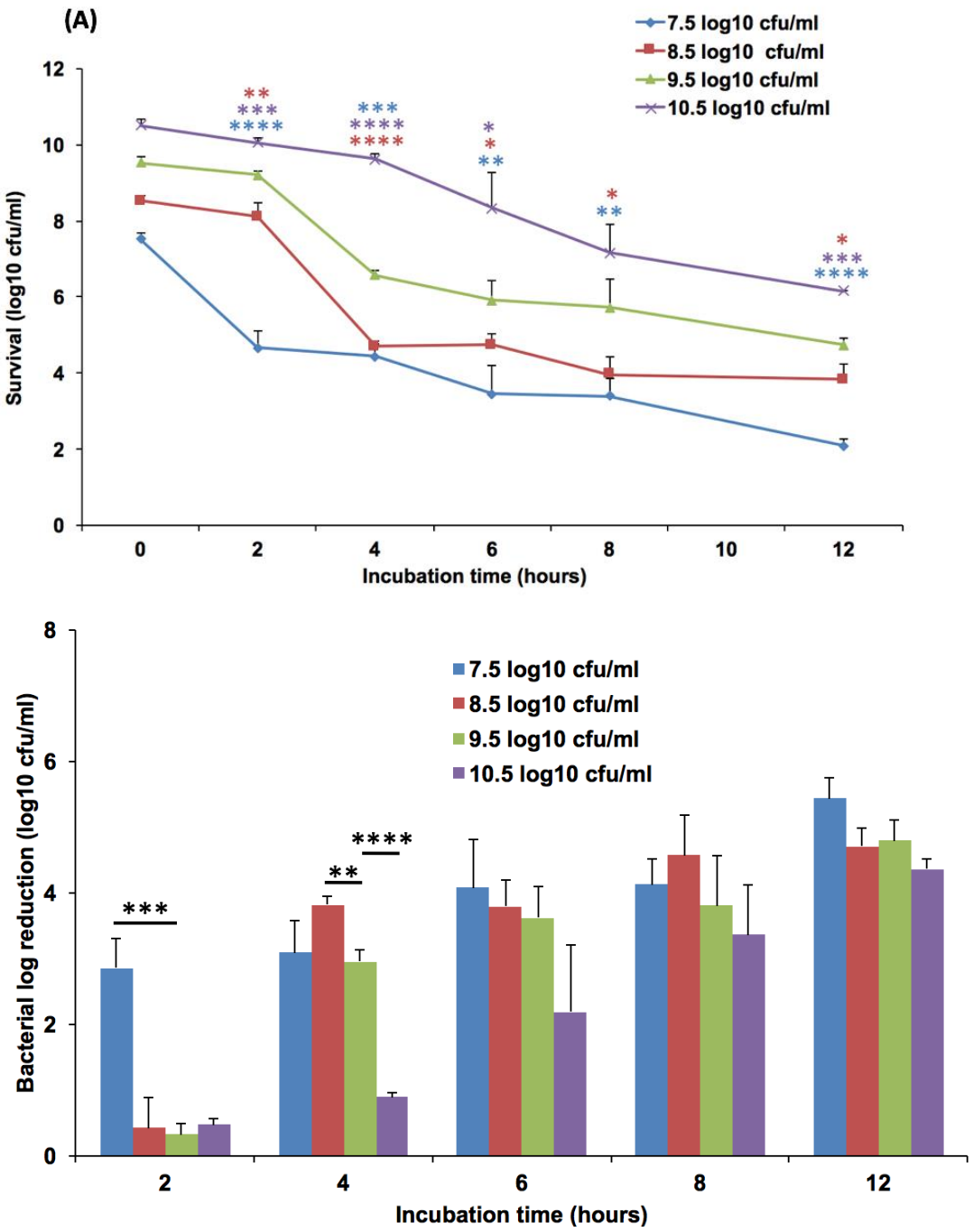


Figure 13. The effects of bacteria concentration and pH 4 on survival of LAB-VP1

(A) The survival of LAB-VP1 concentrated 10 x, undiluted, diluted 1:10, and diluted 1:100 after placed in pH 4 saline solution. The tubes of each concentration were removed at 2, 4, 6, 8 and 12h. (B) The log reduction of LAB-VP1 at pH 4 solution.

2.4.5. Stability of LAB-VP1 in pH 2 saline solutions

Finally, we determined the stability of LAB-VP1 in stomach pH. LAB-VP1 was placed in pH 2 saline to assess the survival after 2 h incubation. Briefly, the 10 times concentrated, undiluted, and diluted 1:10 LAB-VP1 were placed in pH 2 saline (adjusted with HCl) then harvested at 30, 60, 90, and 120m. For undiluted LAB-VP1, 5.2 log reductions were detected after 30 min incubation, and 6.5-7.1 log reductions were observed after 60-120 min incubation. No bacterial survival (8.9 log bacteria reductions) was detected when LAB-VP1 was diluted 10 times after 30 min incubation at pH 2.0. Interestingly, there was no significant titer reduction when LAB-VP1 was concentrated 10 times and incubated at pH 2 for 2 h. This suggests that a higher concentration of LAB-VP1 enhance survival in a stomach acid environment. Since all bacteria were inactivated at pH 2.0 after 30 min incubation when LAB-VP1 was diluted 1:10 we further determined the bacterial survival by reducing the incubation time (3, 6, 12, 15, and 18 min). As shown in Fig. 14C, both 1:10 and 1:100 diluted LAB-VP1 were inactivated after 18 min incubation. Samples from 1:10 dilution had less bacteria reduction compared to those from 1:100 dilution, although there was no significant difference between these two groups, with the exception of samples at 6 min incubation.

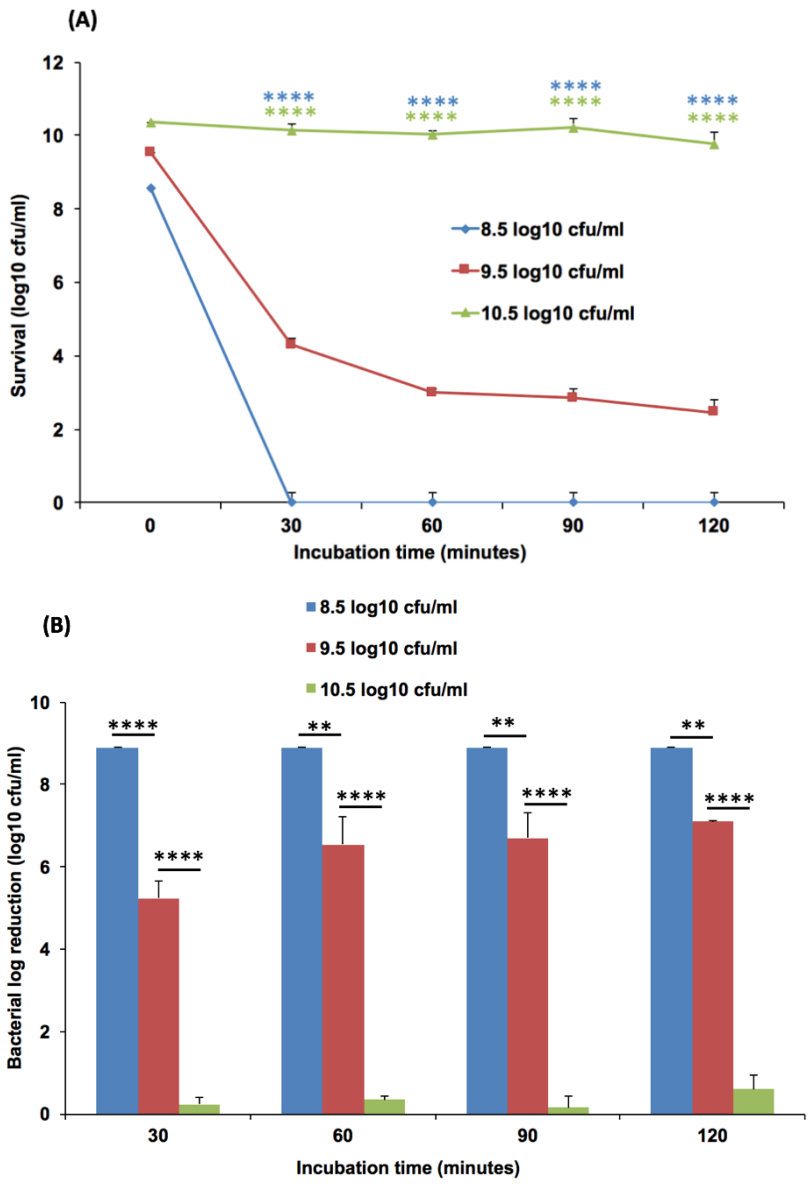
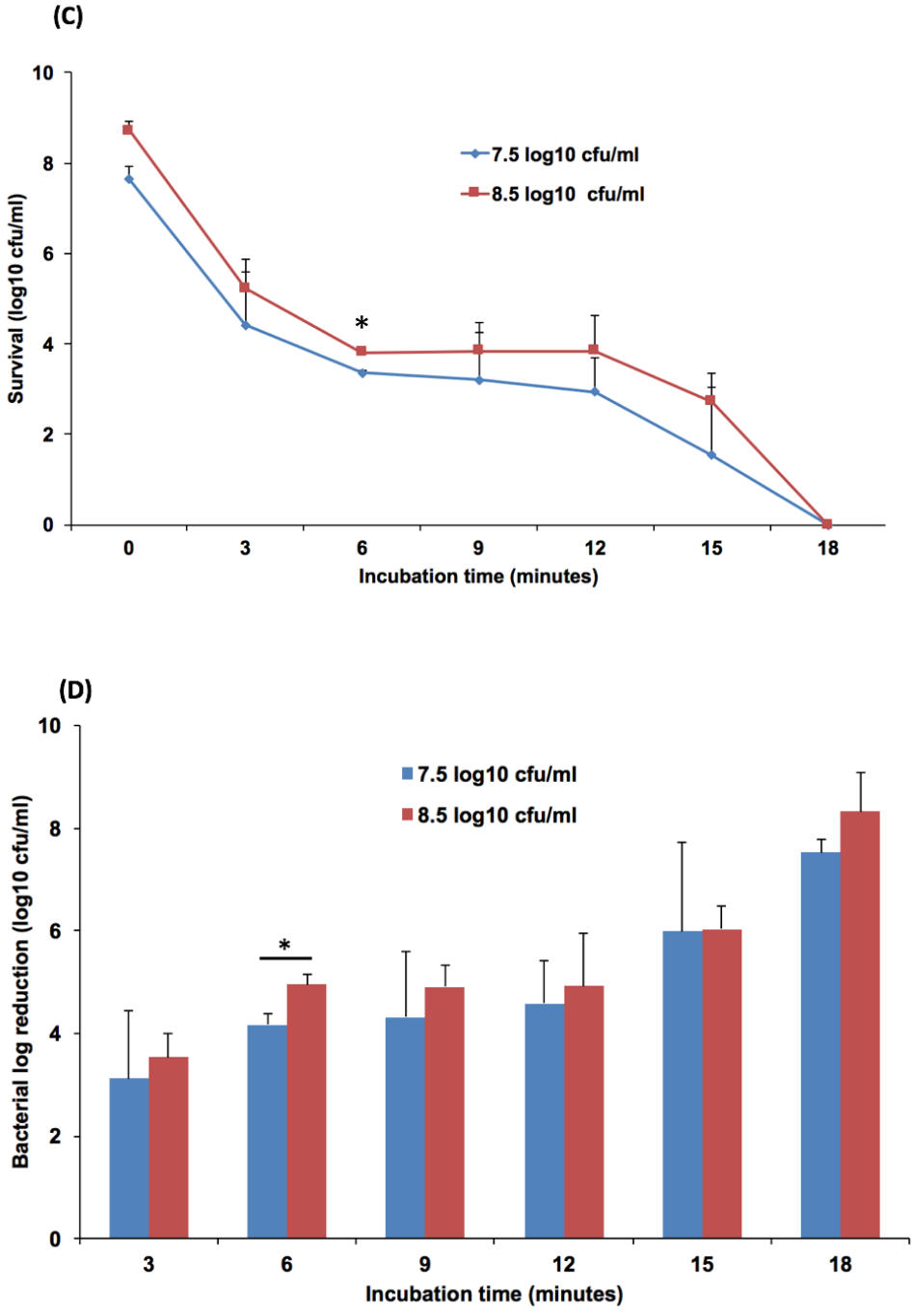


Figure 14. The effects of bacteria concentration and pH 2 on survival of LAB-VP1

(A) The survival of LAB-VP1 concentrated 10x, undiluted, and diluted 1:10 after placed in pH 2 saline solution. The tubes of each concentration were removed at 30, 60, 90, and 120m. (B) The log reduction of LAB-VP1 at pH 2 solution. (C) The survival of LAB-VP1, diluted 1:10 and diluted 1:100 after placed in pH 2 saline solution. The tubes of each concentration were removed at 3, 6, 9, 12, 15, and 18m. (D) The log reduction of LAB-VP1 diluted 1:10 and diluted 1:100 at pH 2 solution.

Figure 14. Continued



2.4.6. Direct comparison of survival of LAB-VP1 in various pH

The above results suggest that the survival of LAB-VP1 was enhanced at a higher pH. Next, we directly compared the survival of LAB-VP1 among a range of pH solutions. The pH stability assays are described previously with the exception of incubation time changing to 12, 24, 36, and 48 h.

We first directly compared the survival of the undiluted LAB-VP1 (at concentration of 10^9 cfu) at pH 8.5, 7.0, and 4.0. As shown in Fig. 15A and 15B, the stability of LAB-VP1 in different pH can be ranked pH 8.5 >pH 7.0 > pH 4.0. Clearly, pH of 4.0 is the most unstable environment for LAB-VP1, and no bacteria survived after 36 h incubation. Next, we compared the stability of LAB-VP1 when they were diluted 100 times (concentration of 10^7 cfu). The survival rate at pH 8.5 was significantly higher than those at pH 4 and pH 7 ($P>0.05$). There was no significant difference between the pH 4 and pH 7 survival ($P>0.05$). Together, these results demonstrated that the survival of LAB-VP1 significantly enhanced at basic pH environment.

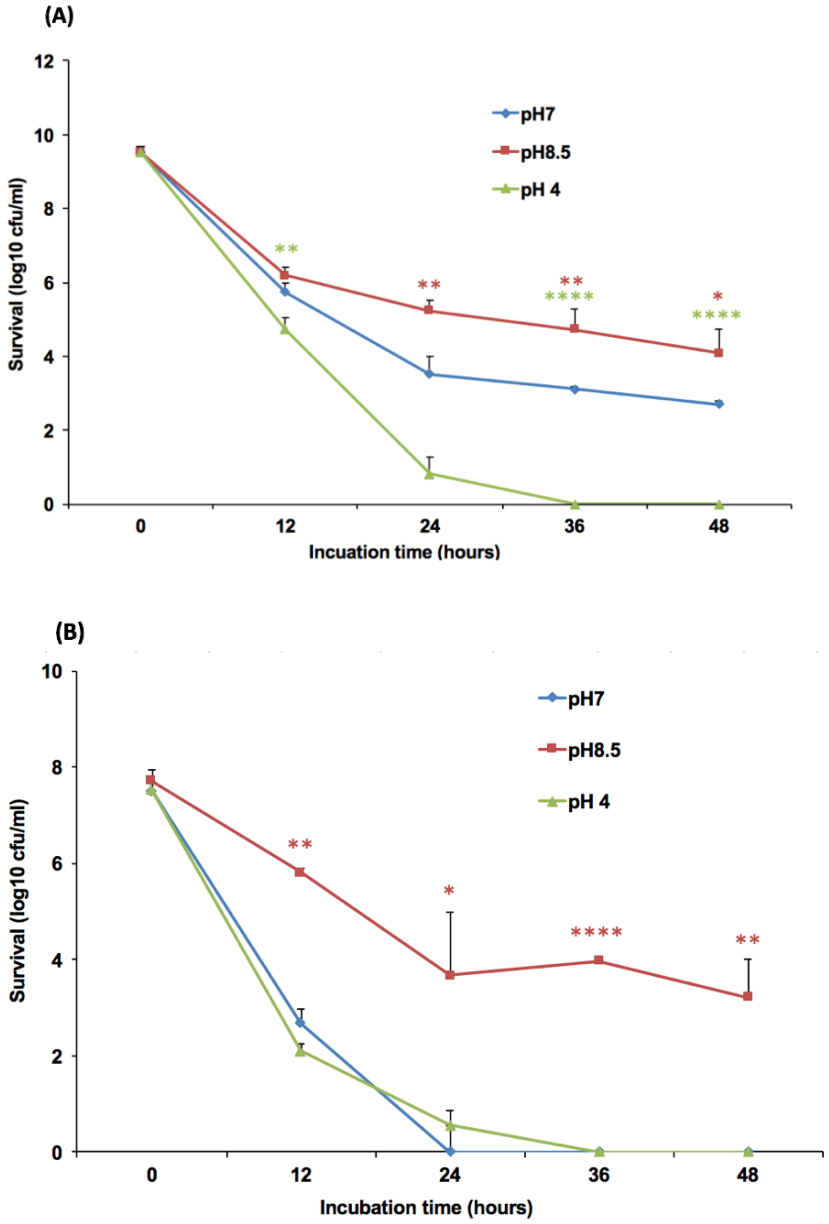


Figure 15. Direct comparison of the stability of LAB-VP1 in various pH saline solutions

(A) The effects of pH on the stability of LAB-VP1 at a concentration of 10^7cfu/ml. The original LAB-VP1 was diluted 100 times (1:100), and was placed in pH 4, 7, and 8.5 saline solutions. The tubes of each concentration were removed at 12, 24, 36, and 48 h. (B) The effects of pH on the stability of LAB-VP1 at a concentration of 10^9cfu/ml. The undiluted LAB-VP1 (concentration of 10^9cfu/ml) were placed in pH 4, 7 and 8.5 saline solutions. The tubes of each concentration were removed at 12, 24, 36 and 48h.

2.4.7. The survival of LAB-VP1 in storage at 4°C

The stability of LAB-VP1 while stored in 4°C was assessed to determine the survival with cold storage for multiple weeks. Briefly, LAB-VP1 of undiluted, diluted 1:10, diluted 1:100 were incubated in pH 7 saline at 4°C for 3, 7, 10, 14, 17, 21, 24, and 28 d, and the survival of LAB-VP1 was determined by bacterial counts. The undiluted LAB-VP1 was significantly more stable than the diluted samples for the first 10 days then the log reduction started to increase. There is an increase in log reduction from day 14 to day 17 in the undiluted as the titer drops close to the diluted titers. Overall, the diluted 1:10 had a better survival rate than the diluted 1:100 although there was no significant difference at some time points. Interestingly, all three concentrations ended with comparable titers after 28 d of incubation (Fig. 16A). This result suggested that storage of LAB-VP1 at cold environment significantly enhanced the stability of bacteria and that stability of LAB-VP1 was concentration-dependent.

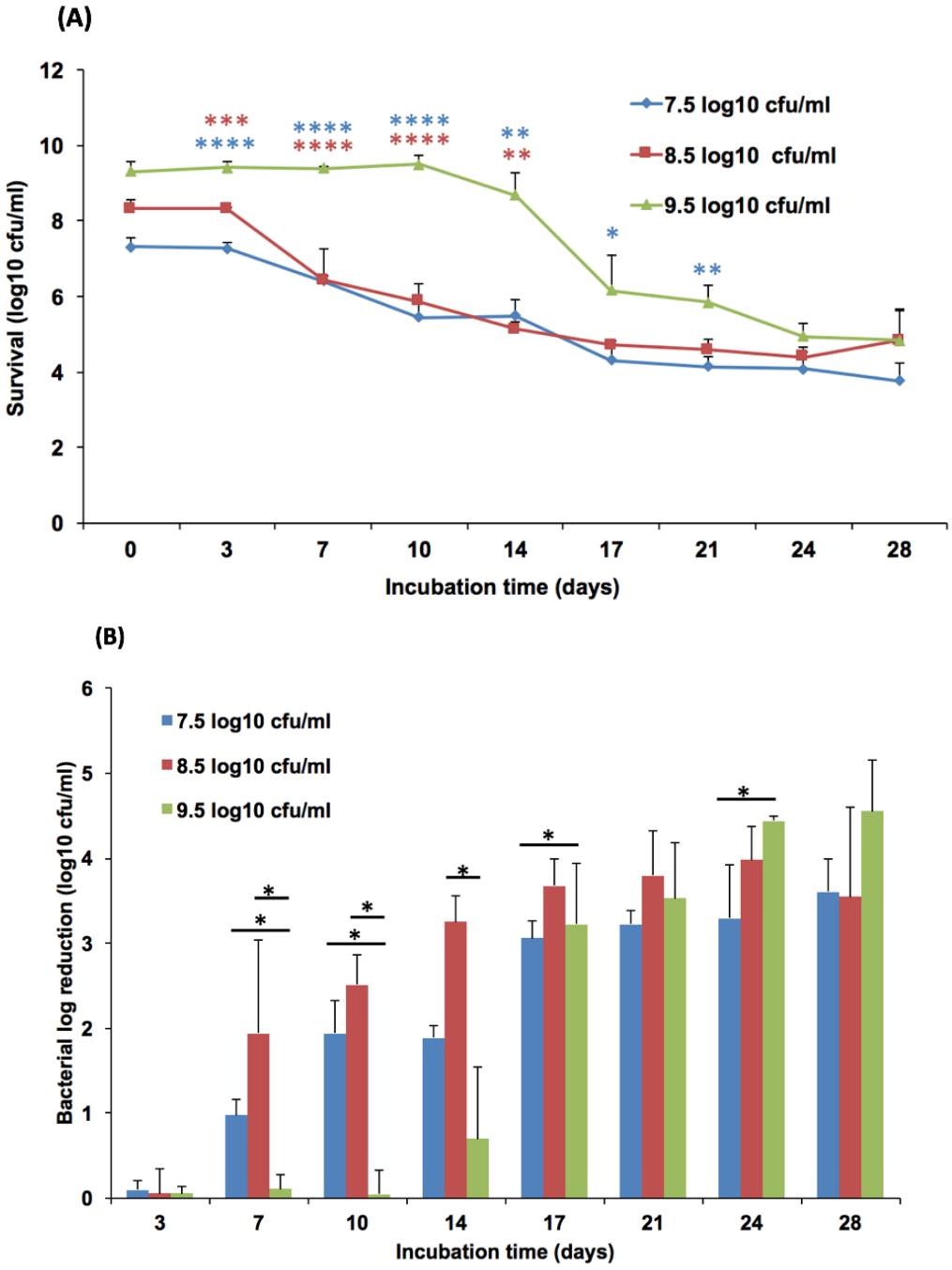


Figure 16. The stability of LAB-VP1 stored in 4°C

(A) The survival of LAB-VP1 stored in 4°C. The concentrated 10x, undiluted, diluted 1:10, and diluted 1:100 LAB-VP1 was placed in pH 7 saline in 4°C at 3, 7, 10, 14, 17, 21, 24, and 28d time points, and bacterial survival was determined (B) The log reduction of LAB-VP1 stored in 4°C.

2.4.8. Enhancement of the stability of LAB-VP1 by microencapsulation

We have developed a novel microencapsulation technology to encapsulate LAB into microparticles. Briefly, the alginate microcapsules with LAB encapsulated in their core were generated using a novel 3-D microfluidic device. The surface of the alginate microcapsule was further modified with chitosan using a layer-by-layer coating approach. The goal of the microencapsulation of probiotic vaccines with alginate and a chitosan coating is to improve their survival in the gastric and intestinal environment and allow for an effective delivery of viable bacterial cells to the colon. Furthermore, the outer layer helps to protect from the harsh environments while keeping minimal contact with the environment through pores. Thus, microencapsulation of probiotics could improve their storage and allows the production of dried food composition that may reduce the dependence on the cold storage. As shown in Fig. 17D and 18 microparticles with the size of 300 μm were observed under a light microscope.

To determine whether microencapsulation can enhance the stability of LAB-VP1, microparticles encapsulating LAB-VP1 with a chitosan and alginate shell were placed in various pH conditions, and the survival rate was compared with nonencapsulated LAB-VP1. Briefly, encapsulated and nonencapsulated LAB-VP1 were placed in pH7 saline or pH 8.5 at 37°C and harvested after 12, 24, 36 and 48 h of incubation. The microparticles were dissolved with 75mM TSC then the bacterial titer was determined. The microparticle encapsulated LAB-VP1 was significantly more stable in pH 7 than nonencapsulated LAB-VP1 ($P<0.01$) (Fig. 17A). After 24 h of incubation, no significant reduction was observed in microparticle encapsulated LAB-

VP1. In contrast, nonencapsulated LAB-VP1 had 4.2 and 6.3 log bacterial reduction after 12 and 24 h of incubation respectively. None of the nonencapsulated LAB-VP1 survived after 36 h of incubation. The chitosan and alginate layering provided protection from the pH environment to allow for significantly higher titers over 48 h. While placed in pH 8.5, there was no significant difference between the encapsulated LAB-VP1 and nonencapsulated LAB-VP1 (Fig. 17B). The microparticles did not significantly enhance the stability of LAB-VP1 at pH 8.5 as LAB-VP1 is already highly stable in basic pH solution.

The microparticles protection is needed most for protection from the acidic pH in the stomach. Briefly, encapsulated and nonencapsulated LAB-VP were placed in pH 2 saline at 37°C and harvested at 3, 6, 9, 12, 15 and 18 min. The microparticles were dissolved with 75mM TSC then the bacterial titer was determined. The microparticles enhanced the stability of LAB-VP1 while in the harsh acidic pH 2 as shown in Fig. 17C. Only 1 log bacteria reduction was observed in LAB-VP1 microparticles whereas 3.3 log bacteria reductions were detected in nonencapsulated LAB-VP1 group at 3 min of incubation. After 15 min of incubation, the LAB-VP1 microparticles had 2 log of bacteria survive compared to 0.2 log survive in nonencapsulated LAB-VP1 group. The chitosan and alginate layering of the encapsulation provided protection from the acidic environment to allow the low starting concentration ($\log_{10}$ 6.5) to survive.

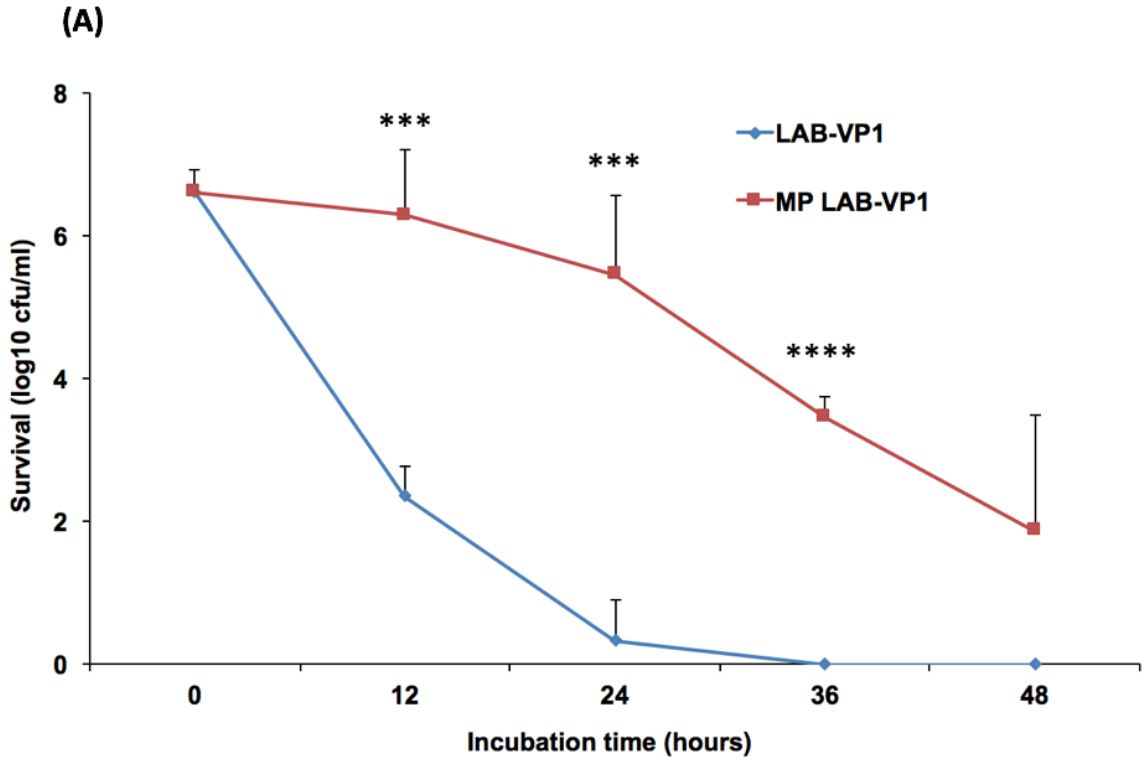
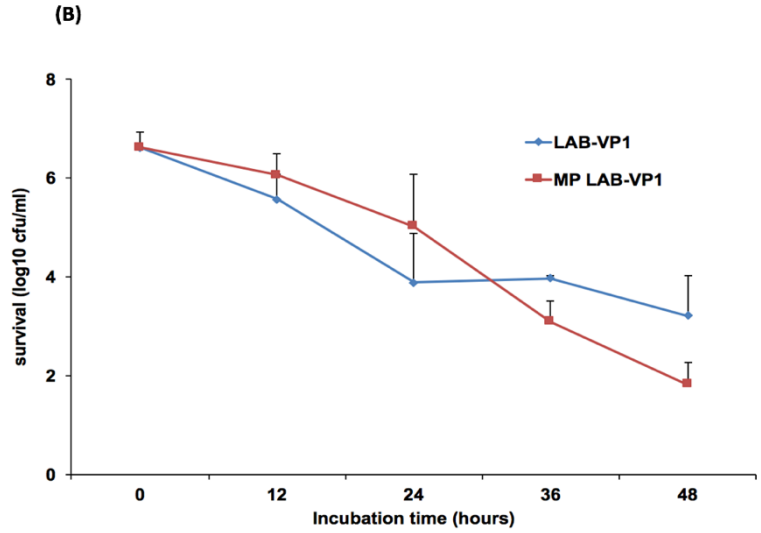
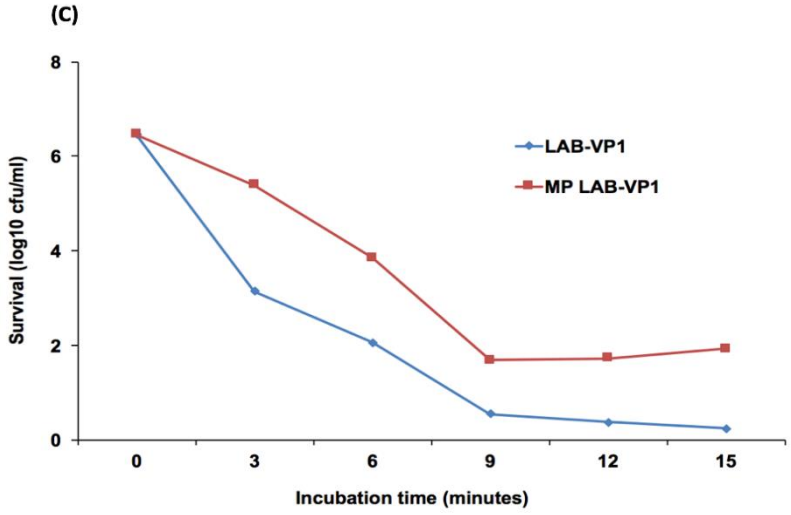


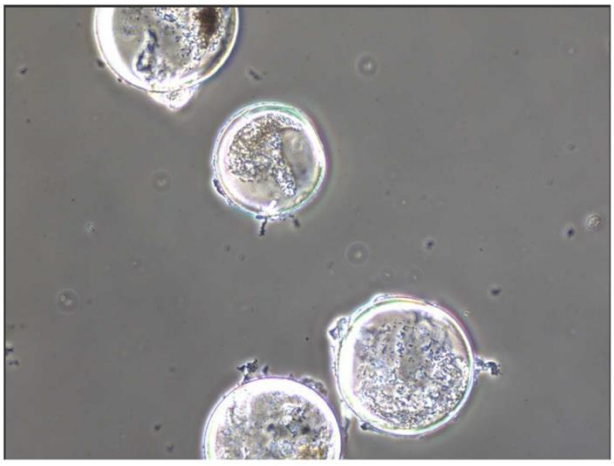
Figure 17. Comparison of the survival of microencapsulated LAB-VP1 and nonencapsulated LAB-VP1 (A) The stability of microencapsulated LAB-VP1 at pH 7.0. LAB-VP1 encapsulated with chitosan and alginate was compared to nonencapsulated LAB-VP1. Equal amounts of LAB-VP1 with or without microencapsulation were placed in pH 7 saline solution and removed at 12, 24, 36, and 48 h. Bacterial survival was determined. (B) The stability of microencapsulated LAB-VP1 at pH 8.5. Equal amounts of LAB-VP1 with or without microencapsulation were placed in pH 8.5 saline solution and removed at 12, 24, 36, and 48 h. (C) The stability of microencapsulated LAB-VP1 at pH 2.0. Equal amounts of LAB-VP1 with or without microencapsulation were placed in pH 2 saline solution and removed at 3, 6, 9, 12, 15, and 18 min. (D) Pictures of chitosan-alginate-chitosan shell microparticles encapsulating LAB-VP1

Figure 17. Continued





(D)



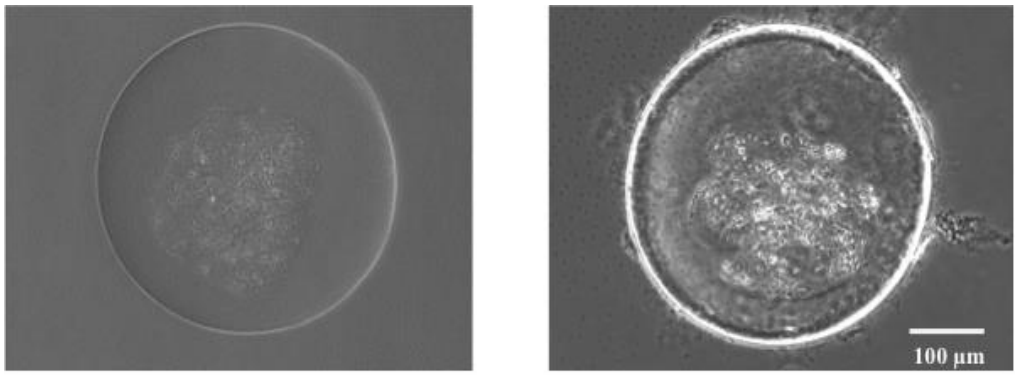


Figure 18. The bacteria-laden microcapsules without coating (left) and with coating (right)

2.4.9. Fecal shedding of LAB in gnotobiotic piglets

Gnotobiotic piglets have been used as vaccination and challenge models for HuNoV since 2006 (S. Cheetham et al., 2007). Previously, our laboratory has successfully tested the efficacy of vesicular stomatitis virus (VSV) vectored HuNoV vaccines (rVSV-VP1) in this model. Specifically, it was found that rVSV-VP1 vaccination induced HuNoV-specific mucosal and humoral immune responses and protected gnotobiotic piglets from HuNoV challenge (Ma & Li, 2011). In this study, we determined whether oral delivery of LAB can be colonized in the pig intestine and whether LAB-based HuNoV vaccine is immunogenic and is capable of protecting from HuNoV challenge in a gnotobiotic piglet model. Experimental design and group information were summarized in Table 3. For these experiments, there were 5 different groups of piglets including LAB-VP1 in microparticles (MP LAB-VP1) 10^9 cfu, LAB-VP1 10^9 cfu, LAB-VP1 10^{12} cfu, LAB-VP1 10^{10} cfu, and LAB-empty vector 10^{12} cfu. The vaccine candidates were administered via oral gavage to 4-day-old piglets. After vaccination, fecal material was collected on post-inoculation day (PID) 6, PID 13, and PID 20 to determine the shedding of LAB in feces.

The fecal LAB titer represented the colonization and growth of LAB in the intestines. The piglets were monitored daily following vaccination and there were no adverse reactions observed for the entirety of the experiment. LAB is a probiotic and is a generally recognized as safe (GRAS) agent therefore, no abnormal reaction was expected following the vaccination. The groups all had comparable amounts of LAB shed in fecal samples collected on PID 6 as shown in Fig. 19A ($P>0.05$). The concentrations ranged from $\log_{10}$ 8.1-2.1 cfu/g feces.

The fecal shedding continued at PID 13 in the majority of the pigs (Fig. 19B). There was no significant difference among these groups ($P>0.05$). The LAB concentration ranged from $\log_{10}$ 7.4-2, which is a similar range to PID 6. There was a piglet from the microparticle group and a piglet from the LAB-VP1 10^{10} group that did not have detectable amount of LAB shedding. Fecal LAB was still detectable at day 20 post-vaccination. The LAB-VP1 concentration ranged from $\log_{10}$ 5.5-2 cfu/g feces, while LAB-empty vector concentration ranged from $\log_{10}$ 7-4 cfu/g feces. Therefore, the LAB shedding in feces continued for at least 3 weeks in the majority of piglets.

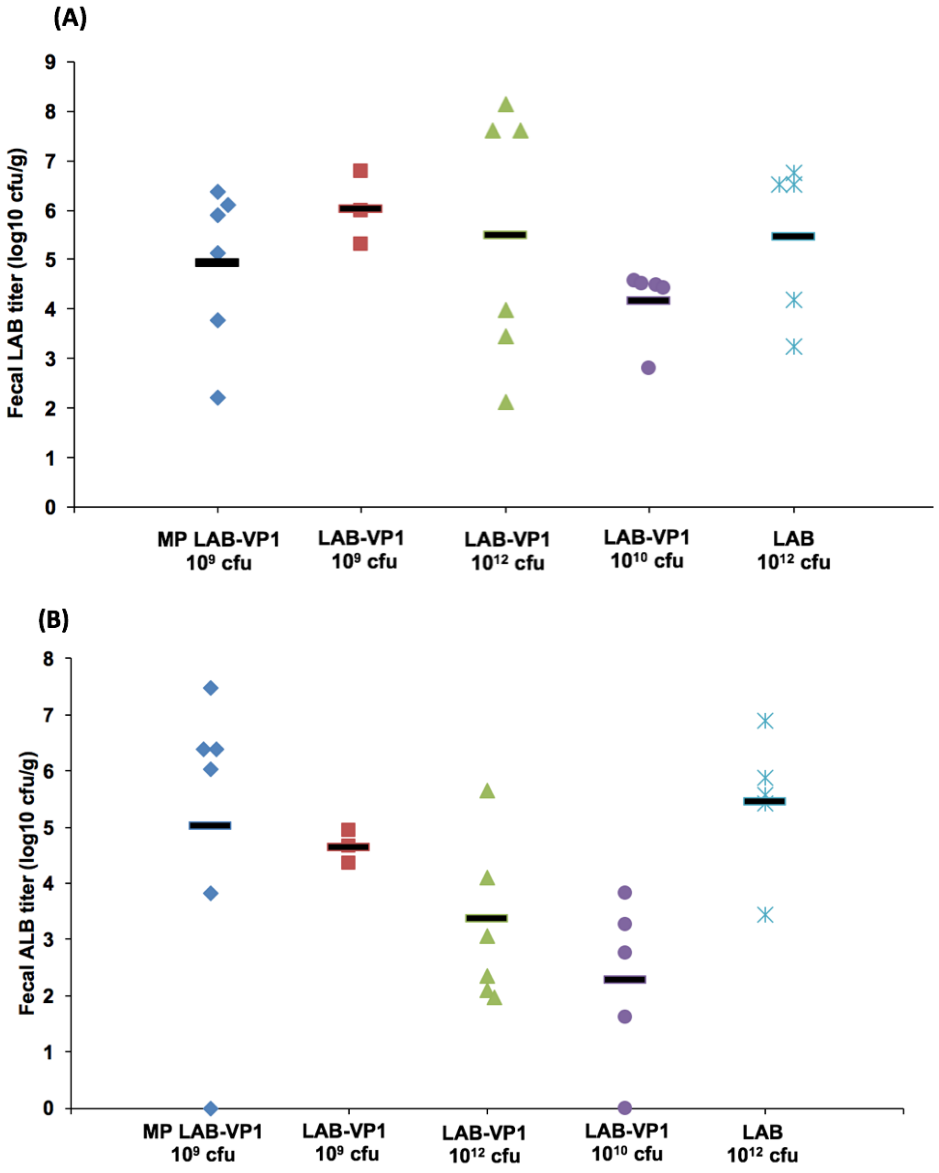
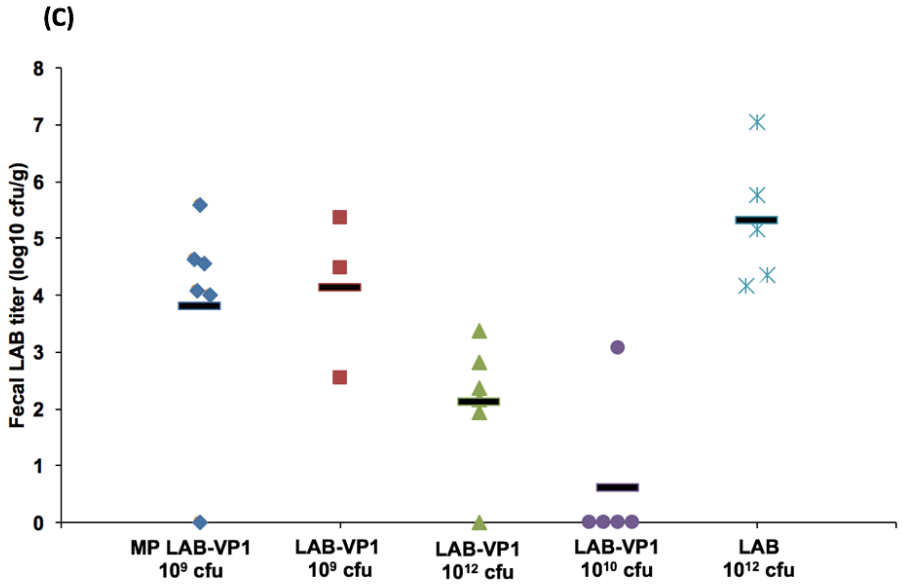


Figure 19. LAB shedding in piglet feces after oral vaccination

(A) LAB titer in piglet feces collected on PID 6. (B) LAB titer in piglet feces collected on PID 13.

(B) LAB titer in piglet feces collected on PID 20.

Figure 19. Continued



2.4.10. LAB titer in the piglet intestine and colon

At day 21 post-vaccination, all piglets were challenged with 10^7 RNA genome copies of HuNoV. At day 5 post-challenge, all piglets were euthanized. During necropsy: intestine sections (duodenum, jejunum, and ileum), spleen, mesenteric lymph nodes, jejunum content, colon content, and blood were collected from each piglet. To determine the amount of LAB present in the intestines, the intestine tissue pieces were scraped with a scalpel, eluted in saline, and the LAB titer was determined by plate count. As shown in Fig. 20A, the ileum had significantly more LAB than the duodenum in the 10^9 cfu LAB-VP1 microparticle group, LAB-VP1 10^{10} cfu group, and LAB 10^{12} cfu group. In addition, for the 10^9 cfu LAB-VP1 microparticle group, the ileum had significantly more LAB- titer than the jejunum. In all the groups the highest titer was in the ileum and the lowest was the duodenum. Among all the groups the duodenum ranges

from log_{10} 2.4-4.5 cfu/g tissue, the jejunum ranges from log_{10} 4-5.5 cfu/g tissue, and the ileum ranges from log_{10} 5-6 cfu/g tissue. The content of the jejunum was collected to determine the amount of LAB. It was found that the LAB concentration was comparable between groups ($P>0.05$), ranging from 5-7 log_{10} cfu/g content (Fig. 20B). Next, we determined the LAB titer in colon content. The concentration of LAB ranged from 4.0-6.6 log_{10} cfu/g feces and was comparable among all the groups ($P>0.05$) (Fig. 20C). Taken together, a significant amount of LAB was detected in different sections of the intestine, jejunum content, and colon content of all groups. These results suggest that LAB may be capable of multiplying in pig intestine and survived for at least 25 days.

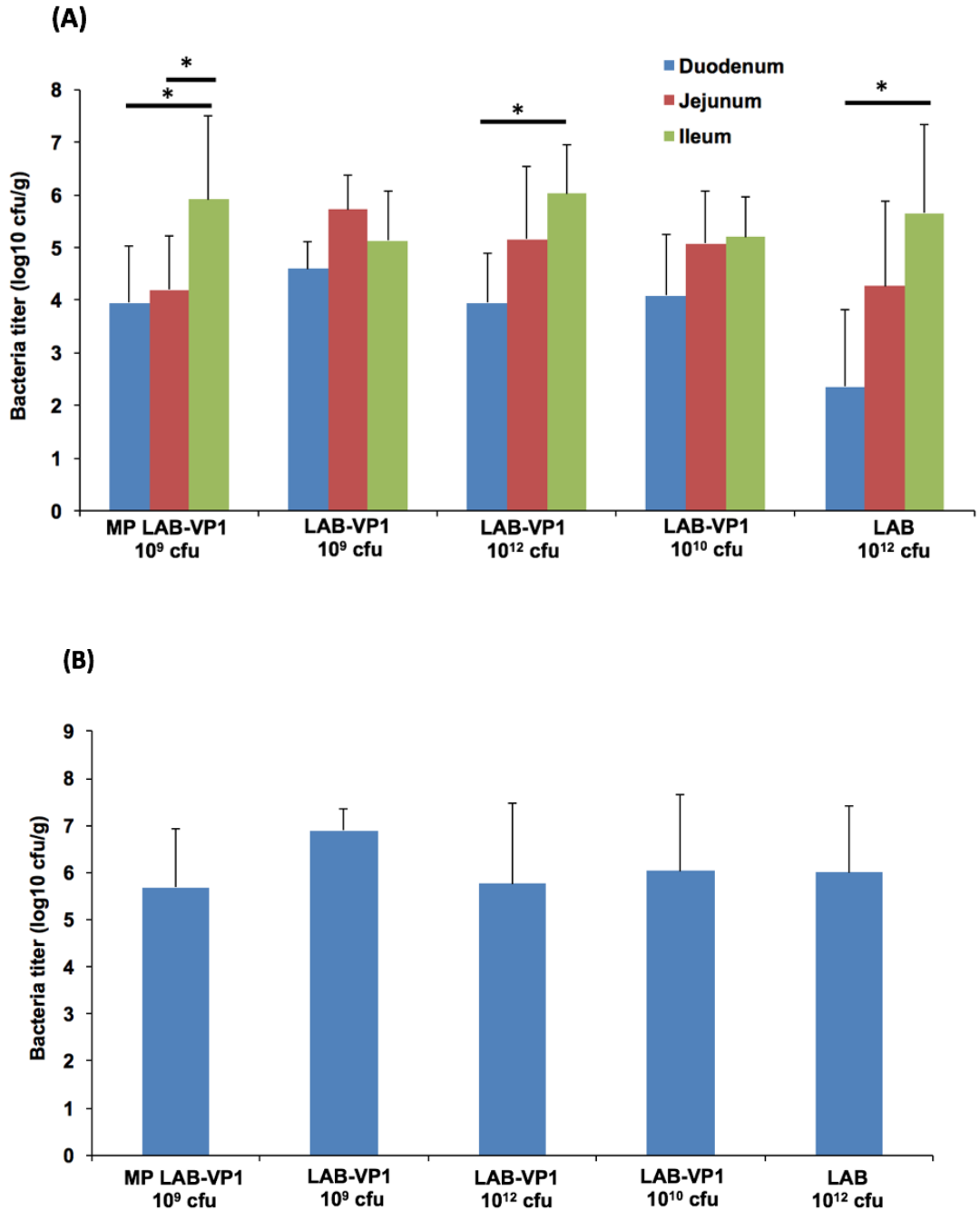
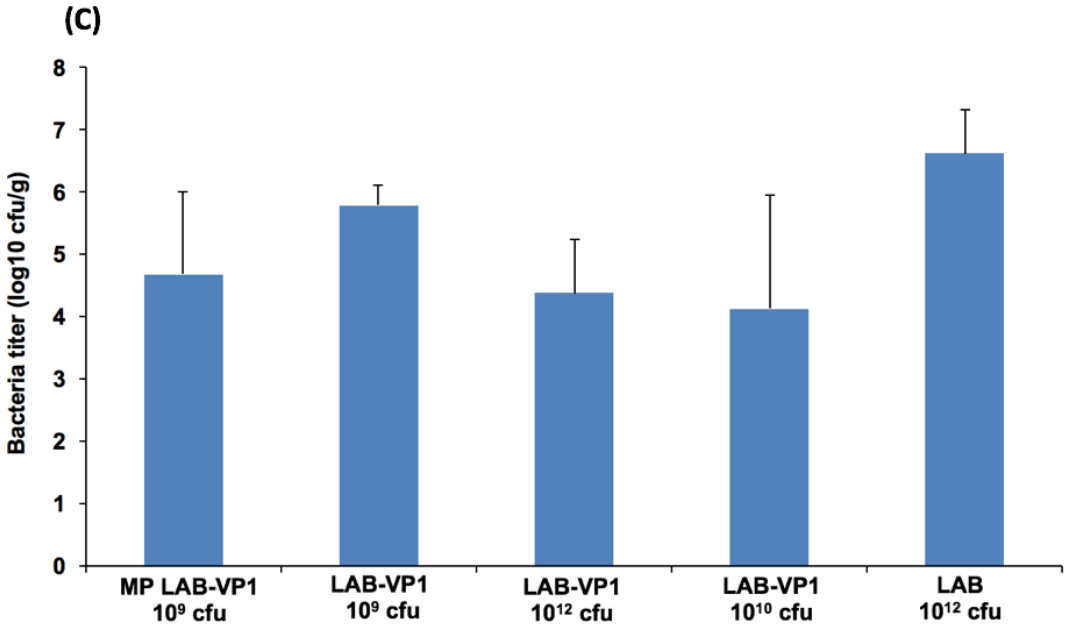


Figure 20. LAB titer in piglet intestine and colon content at necropsy

(A) LAB titer in piglet intestine sections on PCD 5. (B) LAB titer in the piglet jejunum content on PCD 5. (C) LAB titer in the piglet colon content on PCD 5.

Figure 20. Continued



2.4.11. Presence of LAB in spleen and mesenteric lymph nodes

Next, we determined whether LAB can be found in non-gastrointestinal tissue including spleen and mesenteric lymph nodes. Briefly, the spleen and mesenteric lymph nodes were isolated aseptically and homogenized with saline and plated to determine the live LAB titer. Interestingly, 3 out of 6 piglets in microparticle group live LAB with an average titer of 4 $\log_{10}$ cfu/g tissue whereas no live LAB was detectable in all other groups, suggesting that microparticles are likely captured by host immune cells (such as macrophage and dendritic cells) and transported to the mesenteric lymph nodes (Fig. 21A). No live LAB was detected in spleen tissues of all groups.

Since live LAB was not detectable in lymph nodes and spleen tissue from most groups, we next determined whether LAB DNA can be detected in these tissues. To do this, total DNA was

extracted from the homogenized spleen and mesenteric lymph nodes, the LAB DNA was quantified by real-time qPCR using primers annealing to the plasmid in the LAB. As shown in Fig. 21B and 21C, high amounts of LAB DNA copies were detected in both spleen and mesenteric lymph nodes. However, the LAB DNA copies in the spleen was similar among all the groups ($P>0.05$), ranging from 6-11 $\log_{10}$ DNA copies/g tissue. Similarly, no significant difference in LAB DNA copies was observed in the mesenteric lymph nodes among all groups ($P>0.05$). The presence of LAB DNA in spleen and mesenteric lymph nodes suggests that LAB has been captured by immune cells and migrated to these immune organs.

Since high copies of LAB DNA were detected in spleen and mesenteric lymph nodes, we next asked whether these LAB still carrying VP1 gene. To do this, a traditional PCR was carried out using two primers annealing to VP1 gene. As shown in Fig. 22A, 22B, 22C and 22D, all groups except for the LAB vector control group had detectable VP1 gene. This suggested that LAB-VP1 retained VP1 gene, even they had been migrated into spleen and mesenteric lymph nodes.

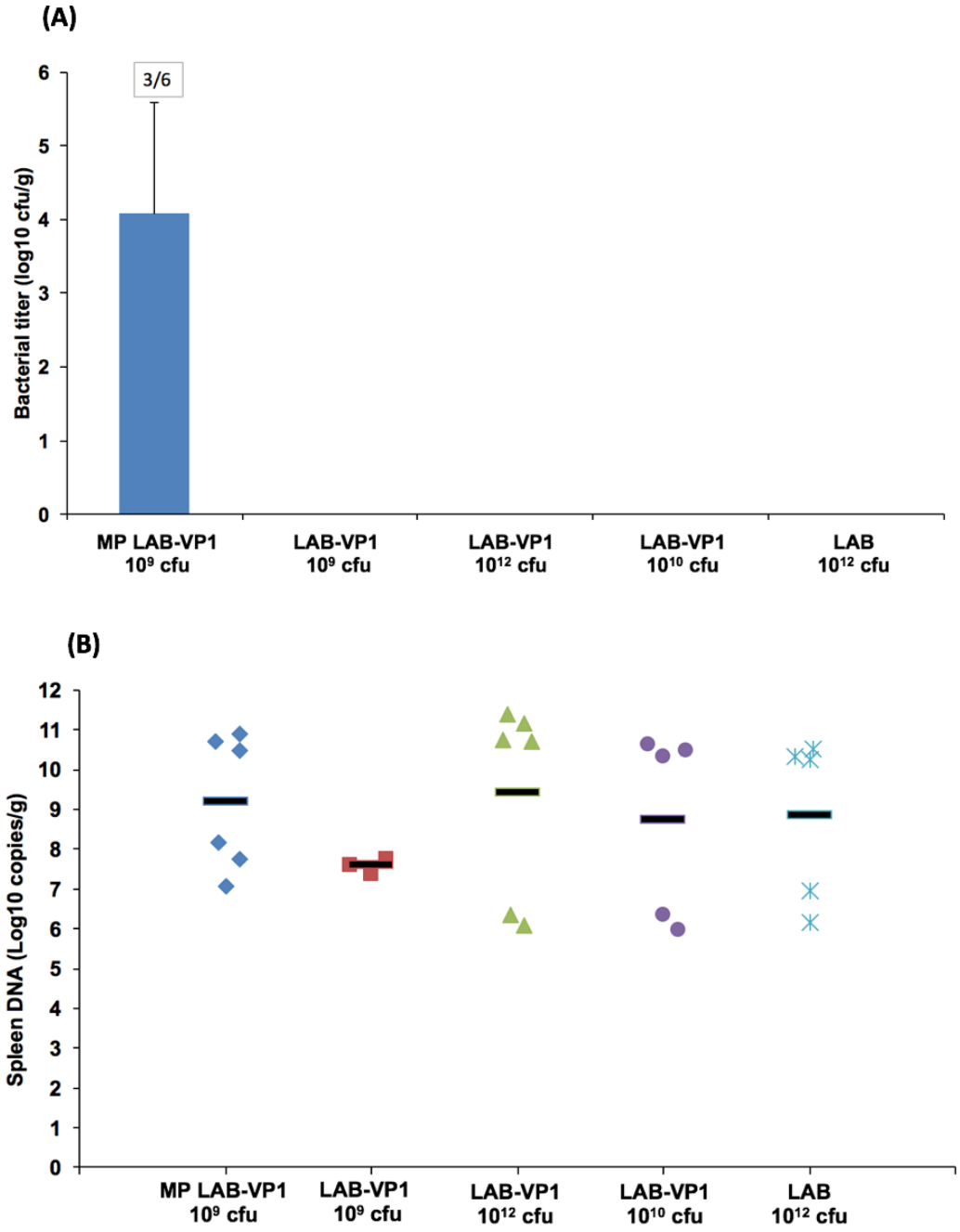
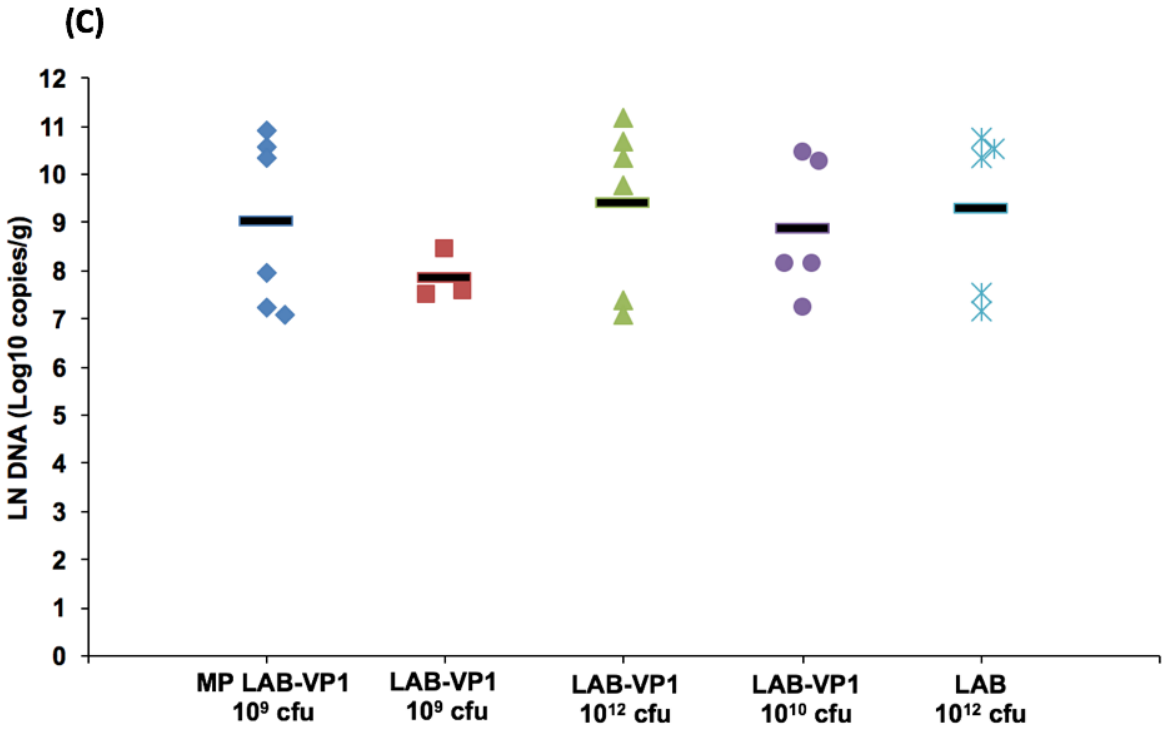


Figure 21. Presence of LAB in mesenteric lymph nodes and spleen of gnotobiotic piglets

(A) The presence of live LAB in the mesenteric lymph node tissue of the microparticle group.

(B) LAB DNA copies in spleen tissue at PCD 5 determined by real-time PCR. (C) LAB DNA copies in the mesenteric lymph node tissue at PCD 5 determined by real-time PCR.

Figure 21. Continued



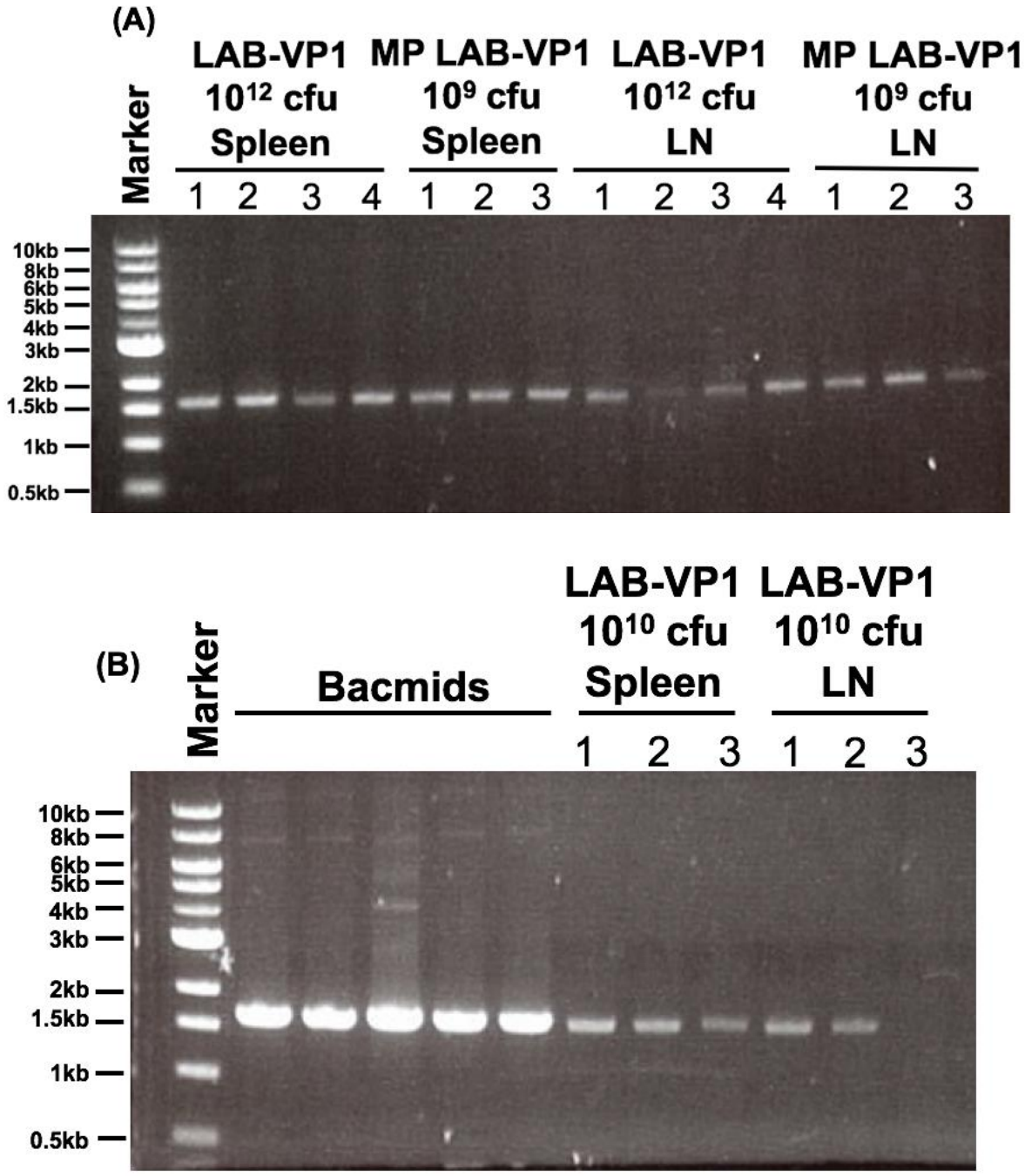
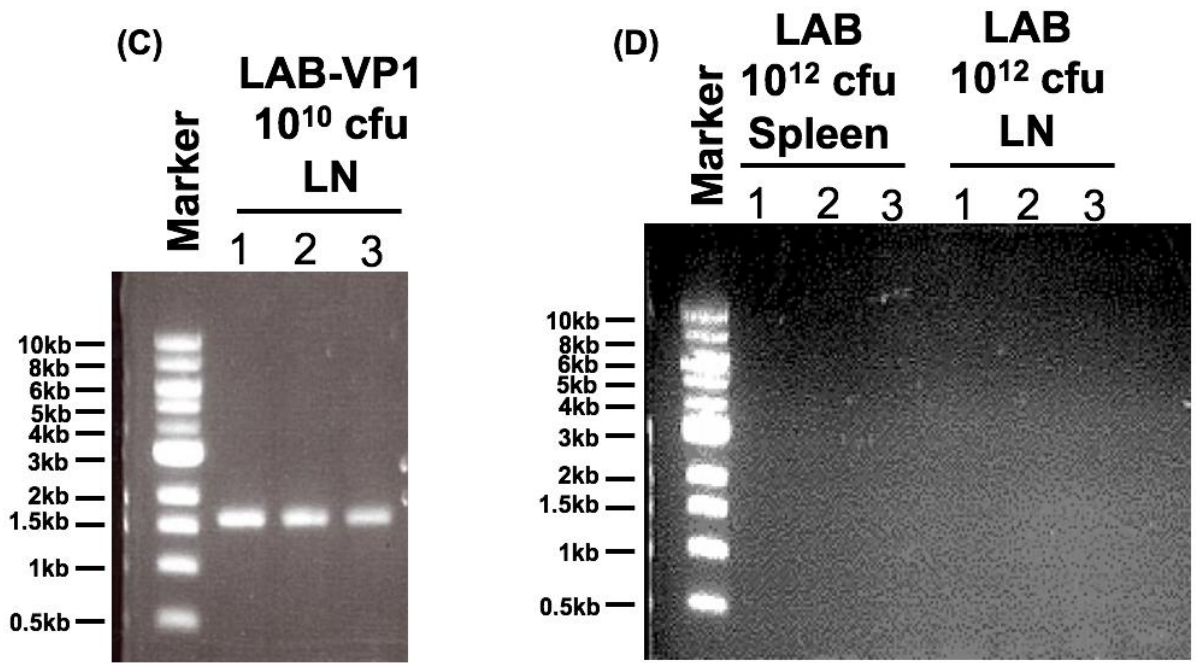


Figure 22. HuNoV VP1 DNA in spleen and lymph nodes of piglets

(A) VP1 at 1.5kb presence in spleen and lymph nodes of LAB-VP1 10^{12} cfu group and MP10^{9} cfu group. (B) VP1 presence in spleen and lymph nodes of LAB-VP1 10^{10} cfu group. (C) VP1 presence in lymph nodes of LAB-VP1 10^{10} cfu group redone. (D) VP1 absence in LAB 10^{12} cfu group.

Figure 22. Continued



2.4.12. Oral vaccination of LAB-based vaccine induced HuNoV-specific IgG response

To determine whether a LAB-based vaccine will trigger -HuNoV-specific IgG responses, serum was isolated from each piglets at days 13 and 20 post-vaccination, and HuNoV-specific IgG titer was detected by ELISA. As shown in Fig. 23A, HuNoV-specific IgG was detectable in all LAB-based HuNoV vaccine groups, but not the LAB control group at 13 post-vaccination. However, there was no significant difference among vaccine groups containing LAB-VP1 ($P>0.05$). At day 20 post-vaccination, HuNoV-specific IgG titer was increased in LAB-VP1 10^{12} cfu group ($P<0.05$). However, the average IgG titer in other groups at day 20 was similar to that at day 13 ($P>0.05$). This result demonstrated that LAB-based HuNoV vaccine triggered HuNoV-specific IgG in gnotobiotic piglets.

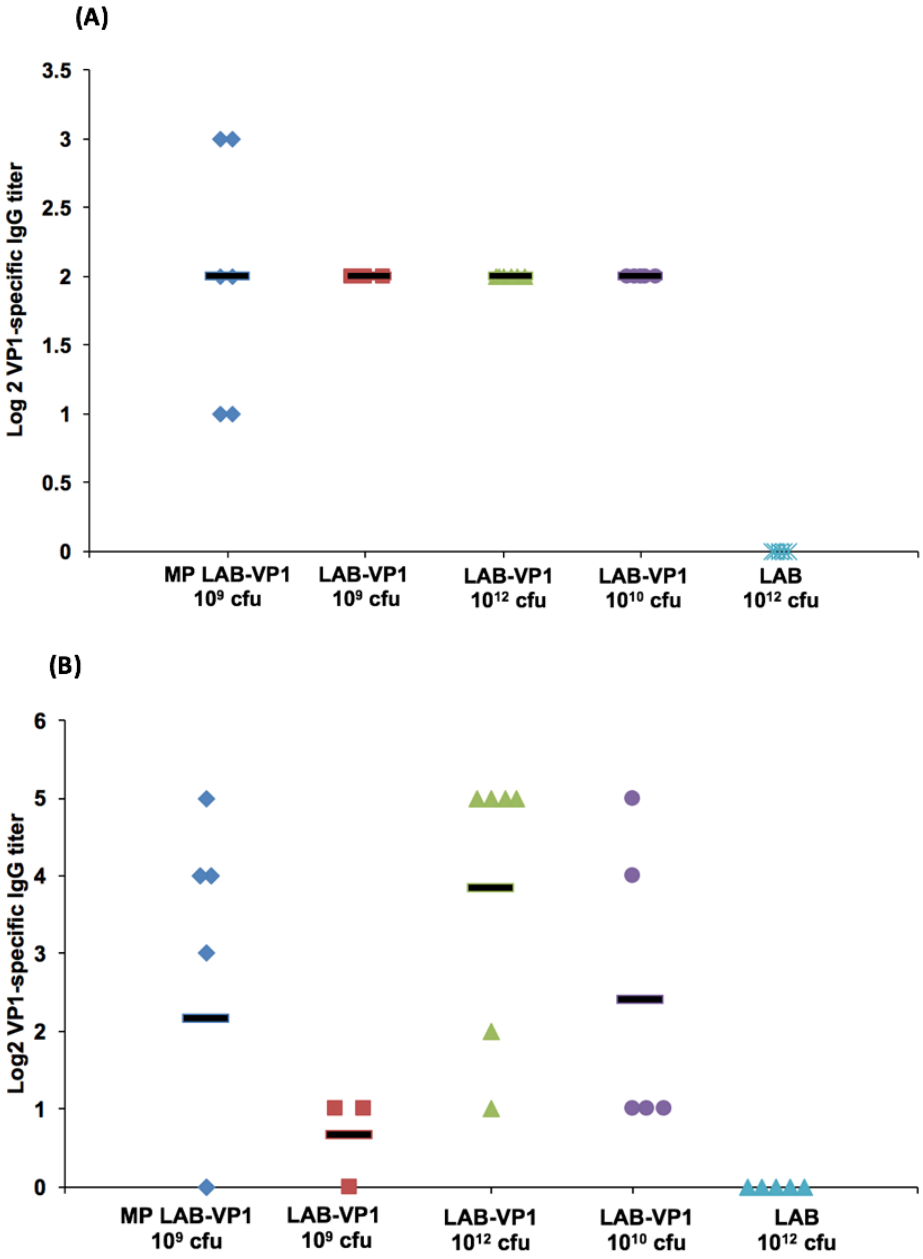


Figure 23. Immune response of IgG from oral vaccination of LAB-VP1 in gnotobiotic piglets

(A) The titer of HuNoV-specific IgG on PID13 measured with ELISA. (B) The titer of HuNoV-specific IgG on PID20 measured with ELISA.

2.4.13. Oral vaccination of LAB-based vaccine induced HuNoV-specific IgA response

To determine whether LAB-based HuNoV vaccine triggered HuNoV-specific IgA, feces were collected from each piglet at days 13 and 20 post-vaccination, and HuNoV-specific IgA responses were detected by ELISA. As shown in Fig. 24A, HuNoV-specific IgA antibody was detectable in some of the piglets vaccinated with a vaccine containing LAB-VP1. One piglet from the LAB-VP1 10^9 cfu and LAB-VP1 10^{12} cfu groups, and two piglets from LAB-VP1 10^{10} group had detectable IgA. However, none of piglets in LAB control group had detectable HuNoV-specific IgA. At day 20 post-vaccination, more piglets developed HuNoV-specific IgA. Three out of 5 piglets in the LAB-VP1 10^{10} cfu group, 3 out 6 piglets in LAB-VP1 10^{12} cfu group, 2 out of 3 piglets in LAB-VP1 10^9 cfu group, and 2 out of 6 in MP LAB-VP1 10^9 cfu group in were positive for HuNoV-specific IgA . There was no significant differences among these groups ($P>0.05$). In contrast, none of piglets in LAB control group were positive for HuNoV-specific IgA. Therefore, LAB-based HuNoV vaccine triggered HuNoV-specific IgA antibody.

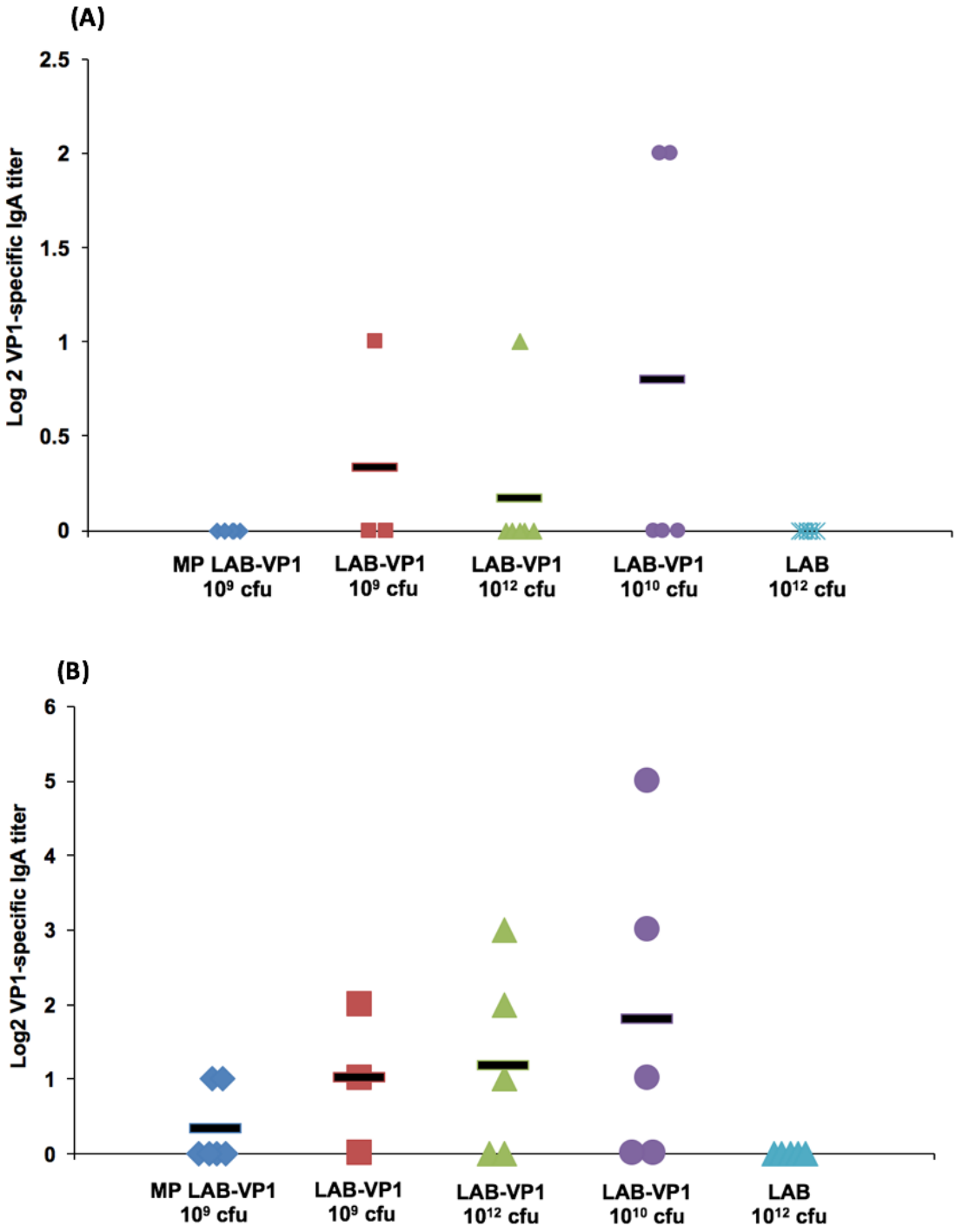


Figure 24. Immune response of IgA from oral vaccination of LAB-VP1 in gnotobiotic piglets

(A) The titer of HuNoV-specific of IgA on PID13 measured with ELISA. (B) The titer of HuNoV-specific IgG on PID20 measured with ELISA.

2.4.14. HuNoV shedding following the challenge in gnotobiotic piglets

To determine whether LAB-based HuNoV vaccine can protect gnotobiotic piglets from HuNoV shedding, fecal samples were collected from each piglet at day 1-5 after challenge with HuNoV GII.4 strain. Total RNA was extracted from fecal samples, and HuNoV RNA was quantified by real-time RT-PCR. The presence and the average titer of viral RNA detected in pig feces at each PID are summarized in Fig. 25. Unfortunately, there was no significant difference in HuNoV shedding among all groups including the LAB vector control group.

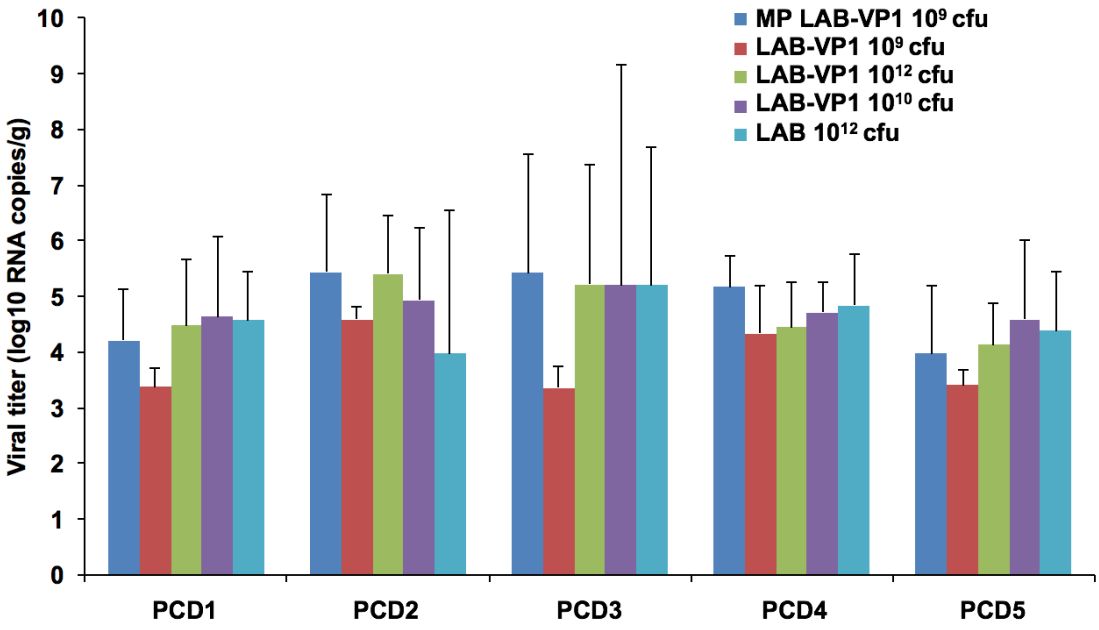


Figure 25. Fecal HuNoV shedding in gnotobiotic piglets after challenge

The fecal samples were collected from each piglet until PID 5 after challenge with HuNoV. Total RNA was extracted, and the HuNoV RNA was quantified by real-time RT-PCR.

2.4.15. LAB-based HuNoV vaccine prevented HuNoV antigen expression in small intestines of gnotobiotic piglets following challenge

Finally, we determined whether HuNoV antigens could be detected in intestinal tissues after challenge. To do this, fresh duodenum, jejunum, and ileum tissues were collected at PID 5, and were subjected to a whole-mount tissue indirect immunofluorescence assay (IFA) using a polyclonal antibody against the VP1 protein of HuNoV. The presence of HuNoV VP1 antigens was visualized by confocal fluorescence microscopy. As shown in Fig. 26, a large number of HuNoV-positive staining (green) cells in duodenum and jejunum tissues from gnotobiotic piglets vaccinated with LAB control were detected. The HuNoV positive staining of cells at villous tips and the adjacent sides of individual villi indicated that the replication of HuNoV occurred in enterocytes and HuNoV antigens were expressed in enterocytes in these cells. In contrast, significant less or fewer HuNoV-positive staining (green) cells were observed in all LAB-based vaccine groups. No antigen expression was detected in negative control (LAB group without anti-HuNoV antibody). Therefore, these data suggest that LAB-based HuNoV diminished or prevented HuNoV replication in pig intestine.

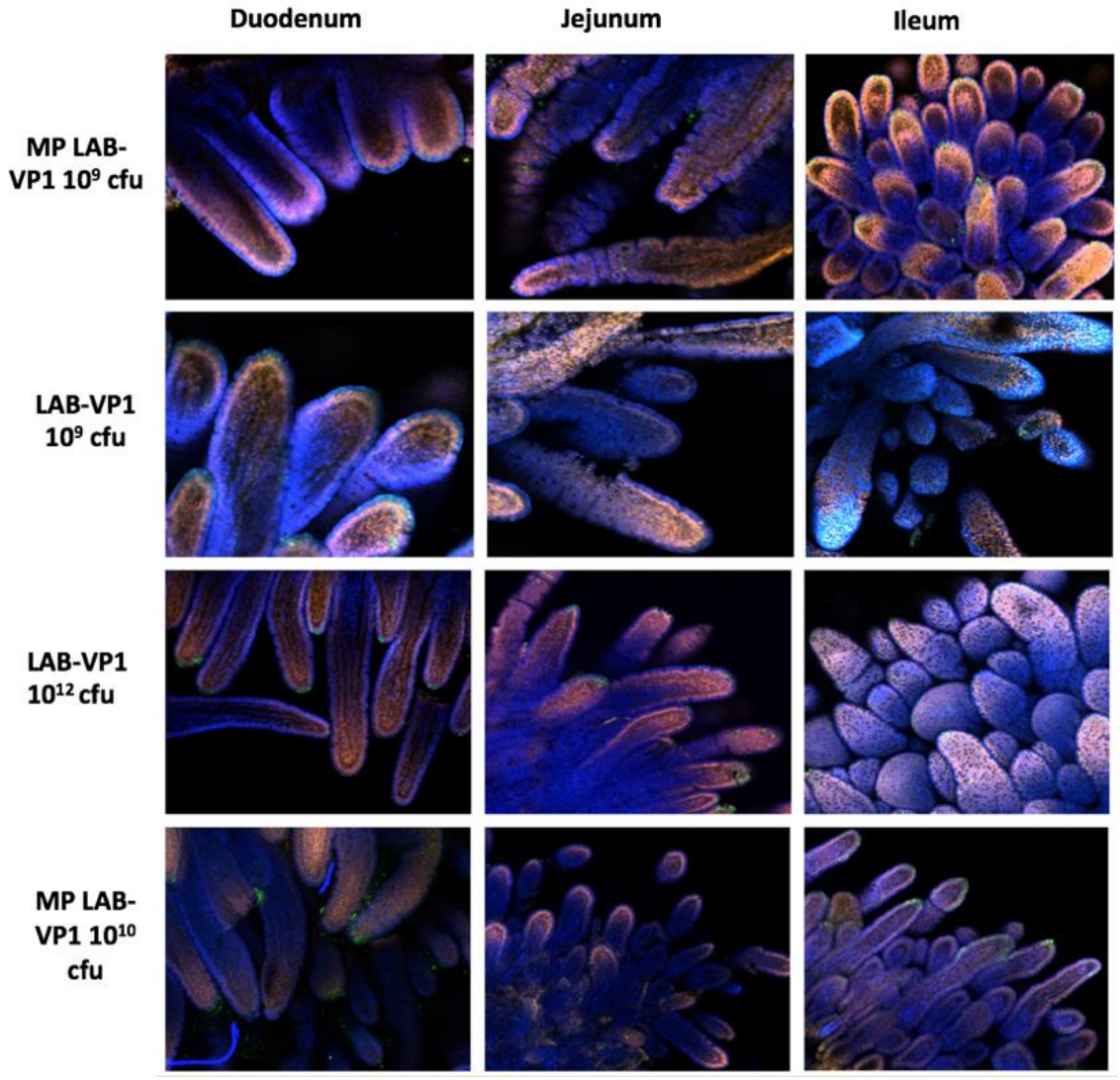
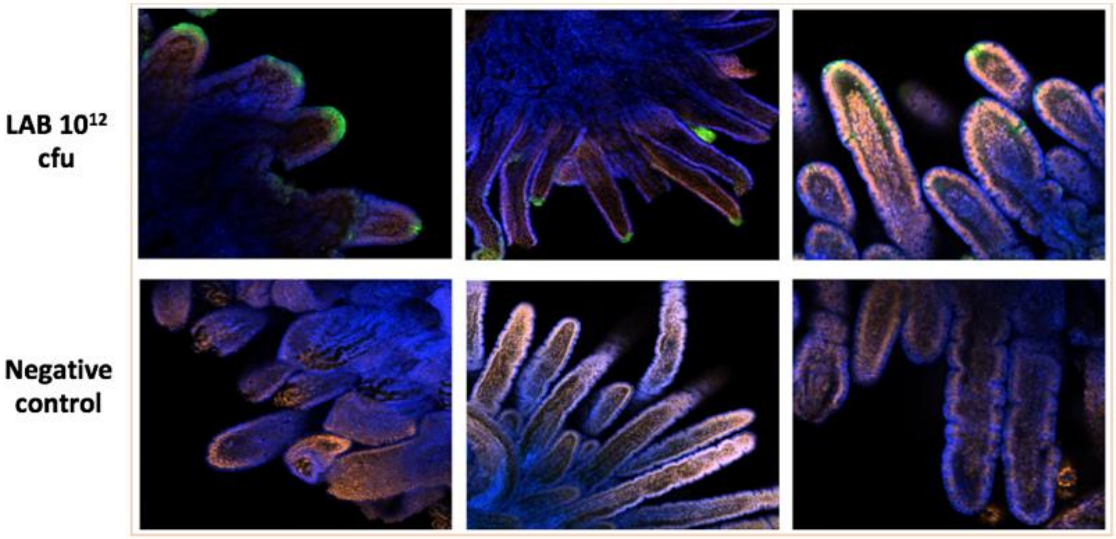


Figure 26. Detection of HuNoV antigen-positive cells in the intestine by IFA

Figure 26. Continued



2.5. Discussion

There is a need for a safe and immunogenic HuNoV vaccine to prevent and control the spread of HuNoV. LAB-based vaccine candidates have been developed for a number of viruses, bacteria, and parasite. However, whether LAB can be used as a vector to deliver HuNoV has not been explored. In this study, we developed a LAB-based HuNoV vaccine candidate. We first showed that HuNoV VP1 can be highly expressed by LAB vector. Then, we developed a novel microencapsulation technology to enhance the stability of LAB. Finally, we showed that oral vaccination of LAB-based HuNoV vaccine triggered HuNoV-specific IgA and IgG responses and prevented HuNoV replication in a novel gnotobiotic pig model. Our data highlights that LAB-based HuNoV is a promising vaccine candidate for HuNoV.

The stability of LAB-VP1 in different pH environments.

The survival of LAB-VP1 in the GI tract has been a challenge due to the harsh acidic stomach acid and alkaline environment in the duodenum. In addition, the enzymes secreted in the digestive tract will likely impact the stability of LAB-VP1. The pH of saliva is usually between 6.5- 7.5. In the stomach, the pH reaches 1.5-2.5. After mixing food and stomach juices, it then enters the duodenum section of small intestine where the pH raises to 7.0 – 8.5. We determined the stability of LAB-VP1 in different pH environments. We demonstrated that LAB-VP1 was significantly more stable when starting in basic solutions (pH 8.5) than neutral and acidic pH ($P<0.05$). Incubation of undiluted LAB-VP1 at pH 8.5 at 37 ^{o}C for 48 h resulted in 5.6

log reduction whereas at pH 7.0 and 4.0 led to 6.5 and 9.5 log reduction respectively. At pH 2.0, the undiluted LAB-VP1 had 5.2 log reductions after 30 min incubation. These results demonstrate that LAB-VP1 was highly susceptible to acidic pH environment. The most convenient and inexpensive cold storage for bacteria is 4°C. As expected, we showed that LAB-VP1 had a much better survival rate in 4 ^{o}C than 37 ^{o}C. The LAB metabolic processes slow down when the temperature drops, thus allowing for elongated survival. When LAB (*Lactobacillus gasseri)* was stored in 4°C, the survival lasted for the entire 28d as was observed with LAB-VP1 (Chávarri et al., 2010). We also found that bacteria concentration affected the survival of LAB-VP1 in stressful conditions. In all cases, a higher concentration of LAB-VP1 in solutions enhanced the survival of bacteria whereas lower concentrations of LAB-VP1 reduced the survival rate. It is known that *Lactobacillus* uses the cell density-dependent quorum sensing system to regulate the expression of related genes to make themselves more adaptable to the surrounding environment (Sturme, Francke, Siezen, de Vos, & Kleerebezem, 2007). It is likely that a higher density of bacterial cells would have a higher quorum sensing activity which enhances cell-cell communication and the survival rate. In addition, a higher concentration of LAB may produce higher amount of metabolites (such as lactic acid, sugar, and other small molecules) which protect the bacteria from autolysis. The difference in strain is another factor that influences the stability of LAB in acidic environments. When *L. lactis* is exposed to acid stress, oxidative stress proteins and heat shock proteins are expressed to help survival within the harsh acidic environment. It was shown that *Lactobacillus acidophilus* JCM 1132c and *Lactococcus lactis* subsp. *lactis* bv. diacetylactis N7 had 0.5 and 4.5 log reduction after treatment with pH 2.5 at 37 °C for 30 min respectively, whereas *Lactococcus lactis* subsp.

cremoris (ATCC 19257) and *Lactococcus lactis* subsp. *lactis* bv. diacetylactis DRC1 were almost completely inactivated at the same condition (Kimoto, Nomura, Kobayashi, Mizumachi, & Okamoto, 2003).

To further enhance stability of LAB-VP1, we developed a novel microfluidic device to encapsulate the lactic acid bacteria (LABs) into microparticles. The bacteria-laden microcapsules were suspended in 0.4% chitosan to obtain the first layer of chitosan, followed by soaking in of 0.15% sodium alginate to obtain the second layer of alginate coating, and then resuspended in 0.4% chitosan to obtain the first layer of chitosan. The final microparticles have chitosan-alginate-chitosan (C-A-C) coating on microcapsules. The alginate layering is from brown seaweed. The chitosan layering is made from chitin, which is found on crustacean shells, when deacetylation occurs and allows the microparticles to bind to the intestinal lining following ingestion. The chitosan and alginate shell of the microparticles provide layers of protection from the harsh pH environments and enhance the stability of LAB-VP1. We found that microencapsulation of LAB-VP1 enhanced the survival rate in an acidic environment. This is consistent with several other studies using other LAB strains. When LAB (*Lactobacillus gasseri*) were encapsulated with chitosan-alginate, they were significantly more stable in pH 2 simulation gastric juice and bile simulation intestinal juice (pH 6) compared to nonencapsulated LAB (Chávarri et al., 2010). Then in Lee, Cha, and Park (2004) study, *Lactobacillus bulgaricus* encapsulated in chitosan-alginate microparticles were also significantly more stable in simulation gastric juice of pH 2 (J. S. Lee, Cha, & Park, 2004). These findings are consistent with

the results from this study with a significantly higher survival of LAB-VP1 microparticles in pH 2 and pH 7. Clearly, microencapsulation of LAB will enhance the stability of LAB *in vitro*.

LAB-VP1 shedding and persistence in gnotobiotic piglets.

Gnotobiotic piglets provide a good animal model to investigate the persistence of LAB-VP1 in the digestive system and evaluate the shedding in their feces. These piglets are germ free, lack microflora, and share many similarities with humans in gastrointestinal structure, physiology, and immunology. The ability for LAB-VP1 to survive in the digestive tract is critical to stimulate an immune response. To evaluate the survival of LAB in the GI tract, newborn gnotobiotic piglets were orally inoculated with various doses either with or without microencapsulation, and fecal samples were taken to quantify LAB titer. We found that high LAB titers were detected at days 6, 13, and 20 post-inoculation. Surprisingly, no significant difference was observed among the different doses of LAB-VP1, ranging from 10^9 cfu to 10^{12} cfu per piglet. At day 25, all piglets were euthanized, and high amounts of LAB were detected in different sections of the small intestine, and contents of the jejunum and colon. This demonstrated that LAB can survive in the pig digestive system and shed in pig feces for at least 25 days. Although we are not able to demonstrate whether LAB-VP1 is capable of colonizing in the gut of the pig, it is clear that LAB survived the harsh acidic pH in the stomach and grew in the small and large intestine. For example, 10^{5-7} cfu/g feces of LAB-VP1 were detected in some piglets inoculated with 10^9cfu of LAB-VP1. Given the fact that a normal piglet produces at least 100 g of feces per day, the amount of LAB-VP1 shedding far exceeds the input LAB-VP1. Thus, bacteria cells must have been multiplying in the pig digestive tracts. Although we do not

understand why LAB-VP1 shedding was similar in all groups, it is possible that a dose of 10^9 cfu is sufficient. Therefore, enough bacteria survived the harsh environment and are capable of multiplying in the intestine. Further studies are needed to investigate the level of LAB shedding in gnotobiotic piglets using a dose lower than 10^9 cfu per piglet.

Currently, it is still controversial whether LAB can colonize in the intestine after oral ingestion. However, it is believed that some LAB strains can establish colonization, for at least short-term, which may be sufficient to deliver and release a foreign antigen to stimulate an immune response. Although LAB has been used as a vector to deliver antigens of many pathogens, most of these studies used small animals (such as mice and rats) to determine the survival and colonization in the gut. The LAB shedding after oral vaccination in mice varies from 12 h to 10 days (Kimoto et al., 2003; Pavan, Desreumaux, & Mercenier, 2003). In humans, it has been shown that oral administration of probiotic bacteria can produce temporary colonization of the intestine in patients with a fully developed gut microflora. Several probiotics have been shown to be able to attach to the human intestinal mucosa. For example, oral administration of *Lactobacillus* GG into infants can result in a 2- to 12-week fecal recovery of the administered strain in feces (Schultz et al., 2004). This suggests that probiotics are capable of multiplying and surviving in the normal intestinal tract. Similarly, in our study, we found that a significant amount of LAB shed in the feces for at least 25 days in gnotobiotic piglets. Future studies will be needed to determine how long LAB-VP1 can survive in gnotobiotic piglets.

Immunogenicity of LAB-based HuNoV vaccine in gnotobiotic piglets.

Despite major efforts, there is no FDA approved HuNoV vaccine to protect people from HuNoV infection. This is due, in major part, to the fact that it lacks an efficient culture system and a small animal model to evaluate the efficacy of vaccine. To date, most HuNoV vaccine studies have used HuNoV virus-like particles (VLPs) as immunogen(s). Oral or intranasal immunization with VLPs induces variable humoral, mucosal, and cellular immunity. The safety concern of viral vectored vaccine candidates prevented the clinical trials in humans. Although, the VLP-based vaccine candidate is promising, there is a need to explore alternative strategies to develop a HuNoV vaccine.

An ideal HuNoV vaccine should be safe, stable, inexpensive, easy to deliver, and induce robust humoral, mucosal, and cellular immune responses at sites where pathogens interact with the host. Therefore, this can generate immediate (innate) and long-term (acquired) immune barriers against infection. Food grade LAB is an excellent vector to deliver a HuNoV vaccine. First, LABs are natural probiotics which are safe for human consumption. Second, LAB can survive passage through gastric acid and are able to grow in the gut as well as provide long-term boost effects of target vaccines. Third, high levels of antigen expression can be achieved using LAB as the vehicle. Fourth, LAB-based vaccines can elicit both mucosal and systemic immune responses which have been shown for many other pathogens. Fifth, LAB expressed antigens can be absorbed into Peyer's patches, the inductive sites of the mucosal immune system. Finally, LAB can be easily grown and is inexpensive, thus can facilitate the large scale production of vaccines (S. H. Kim et al., 2016).

In our study, we showed that HuNoV VP1 can be highly expressed by a LAB vector. A large number of VP1 can be detected in LAB-VP1 cell lysates and supernatants. These VP1 proteins will likely be captured by antigen presenting cells which in turn generate HuNoV-specific immunity. Using a unique gnotobiotic piglet model, we showed that a LAB-based vaccine triggered HuNoV-specific fecal IgA and serum IgG response as early as at day 13 post-vaccination and was increased at day 20 post-vaccination. Previously, it has been shown that gnotobiotic piglets can be infected by HuNoV. However, the robustness of infection will depend on the strain of HuNoV, the inoculation level, age, and the level of HBGA expression in the pig's intestine. It was shown that gnotobiotic piglets infected by some of HuNoV strains can develop mild diarrhea and HuNoV shedding in feces. At the necropsy, the HuNoV antigen expression can be detected in the small intestine but not the large intestine. Within the small intestine, the duodenum section typically had significantly more HuNoV expression than the jejunum and ileum sections. In our study, we did not observe a significant difference in HuNoV shedding in feces after the HuNoV challenge. Importantly, we found that LAB control group had a large number of HuNoV antigen expression in the duodenum, and had relatively less HuNoV antigen expression in the jejunum and ileum section. Importantly, all groups received LAB-based vaccines containing LAB-VP1 has significantly less or no HuNoV expression in duodenum sections, and essentially no antigens were detected in the jejunum and ileum sections. These results demonstrate that the LAB-based HuNoV vaccine prevents HuNoV replication in the small intestine.

In our study, we did not observe a dose effect for the LAB-based HuNoV vaccine. We used three doses of LAB-based vaccines, 10^9, 10^{10}, and 10^{12} cfu per piglet for vaccination. For comparison, we also included a group of piglets receiving 10^9 cfu of LAB-VP1 with microparticles. This was administered with the goal of enhancing the stability of LAB-VP1 as well as testing the potential adjuvant effect of chitosan (Wittaya-areekul, Kruenate, & Prahsarn, 2006). Unfortunately, we did not observe a significant difference in HuNoV-specific IgA and IgG responses within these groups. In the current experiment, we only tested the efficacy of a single dose vaccination within three weeks after vaccination. Future experiments should examine the long term immune responses and the effects of booster vaccination. Interestingly, live LAB-VP1 bacteria were detected in mesenteric lymph nodes of piglets receiving 10^9 cfu of LAB-VP1 with microparticles. However, live bacteria were negative for all other groups, including the 10^{12} cfu group receiving a 1000 times higher dose of LAB-VP1. It is likely that the composition of the microparticle (such as chitosan) may facilitate the attachment of LAB-VP1 to the intestine which can be captured by antigen-presenting cells such as macrophages and dendritic cells. Since the size of microparticles ranges from 300 to 400 μm, it may not be directly phagocytized by these antigen presenting cells. However, the association of chitosan with LAB-VP1 bacteria after degradation of microparticles may be phagocytized and transported to the lymph nodes. Interestingly, when LAB DNA in the spleen and lymph nodes was quantified by qPCR, all groups had similar levels of LAB DNA copies. This suggests that intestinal LAB can be captured by antigen-presenting cells. Future studies are needed to investigate the mechanisms of antigen presentation in gnotobiotic piglets.

In conclusion, we have developed a novel “live” LAB-based vaccine candidate for HuNoV. We found that (1) HuNoV VP1 was highly expressed by a LAB vector; (2) microencapsulation enhanced the stability of the LAB-based vaccine candidate; and (3) LAB-based HuNoV vaccine is immunogenic in gnotobiotic piglets. This innovative vaccine strategy will also shed light on vaccine development for other noncultivable food- and water-borne viruses.

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