Development of Recombinant *Bacillus subtilis* Spore Vaccines Based on Thymine Auxotrophy as a Biocontainment Strategy

Paidamoyo Magret Katsande

Thesis submitted to Royal Holloway, University of London in fulfilment of the requirements for the degree of Doctor of Philosophy

School of Biological Sciences
Royal Holloway, University of London
06 July 2023

Declaration of Authorship

I declare this work was carried out in accordance with the regulations of the University of London. I declare that this submission is my own work, and does not represent the work of others, published or unpublished. Collaboratory work in this thesis is acknowledged and clearly stated in the text. No part of this thesis has been submitted for a higher degree at another university or institution.

Signed: (Paidamoyo Magret Katsande)

Date 06/07/2023

Acknowledgements

I would like to express my sincere gratitude to everyone who has supported me throughout this journey of completing my PhD thesis. Without their guidance, encouragement, and assistance, this achievement would not have been possible. However, I also want to take a moment to acknowledge my own efforts and perseverance throughout this journey. I dedicated countless hours to conducting research, analysing data, and writing this thesis. I am proud of the determination and resilience I exhibited in overcoming challenges and obstacles along the way. This thesis stands as a testament to my hard work, passion for knowledge, and commitment to academic excellence.

I extend my gratitude to my supervisor, Prof. Simon Cutting, for providing me with the opportunity to pursue a PhD in his lab and for his support. I would like to express my appreciation to Dr. Hong Huynh, Dr. William Ferreira, Dr. Katie Lloyd-Jones, Raghunath Mandal, Dr. Laurence Bindschedler, and Prof. Linda Popplewell for their invaluable help and companionship during this research endeavor. Special thanks are due to Prof. Joaquim Segalés for facilitating the BSL-3 SARS-CoV-2 challenge study. I am grateful to Dr. Van Duy Nguyen and for his support in the *Helicobacter pylori* challenge study.

Lastly, I am deeply grateful to my family – my mum, dad, brother, and Sam – for their unwavering support and boundless patience throughout this journey. Their love and encouragement have been my greatest source of strength. In particular, I extend my heartfelt thanks to my mum, whose role as my audience and editor has been invaluable. Her consistent motivation and belief in me have been a guiding light, inspiring me to persevere through every challenge.

Abstract

Bacillus subtilis displaying heterologous antigens show promising efficacy for mucosal immunisation against various viral and bacterial pathogens. In this work, THY-X-CISE system was used to engineer thymine auxotrophic *B. subtilis* strains to express immunogenic antigens from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and *Helicobacter pylori* on their spore surface. This system ensures containment of genetically modified (GM) Bacillus spores by inducing cell death upon exogenous thymine depletion. It also eliminates the need for antibiotic resistance gene markers, relying solely on thymine auxotrophy for positive selection.

The immunogenicity and efficacy of thymine auxotrophic spores against SARS-CoV-2 and *H. pylori* were evaluated, along with their ability to induce mucosal immune responses. In murine experiments, a prime-mucosal spore boost strategy using thymine auxotrophic spores expressing SARS-CoV-2 antigens evoked antigen-specific mucosal IgA and is potentially protective, as shown in a hamster model of SARS-CoV-2 infection. Additionally, oral immunisations with thymine auxotrophic spores displaying *H. pylori* antigens induced mucosal responses and significantly reduced *H. pylori* colonisation in mice following challenge.

In summary, the THY-X-CISE spore vaccine platform is attractive due to its potential for biological containment, rapid and cost-efficient vaccine production, and heat stability. Utilised as a mucosal booster or prophylactic vaccine, spore vaccines could benefit in addressing current and future emerging diseases, enhancing both systemic and mucosal immunity across various viral and bacterial diseases.

Tables of Contents

List of Figures

List of Tables

Abbreviations

µg - Microgram

µl - Microlitre

Ab^R - antibiotic resistance

ACE2 - angiotensin-converting enzyme 2

ACK - ammonium-chloride-potassium

ADCC – antibody-dependent cellular cytotoxicity

AI - artificial intelligence

AMR – antimicrobial resistance

***amyE* –** alpha-amylase

APCs - antigen-presenting cells

AprE - subtilisin E

ARDS - acute respiratory distress syndrome

ASR - age-standardised rate

BAL - bronchoalveolar lavage

BHI - brain heart infusion

***bla* -** ampicillin resistance

BSA - bovine serum albumin

BSL-3 - Biosafety level-3

CAA - Casamino acids

CagA - cytotoxin-associated gene A

CDC - Centers for Disease Control and Prevention

CFU - colony forming unit(s)

cGAMP - Cyclic guanosine monophosphate–adenosine monophosphate

CoV - coronavirus

COVID-19 - coronavirus infectious disease 2019

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

CT - cholera toxin

CTB - cholera toxin B subunit

CVST - cerebral venous sinus thrombosis

DC – Dendritic cells

DHF – dihydrofolate

DHFR – dihydrofolate reductase enzyme

DNA - deoxyribonucleic acid

DNA - deoxyribonucleic acid

DSM - difco sporulation media

dsRNA - double-stranded ribonucleic acid

dTMP – deoxythymidine monophosphate

dUMP – deoxyuridine monophosphate

ECDC - European Centre for Disease Prevention and Control

EEA – European Economic Area

ELISA – enzyme-linked immunosorbent assay

EUA – emergency use authorisation(s)

FBS – fetal bovine serum

FDA - Food and Drug Administration

FlaA – flagellar antigen A

FlaB – flagellar antigen B

FliD - Flagellar hook-associated protein 2

GI – gastrointestinal

GM – genetically modified

GMO – genetically modified organism

H_2SO_4 - Sulphuric Acid

HBA – horse blood agar

HGT – horizontal gene transfer

HP – *H. pylori*

HR – heptad repeat

i.g. – intragastric

i.m. – intramuscular

i.n – intranasal

i.p. - intraperitoneal

IARC – International Agency for Research on Cancer

IBV – infectious bronchitis virus

ICAM1 – intercellular adhesion molecule-1

IFN- interferon

Ig - immunoglobulin

IgA - immunoglobulin isotype A

IgG - immunoglobulin isotype

IL – interleukin

ILA - indole-3-lactic acid

IPTG - isopropyl β-D-thiogalactopyranoside

ISG - interferon-stimulated gene(s)

IU – infectious units

***kan* –** kanamycin

kDa – kilodaltons

KF – flagellin

LAIV – live attenuated influenza vaccine

LB - Luria Bertani

LTB – heat liable toxin

Ly6e – lymphocyte antigen 6 complex family member E

MAbs – monoclonal antibodies

MCS – multiple cloning site

MERS - Middle East respiratory syndrome

mRNA – messenger ribonucleic acid

N – nucleocapsid

nABs – neutralising antibodies

NK - Natural Killer

NLRs – nucleotide-binding oligomerisation domain-like receptors

OD - optical density

Omi – omicron

OP - oropharyngeal

ORF – open reading frame

PAbs – polyclonal antibodies

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PHSM – Public Health and Social Measures

PKR – protein kinase R

PoCT – point-of-care assays

PPV – positive predictive value

PRR – pattern recognition receptor(s)

qPCR - quantitative polymerase chain reaction

rAD – recombinant adenovirus

RBD – receptor binding domain

rN – recombinant nucleocapsid

ROS – Reactive oxygen species

rS – recombinant spike

S – spike

SARS - Severe Acute Respiratory Syndrome

SASPs – small acid-soluble proteins

SAT – stool antigen test

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

SMM - Spizizen's minimal medium

SSR – site-specific recombination

ssRNA - single-stranded RNA

T4SS – type IV secretion system

Tcd - Clostridial toxin

Th – T-helper

THF – tetrahydrofolate

THFR – tetrahydrofolate reductase

***thrC* –** threonine

Thy – Thymidylate synthase

TLR - toll-like receptors

Tm – trimethoprim

TMB – 3,3',5,5'-Tetramethylbenzidine

TMP – trimethoprim

TMPRSS2 – transmembrane protease serine protease 2

TNF – tumour necrosis factor

TRM – tissue-resident memory T cell(s)

TSase – thymidylate synthase

TTFC – tetanus toxin fragment C

UBT – urea breath test

***ure* –** urease

UreA – urease subunit A

UreB – urease subunit B

URT – upper respiratory tract

v/v - Volume per volume

VacA - Vacuolating cytotoxin A

VOCs – variants of concern

w/v - weight per volume

WHO – world health organisation

WT – wild type

Wuh – Wuhan

CHAPTER 1: Introduction

1.1 Growth and sporulation of *Bacillus subtilis*

Bacillus subtilis is a rod-shaped, Gram-positive spore-forming bacteria that was originally described as *Vibrio subtilis* in 1835 by Christian Gottfried Ehrenberg (Ehrenberg, 1835) and was renamed in 1872 by Ferdinand Cohn (Cohn, 1872). It is classified as an obligate aerobic bacterium that is ubiquitous, but commonly found in the soil (Nicholson, 2002). *B. subtilis* is not pathogenic or toxigenic and is considered safe for human consumption as a dietary supplement and is currently used as a probiotic supplement in animal feeds (Cutting, 2011; Sorokulova *et al*., 2008).

B. subtilis is recognised as a model prokaryote that offers a unique model system to understand cell division because of its ability to undergo symmetrical cell division (Levin *et al*., 1998) and asymmetrical cell division (Ben-Yehuda & Losick, 2002). During vegetative growth, the rod-shaped bacterium replicates via a binary fission process which precisely occurs in the midpoint of the rod, resulting in the formation of two identical daughter cells, and this process typically occurs when the conditions are favourable (Angert, 2005; Levin *et al*., 1998). Sporulation occurs when the vegetative cell is exposed to nutrient-deprived conditions and takes approximately 8 hours to complete and is controlled by a genetically regulated process (Hosoya *et al*., 2007). Sporulation is initiated through asymmetrical cell division in which a septum forms in the polar region of the vegetative cell. This process produces two distinct morphological cell types that are genetically identical: a larger mother cell and a smaller cell type referred to as a forespore. These cells remain in close contact and are connected by the external cell wall. Subsequently, the mother cell engulfs and encloses its membrane around the forespore,

which leads to the formation of an outer coat. The forespore eventually matures into a dormant spore that is partially dehydrated. Programmed cell lysis of the mother cells leads to the release of the mature spore into the environment (Hosoya *et al.*, 2007; Khanna *et al.*, 2020; Riley *et al.*, 2021; I. S. Tan & Ramamurthi, 2014). A diagrammatic representation of this cycle is displayed in Figure 1.1. Exposure to favourable conditions triggers the spore to germinate and resume the vegetative cell cycle (Cano & Borucki, 1995; Nicholson *et al.*, 2000).

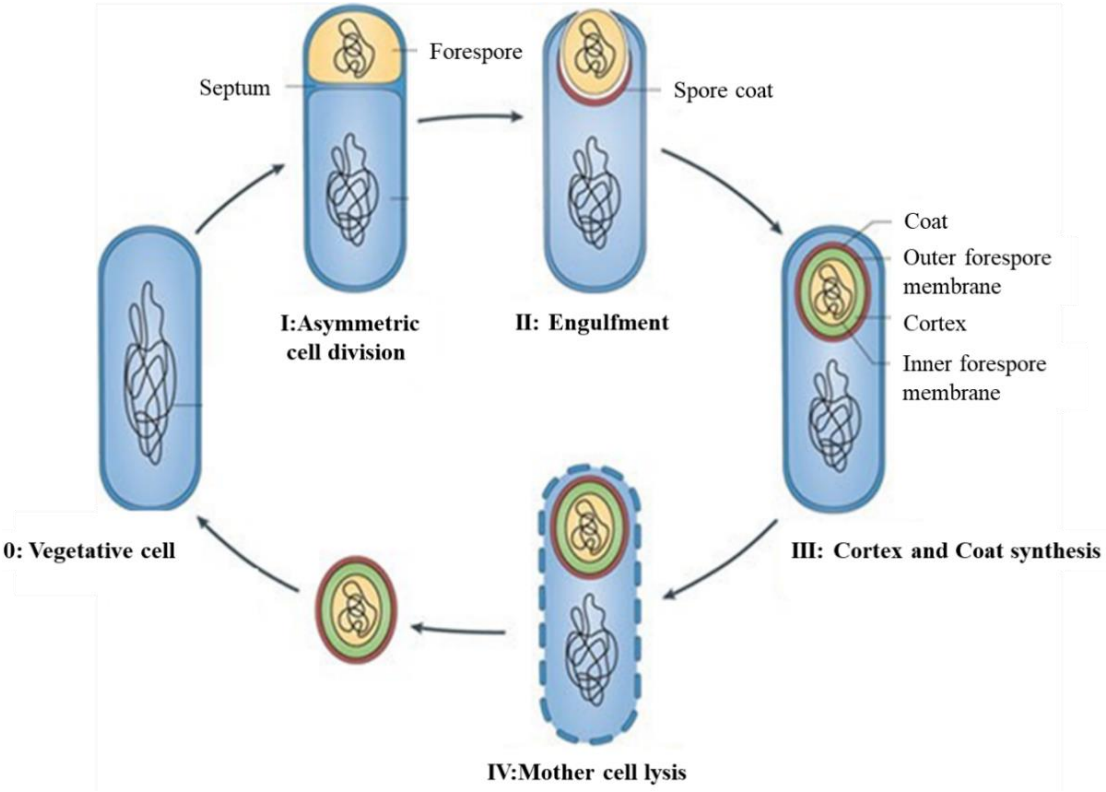

Figure 1.1. The sporulation cycle in *B. subtilis.* (Stage 0) The chromosome replicates in the vegetative cell. (Stage I) Asymmetric cell division and septum formation occur resulting in two compartments, the smaller forespore and the larger mother cell. DNA translocase pumps one copy of the chromosome into the forespore. (Stage II) The mother cells engulf the forespore by enclosing its membrane around the forespore. (Stage III) The cortex and coat are synthesised forming a double membrane forespore in the mother cell. (Stage IV) The forespore undergoes maturation, followed by lysis of the mother cell and the spore is released into the environment. Modified from (McKenney *et al.*, 2013).

1.2 *B. subtilis* spore structure

The spore has an elliptical shape and is made up of three major layers which can be visualised by transmission electron microscopy, illustrated in Figure 1.2. There are many factors contributing to the survival and dormancy state of the spore, including the most inner core which contains a condensed and inactive chromosome that is bound by small acid-soluble proteins (SASPs) that protect the DNA from damage (B. Setlow & Setlow, 1987). There is evidence suggesting that the absence of SASPS makes spores more susceptible to DNA damage following exposure to UV radiation (B. Setlow & Setlow, 1987) and hydrogen peroxide (B. Setlow & Setlow, 1993). The cortex comprises a thin peptidoglycan layer that surrounds the inner core of the spore. Although, the precise role of the cortex remains unclear, it has been proposed that the cortex maintains the partially dehydrated state of the spore. The ability of spores to survive extreme temperature conditions is attributable to the spores partially dehydrated state. This dehydration process reinforces the spores resilience and enhances its resistance to extreme temperatures (Imae *et al*., 1976; Mallidis & Scholefield, 1987; McKenney *et al*., 2013; Ulanowski & Ludlow, 1993). Lastly, the proteinaceous spore coat is assembled around the cortex and is further divided into two layers, a thin inner coat (75 nm wide) and a thick outer coat (70-200 nm wide) (Driks, 1999). The spore coat also contributes to spore survival by providing protection against enzymatic assaults such as lysozymes (Driks, 1999; Moir, 1981; Zheng *et al*., 1988).

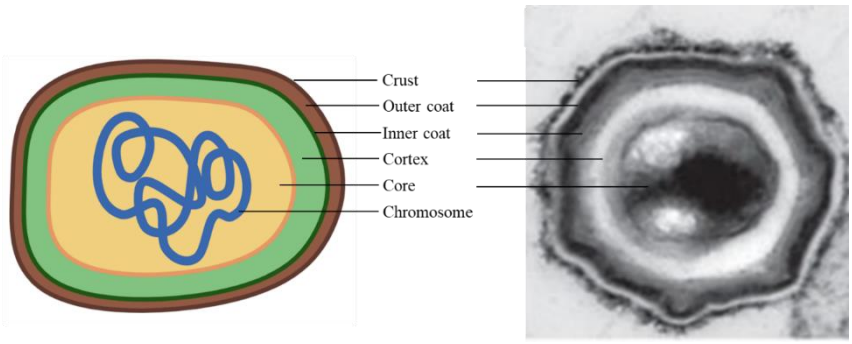

Figure 1.2. Schematic diagram and electron micrograph of *B. subtilis* spore. The spore structure consists of multiple layers that surround the genome, which is located in the central core. The spore coat consists of three layers: the inner coat, the outer coat and the crust (McKenney *et al*., 2013). The electron micrograph was reproduced from (McKenney *et al*., 2010).

1.2.1 Spore coat

The spore coat develops late in sporulation, precisely during forespore maturation. This layer completely degrades when the released spore germinates (Zilhão *et al.*, 2005). The coat is made up of two distinct layers, a lamella-like inner coat and an electron-dense outer coat (P. Setlow, 2006). The spore coat is a proteinaceous structure that contributes to approximately 10% of a single spore's total dry weight and 25% of its total protein (Munoz *et al.*, 1978). There are approximately 70 proteins, and only a small subset of these proteins are required for coat morphogenesis (Henriques & Moran, 2007; Munoz *et al.*, 1978). For instance, GerE is responsible for the formation of the lamella-like inner coat layer. *B. subtilis* spores carrying a *gerE* mutation have a defective inner coat, as evidenced by an increase in the sensitivity of these spores to lysozyme (Henriques & Moran, 2007; Moir, 1981). GerE also plays a role in regulating the expression of other morphogenic proteins including CotX, CotY and CotZ. The CotXYZ proteins are responsible for the formation of the crust layer that surrounds the outer coat, and it has

been reported that these proteins might influence the way the spore interacts with the environment. However, the exact mechanisms by which CotXYZ impacts the spores physiology and behaviour are not fully understood (McKenney *et al.*, 2010; Moir, 1981; Sacco *et al.*, 1995; J. Zhang *et al.*, 1993; Zheng *et al.*, 1988). Lastly, CotE is a 21 kDa morphogenic protein that is expressed during the early stages of spore coat formation and assembles onto the coat. It is responsible for the formation of the outer coat and regulates the expression of outer coat proteins including CotA, CotB, CotC and CotG (Driks, 1999; Zheng *et al.*, 1988). Overall, the coat protects spores against environmental stress factors including (but not limited to) nutrient starvation, acidity, elevated temperature, radiation, desiccation and microorganisms that ingest and digest bacteria (Errington, 2003; Nicholson *et al.*, 2000, 2002; P. Setlow & Johnson, 2019; Stragier & Losick, 1996). It also provides protection against lytic enzymes that target and degrade the spore cortex. Such enzymes are produced by predatory organisms including bacteriophages and unicellular eukaryotes (Driks, 1999). In addition to providing resistance to extreme environmental conditions, the spore coat is also involved in the process of germination, which is when a spore responds to nutrients in the environment and transitions from dormancy to a metabolically active vegetative state (Driks, 1999).

1.2.2 *B. subtilis* spore surface display of recombinant proteins

Microbial surface display systems refer to the use of genetic engineering tools to display heterologous proteins and peptides on the surface of biological entities (J. Kim & Schumann, 2009). A prominent surface display system referred to as the phage display system, was first developed by George Smith in 1985. This system entails fusing a heterologous gene with a phage capsid gene, followed by its integration into the genome

of the filamentous phage. Upon expression, the fusion protein is then displayed on the surface of the phage (G. P. Smith, 1985). Since then, surface display systems have expanded to various biological entities such as bacterial spores (Isticato *et al.*, 2001), non-spore forming bacteria (Freudl *et al.*, 1986), yeast (Boder & Wittrup, 1997) and insect cells (Y. Wang *et al.*, 2005). These systems have found applications in biomedical and biotechnological fields. Some of these applications involve developing whole-cell biocatalysts by immobilising enzymes, creating biosensors, producing bioabsorbents for the removal of hazardous chemicals and heavy metals, and facilitating the delivery of vaccines (Braun *et al.*, 2018; H. Chen *et al.*, 2017; Han *et al.*, 2018; Tafakori *et al.*, 2012). While microbial surface display systems have become increasingly versatile and useful for various biomedical and biotechnological purposes, it is important to acknowledge the potential drawbacks associated with these systems. For example phage (J. Castillo *et al.*, 2001), yeast (S. Park *et al.*, 2006) and bacterial (Georgiou *et al.*, 1997) display systems have limitations in the size of proteins that can be displayed on the surface, large proteins can affect the structure of the anchoring protein on the surface. Furthermore, bacterial surface display systems lack post-translational modification processes that eukaryotic proteins require for correct folding. Although yeast surface display systems offer many advantages such as correct protein folding and glycosylation of proteins, they are also prone to excessive protein glycosylation which might contribute to the misfolding of muti-subunit proteins (S. Park *et al.*, 2006; Shukla *et al.*, 2017; Spadiut *et al.*, 2014). In spite of this, *B. subtilis* spore surface display systems, have been extensively studied and offer a promising approach for presenting heterologous proteins on the surface with high stability and activity (Petrillo *et al.*, 2020). To successfully display heterologous proteins on the spore surface, several factors must be considered. Firstly, a translational fusion

must be constructed by fusing the heterologous gene to the coding gene of an anchor protein (e.g. CotB, CotC, and CotG) (J.-H. Kim *et al*., 2005; Ning *et al*., 2011). Secondly, the fusion gene must be integrated into the coding sequence of a non-essential gene such as the alpha-amylase (*amyE*) locus of *B. subtilis* which encodes a non-essential α-amylase. Successful recombination and integration of the fusion gene at the *amyE* locus disrupts the *amyE* gene and prevents the production of α-amylase (Shimotsu & Henner, 1986). The threonine (*thrC*) locus is another integration site and the insertion of a fusion gene at this locus disrupts the *thrC* gene resulting in threonine auxotrophy (A. M. Guérout-Fleury *et al*., 1996; Ricca & Cutting, 2003). Additionally, an important factor that determines whether heterologous proteins are assembled correctly on the spore surface without loss of protein function is the site of fusion. Heterologous proteins can be genetically fused to the N- terminus, C- terminus or the middle (sandwich fusion) of an anchor protein (Isticato *et al*., 2001; Ricca & Cutting, 2003). A study showed that when the C-terminal fragment of the tetanus toxin (TTFC) was fused to the C-terminal end of a full-length CotB (380- amino acids), the heterologous protein failed to correctly assemble on the spore surface (Isticato *et al*., 2001). It is proposed that failure to assemble on the spore surface could be due to the three 27-amino acid repeats of CotB. Deletion of the three 27-amino acid repeats (CotBΔ105 has 275- amino acids) and fusion of TTFC to CotBΔ105 (CotBΔ105-TTFC) resulted in the correct assembly of TTFC on the spore surface. Additionally, fusion of TTFC to the N-terminal and the middle of CotBΔ105 resulted in the correct assembly of the protein on the spore surface. This suggests that the construction of a stable fusion (CotB-heterologous protein) is achieved by deleting the three 27-amino acid repeats of CotB (Isticato *et al*, 2001). CotC is another surface protein that can be used as an anchor protein and successful spore surface display depends on the

location in which the heterologous protein is fused to. For instance, there was a 5-fold increase in the efficiency of display when TTFC was fused to the N-terminal end of CotC rather than the C-terminal end of CotC (Isticato *et al*., 2007).

1.3 Mucosal delivery of recombinant *B. subtilis* spore vaccines

Genetically engineered *B. subtilis* spores displaying heterologous antigens on their surface have been shown to offer promise as a platform for the mucosal delivery of vaccine antigens (Duc *et al*., 2003; S. Lee *et al*., 2010; Permpoonpattana *et al*., 2011; Reljic *et al*., 2013). The safety of *B. subtilis* is well characterised, given its widespread use as a probiotic. It is non-pathogenic and lacks indigenous virulence genes that are found in the pathogenic bacteria of the genus *Bacillus*, rendering it an ideal model host for vaccine delivery systems (de Souza et al., 2014; Hong et al., 2005; Sorokulova et al., 2008).

Mucosal surfaces including, those of the nasopharyngeal, pulmonary, and gastrointestinal tracts are natural barriers to infection and are constantly exposed to foreign antigens. Most vaccines are currently delivered systemically, targeting pathogens that have already surpassed the mucosal barrier, and as a result, systemic immunisations typically induce the production of systemic immunoglobulin G (sIgG), which are less effective against mucosal infections (Bleier *et al*., 2021). The development of vaccines that elicit a strong immune response against mucosal infections could be an effective strategy to prevent infection at the mucosal surfaces (Vela Ramirez *et al*., 2017; Woodrow *et al*., 2012). Mucosal vaccines elicit immune protection by stimulating the production of both secretory immunoglobulin A (sIgA) at the mucosal site of delivery and IgG at distant

organs (Amuguni *et al*., 2012; Y. Du *et al*., 2021; Hoang *et al*., 2008; Jearanaiwitayakul *et al*., 2021; Permpoonpattana *et al*., 2011; Rossi *et al*., 2014; Y. Yang *et al*., 2018). The development of mucosal vaccines faces numerous challenges that are linked to the innate defence mechanisms of mucosal surfaces, including pH, enzymes and biological barriers (mucus). These factors limit the uptake of vaccine antigens, thereby impeding vaccine efficacy. The successful induction of both sIgA and IgG antibodies requires the development of mucosal vaccines that can efficiently deliver stable and active vaccine antigens across mucosal barriers (Alu *et al*., 2022; Vela Ramirez *et al*., 2017).

B. subtilis spores have been shown to carry natural mucosal adjuvant properties (Barnes *et al*., 2007) and have also been shown to act as immunomodulators and specifically by inducing innate immunity (Song *et al*., 2012). Spores of *B. subtilis* have been successfully used as a platform for delivering recombinant vaccine antigens via the oral and nasal route (Table 1.1). A study showed that the oral administration of recombinant spores expressing a C-terminal domain of *Clostridium difficile* toxin A ($TcdA_{26–39}$) generated sIgA antibodies which protected against *C. difficile* infection (CDI) (Hong *et al*., 2017). In contrast, parenteral administration of toxoids A and B generated IgG antibodies but failed to provide protection against CDI (Kociolek & Gerding, 2016; Kotloff *et al*., 2001). Copland *et al*, (2018) showed that intranasal boosting of BCG primed mice with *B. subtilis* spores genetically engineered to express Ag85B and ACR (spore-FP1) on the spore surface showed increased levels of antigen-specific IgG in the serum and IgA in the lung mucosal surfaces. Additionally, the immunisation of BCG-primed mice with spore-FP1 showed enhanced protection in a low-dose aerosol *Mycobacterium tuberculosis* challenge model, compared to BCG alone (Copland *et al*., 2018). Together this

demonstrates the potential applicability of recombinant spores as mucosal vaccines but also offers insight into the mechanisms of *B. subtilis* spores as a platform for the development of mucosal vaccines.

Table 1.1. Examples of studies demonstrating the production of antigen-specific antibodies and/or protection following immunisations with recombinant *Bacillus* spores.

Target pathogen	Route of delivery	Animal model	Immune response or protection	Efficacy	Reference
C. difficile	Oral	Hamster	Protective and cross-reactive neutralising polymeric antibodies	75%	(Permpoonpattana *et al*., 2011)
Influenza (H5N2)	Nasal	Mouse	Protective mucosal, systemic and cell-mediated responses	100%	(Song *et al*., 2012)
Influenza (H1N1)	Nasal	Mouse	Protective lung mucosal and systemic immune responses	100%	(D. Ma *et al*., 2023)
Clostridium tetani	Sublingual and Oral	Hamster	Protective systemic and mucosal responses and neutralising antibodies to toxins	100%	(Hong *et al*., 2017)
B. anthracis	i.p injection	Mouse	Protective serum antibody responses	100%	(Duc *et al*., 2007)
	i.p injection Oral and Nasal	Mouse	Protective toxin neutralising antibody in sera, and IgA in saliva	90 to 100%	(Y. Oh *et al*., 2020)
H. pylori	Oral	Mouse	Protective serum IgG and faecal IgA and reduction in bacterial load	90.5%	(Z. Zhou *et al*., 2017a)
White spot syndrome virus	Oral	Shrimp	N/A	65%	(A. T. V. Nguyen *et al*., 2014)
S.enterica Typhi	s.c. injection	Mouse	Protective serum antibody responses	50%	(Ghorbani *et al*., 2021)

1.4 *Helicobacter pylori* as a human pathogen

The main functions of the stomach are to initiate digestion and to act as a defensive barrier against orally ingested infectious microorganisms (Martinsen *et al*., 2005). This is achieved by its ability to secrete gastric juice which is composed of gastric acid and pepsin, a proteolytic enzyme (Goo *et al*., 2010; Schubert, 2011). This creates a hostile gastric environment, and as a result, the gastric mucosa was formerly considered sterile. However, the concept of gastric microbiology changed in 1982, when Barry Marshall and Robin Warren discovered and isolated the Gram-negative bacterium *Helicobacter pylori* from the gastric mucosa (B. Marshall & Warren, 1984). Following this discovery, Marshall and Warren applied Koch's postulate method by orally inoculating a culture of *H. pylori* and subsequently developed gastritis (B. J. Marshall *et al*., 1985). Later, Thomas Borody confirmed the link between *H. pylori* and peptic ulcers by developing a triple therapy regimen consisting of bismuth, metronidazole and tetracycline which was shown to cure gastritis and eradicate *H. pylori* infection, thus preventing the recurrence of peptic ulcers (Borody *et al*., 1987, 1989).

H. pylori was formerly known as *Campylobacter pyloridis* and was originally assigned the *Campylobacter* genus due to its broad phenotypical similarities with *C. jejuni*. Subsequent genomic, phylogenetic and ultrastructural analysis revealed that this bacterium did not belong to this genus, and this prompted the creation of the genus *Helicobacter* and the species *Helicobacter pylori* in 1989 (Goodwin *et al*., 1989). *H. pylori* is a microaerophilic bacterium that belongs to the taxonomic Class of Epsilonproteobacteria, of the phylum Proteobacteria (Tomb *et al*., 1997). It is a spiral-

shaped bacterium measuring approximately 2 to 4 μm in length and 0.5 to 1 μm in width, and contains 5 to 7 polar-sheathed flagella essential for motility (Goodwin *et al.*, 1986).

In 1994, the International Agency for Research on Cancer (IARC), classified *H. pylori* as a class I human carcinogen (IARC, 1994). This was later confirmed by a succession of studies that investigated the correlation between *H. pylori* infection and gastric cancer using animal models of infection. Mongolian gerbils are considered one of the best models to study the effect of *H. pylori* infection because it recapitulates many features of *H. pylori* induced gastritis, peptic ulcers, intestinal metaplasia and carcinogenesis (Hirayama *et al.*, 1996; Honda *et al*., 1998; Matsumoto *et al.*, 1997; Noto *et al.*, 2016; Ohkusa *et al*., 2003; Yokota *et al*., 1991). Watanabe *et al*, evidenced that Mongolian gerbils orally inoculated with *H. pylori* developed chronic gastritis which progressed to ulcers and eventually 37% of infected animals developed adenocarcinoma after 62 weeks of infection (Watanabe *et al*., 1998). This was further supported by longitudinal cohort studies (Chow *et al*., 1998; Hansen *et al*., 1999; Hansson *et al*., 1996; Uemura *et al*., 2001). Hansen *et al*, performed a nested case-control study consisting of over 100 000 patients who were followed-up for over 12 years. They showed that *H. pylori* infection increases the risk of developing distal gastric cancer by nine-fold compared to *H. pylori*-negative individuals (Hansen *et al*., 1999). Another study by Uemura *et al*, evaluated 1, 526 Japanese patients with pre-existing ulcers at the time of enrolment. In this study, approximately 82% of these individuals were confirmed to have *H. pylori* infection, and of these individuals, 3% developed gastric cancer (Uemura *et al*., 2001). These studies provide strong evidence that *H. pylori* infection increases the risk of developing gastric cancer.

1.5 *H. pylori* infection

1.5.1 The disease

H. pylori is one of the most successful human pathogens colonising the gastric mucosa of approximately 50% of the global population. The prevalence of infection is variable, ranging from 35% to 90% depending on the population diversity and geographic location (Zabala Torrres *et al*., 2017). Despite a decline in the prevalence of *H. pylori* infection in Western countries, due to improved living standards and sanitation (Eusebi *et al*., 2014), developing countries continue to report high infection rates. This might be attributed to factors such as poor sanitation and low socioeconomic status (Awuku *et al*., 2017). In particular, living in rural areas (Hanafi & Mohamed, 2013; Vilaichone *et al*., 2013), crowded homes (Dorji *et al*., 2014; Hanafi & Mohamed, 2013) and consumption of contaminated water (Ozaydin *et al*., 2013) have been identified as significant factors that increase the risk of acquiring *H. pylori* infection. Cumulative findings from meta-analytic studies have reported that approximately 4.4 billion individuals worldwide are infected with *H. pylori* (Hooi *et al*., 2017). The areas that have reported the highest prevalence of *H. pylori* infection include Africa (70.1%), Southern Asia particularly in Pakistan and India (81% and 63.5%, respectively), Southeast Asia (Vietnam, >70%) and Western Asia (Turkey, 77.2%) (Binh *et al*., 2013; Hooi *et al*., 2017; T. L. Nguyen *et al*., 2010; S. Smith *et al*., 2019).

Initial colonisation of the gastric mucosa typically occurs in infancy or early childhood (Thomas *et al*., 1999) and has widespread health implications and represents a significant public health concern (Hooi *et al*., 2017). In 2006, a community-based study conducted

in Vietnam evidenced that the transmission of *H. pylori* is common within families, and primarily through faecal-oral and oral-oral routes (V. B. Nguyen *et al*., 2006). Nevertheless, the exact reasons why the initial colonisation of *H. pylori* is more frequent among children and less frequent among adults remain largely unknown. However, various works of literature speculate that the gastric environment in children is more favourable for *H. pylori* colonisation and transmission (Pérez-Pérez *et al*., 2003; Rothenbacher *et al*., 2000; Rowland *et al*., 2006). One factor that may contribute to this is that younger children have slower postprandial reacidification (higher gastric pH) and lower pepsin secretion and activation compared to adults (Bücker *et al*., 2012; Gan *et al*., 2018). Bücker *et al,* demonstrated that the initial colonisation of *H. pylori* was significantly higher in Mongolian gerbils under conditions simulating the postprandial reacidification and pepsin activation profiles in young children, further supporting the statement above (Bücker *et al*., 2012).

While the estimated risk of developing overt clinical diseases such as peptic ulcer disease and gastric cancer is low, typically ranging from 10-20% (Dorer *et al*., 2009; Hooi *et al*., 2017), the majority of infected individuals (80-90%) exhibit subclinical disease with varying degrees of mucosal inflammation (Alexander *et al*., 2021; Peek Jr & Crabtree, 2006). If left untreated, persistent *H. pylori* infection can cause long-term damage to the gastric mucosa and increase the risk of developing various gastric diseases, such as chronic gastritis and peptic ulcers. In severe cases it may also lead to the development of gastric malignancies such as gastric adenocarcinoma and mucosal-associated lymphoid tissue lymphoma (Correa & Piazuelo, 2008; J. S. Park *et al*., 2020; Seo *et al*., 2017; Zabala Torrres *et al*., 2017).

1.5.2 Clinical presentation of *H. pylori*-associated disease

Correa's cascade is a multistep model developed by Correa that describes the various premalignant stages leading to gastric cancer (Correa, 1992). According to the model, *H. pylori*-induced inflammation initiates the process, which progresses to chronic gastritis, followed by atrophy, intestinal metaplasia, dysplasia, and ultimately gastric cancer (Correa, 1992; Correa & Piazuelo, 2012). Initial infection with *H. pylori* induces an acute inflammatory response via the activation of transcription factors and secretion of proinflammatory cytokines (Keates *et al*., 1999). This inflammatory response is initiated by recognition of *H. pylori* by toll-like receptors (TLRs), which trigger the activation of nuclear factor kappa B (NF-κB). Activated NF-κB, in turn, triggers the secretion of proinflammatory cytokines from both epithelial cells and the lamina propria (Lamb & Chen, 2013). Additionally, the translocation of *H. pylori* activated NF-κB also plays a role in the activation of $CD4^+$ T cells. Specifically, the activation of NF-κB promotes the differentiation of $CD4^+$ into Th1 subtype, which produces more proinflammatory cytokines such as IFN-γ and TNF-α (Scott Algood, 2020; J. Xie *et al*., 2020; Zhu *et al*., 2010). *H. pylori* also activates the gastric epithelium to produce IL-8. IL-8 is a crucial cytokine that plays a significant role in regulating the recruitment and activation of neutrophils in the gastric epithelium and the underlying lamina propria, where they induce gastric tissue damage (Maeda *et al*., 2000; Torok *et al*., 2005). When the host's immune response fails to eliminate the infection, and proactive inflammation persists in the gastric mucosa, it leads to chronic gastritis. This type of gastritis is characterised by the accumulation of mononuclear leukocytes such as T and B lymphocytes, plasma cells and macrophages in the lamina propria. These immune cells produce proinflammatory

cytokines such as interleukin-1β (IL-1β) and TNF-α, which inhibits the secretion of gastric acid. Therefore, an increase in the gastric mucosal production of IL-8, TNF-α and IL-1β contributes to enhanced gastric inflammation and inhibits parietal cell acid secretion (Noach *et al*., 1994; Peek & Blaser, 2002; Rizwan *et al*., 2008; Sugimoto *et al*., 2012). Data from subjects who deliberately or accidentally ingested *H. pylori* showed that the early stage of colonisation might be associated with non-specific symptoms, including (but not limited to) fullness, nausea, vomiting and diarrhoea. Additionally, reports from these subjects showed the inflammation was restricted to the proximal and distal gastric mucosa and this stage of infection is often associated with hypochlorhydria, a condition characterised by low levels of stomach acid (Graham *et al*., 1988, 2004; Morris & Nicholson, 1987; Sobala *et al*., 1991). Persistent colonisation of the gastric mucosa by *H. pylori* results in chronic gastritis and atrophic gastritis. Over time, the resulting active inflammatory response further exacerbates hypochlorhydria. Specifically, increased gastric production of IL-1β and TNF-α, which are potent inhibitors of gastric acid secretion (El-Omar *et al*., 1997; Ruiz *et al*., 1996; Sugimoto, Furuta, *et al*., 2009). Atrophic gastritis is defined as a severe form of chronic inflammation that causes the loss of gastric glandular cells. This leads to changes in the gastric mucosa, which begins to resemble the intestinal epithelium. As the disease progresses, it can result in the development of intestinal metaplasia, which has been identified as an essential precancerous lesion that predisposes an individual to gastric cancer. Intestinal metaplasia results in further replacement of damaged gastric epithelial cells with fibrosis and intestinal-type epithelium (Correa *et al*., 2010; Jencks *et al*., 2018; Kuipers *et al*., 1995). The extent and severity of gastric atrophy is a determining factor for gastric cancer development and can increase the risk of gastric cancer by 5 to 90 fold (Sipponen *et al*.,

1985). A study reported that individuals with pre-existing severe hypochlorhydria had an increased risk of developing atrophic gastritis when they received treatment containing a proton pump inhibitor (omezaprole) (Kuipers *et al*., 1996). Additionally, a randomised prospective study showed that anti-*H pylori* therapy (amoxicillin, metronidazole, and bismuth subsalicylate) significantly reduced the presence of precancerous gastric lesions in subjects with histological diagnosis of precancerous lesions (Mera *et al*., 2005). These studies demonstrate a clear relationship between *H. pylori* infection and an increased risk of developing severe gastric disease and gastric cancer. There are two distinct histological variants of gastric cancer that have been identified, the first is diffuse-type adenocarcinoma which consists of individually infiltrating neoplastic cells that do not form glandular structures and is more common in younger people, especially females (Chon *et al*., 2017). The second is intestinal-type adenocarcinoma which is more prevalent in older men (Y. Choi *et al*., 2022) and progresses through a series of gastric lesions as shown in the Correa´s cascade (Correa, 1992; Correa & Piazuelo, 2012; Parsonnet *et al*., 1991).

1.6 *H. pylori* virulence factors

1.6.1 Role of urease in colonisation

One of the most remarkable characteristics of *H. pylori* is its ability to persist for decades in the stomach. To overcome the barrier functions of the human stomach, the bacterium has developed adaptations, and one such adaptation is its capacity to produce functional urease. The urease-encoding region of the *H. pylori* genome consists of two gene clusters, the first is *ureAB* genes encoding two structural subunits, UreA (26.5 kDa) and UreB

(61.9 kDa). UreA subunit produced by *H. pylori* is unusual because it is encoded by a single gene *ureA*, whereas in other bacterial species, it is encoded by two separate genes (Jabri *et al.*, 1995; Lam *et al.*, 2010). UreA and UreB form an apoenzyme, which is inactive in the absence of nickel ions. Activation of the UreAB apoenzyme is dependent on nickel ions under a process involving five accessory proteins UreIEFGH, encoded by *ureI*, *ureE*, *ureF*, *ureG* and *ureH* located in the second gene cluster (Akada *et al.*, 2000; Cussac *et al.*, 1992). Accessory proteins UreEFGH share homology with other bacterial species, whereas UreI is unique to *H. pylori* (Cruz-Ramos *et al.*, 1997; Mulrooney & Hausinger, 1990; I. S. Park *et al.*, 1994). It has been proposed that accessory proteins interact with UreAB apoenzyme and deliver nickel ions to the active site located in UreB in an energy-dependent manner (Benini *et al.*, 1999; Nim & Wong, 2019). The catalytically active urease enzyme produced by *H. pylori* is responsible for catalysing the conversion of urea to ammonia and carbon dioxide (CO_2), thereby temporarily neutralising the acidic environment in the gastric region surrounding the bacterium (Weeks *et al.*, 2000). To establish a persistent infection in the gastric mucosa, *H. pylori* must penetrate deep in the gastric pits, where the pH is near neutral (as depicted in Figure 1.4). This process is dependent on motility, which is achieved through its flagella. *H. pylori* has a conserved signal transduction system, which consists of signal transduction proteins from the Che family. CheY is a particularly important protein, it is a chemotaxis response regulator which interacts with the flagellar motor, and controls flagella rotation. Notably, chemoreceptors participate in this signalling system and are critical for detecting chemoattractants or repellents. Previous research has identified various *H. pylori* chemoreceptors, including TlpA (responds to arginine and acid), TlpB (urea and acid), TlpC (lactate), and TlpD (ROS and acid) (Cerda *et al.*, 2011; Collins *et al.*, 2016; J. Y.

Huang *et al*., 2015; Machuca *et al*., 2017; Schweinitzer *et al*., 2008). In a mouse model of *H. pylori* infection, the genetic inactivation of *tlpA* or *tlpB* in *H. pylori* led to a marked decreased in inflammation, after 3 to 6 months of infection. Although, TlpC and TlpD are recognised as chemoreceptors, genetic inactivation of either *tlpC* or *tlpD* did not have an effect and was shown to behave like the wild-type strain of *H. pylori* (Williams *et al*., 2007). In a gerbil model of *H. pylori* infection, the absence of chemotaxis response regulator, CheY, caused complete failure of *H. pylori* colonisation in the stomachs (McGee *et al*., 2005). These findings suggests that TlpA and TlpB play critical roles in sensing specific signals in the stomach and regulating inflammation. CheY is essential for colonisation, by regulating the motility of *H. pylori* towards the gastric epithelium, where attachment and subsequent persistence contributes to tissue damage over time (Foynes *et al*., 2000; McGee *et al*., 2005; Terry *et al*., 2005; Williams *et al*., 2007).

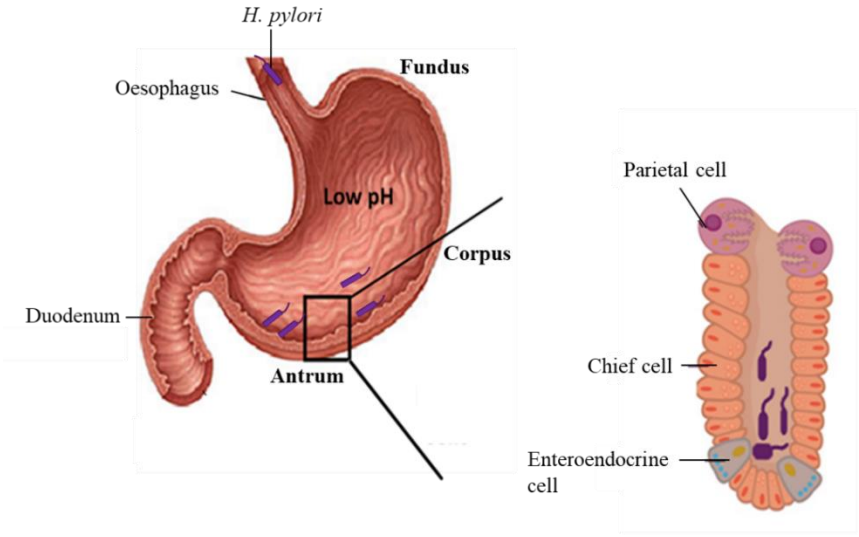

Figure 1.4. *H. pylori* colonisation of the stomach. (Left) Shows the different regions of the stomach, the fundus, corpus and antrum: *H. pylori* primarily colonises the antrum region. (Right) Shows a close-up schematic diagram of *H. pylori* colonisation within the mucus layer, deep in the gastric pits. Here, *H. pylori* can use sialic acid-binding adhesin and/or blood-antigen binding protein A to adhere to gastric epithelial cells. Parietal cells secrete gastric acid, and chief cells and enteroendocrine cells secrete digestive enzymes and hormones, respectively. Modified from (Keilberg & Ottemann, 2016).

1.6.2 Cytotoxin-associated gene A

The cytotoxin-associated gene A pathogenicity island (*cag*PAI) is a major virulent determinant of *H. pylori* virulence (Tomb *et al*., 1997), and is present in nearly 70% of all *H. pylori* strains isolated worldwide. Interestingly, 95% of East-Asian *H. pylori* strains possess a fully functional CagPAI, compared to 60% of Western isolates (Olbermann *et al*., 2010). Most importantly, CagA is an immunodominant antigen that is well-characterised. Numerous studies have correlated this antigen with disease risk and it has been shown to contribute to the severity of inflammation which strengthens its oncogenic potential (Censini *et al*., 1996; Montecucco & Rappuoli, 2001). CagA is encoded by

genes found on the 37 kb chromosomal pathogenicity island (CagPAI). There are approximately 32 genes located in the CagPAI that encodes for the CagA effector protein and structural components of the type IV secretion system (T4SS) (Asahi *et al.*, 2000; Odenbreit *et al.*, 2000; Tomb *et al.*, 1997). The T4SS forms a syringe-like structure to deliver the CagA protein into the hosts gastric epithelial cells where it is phosphorylated by Src family kinase. Phosphorylated CagA form complexes with cell signalling pathways such as Grb2 and SHP-2. These pathways induce morphological changes in host epithelial cells, leading to the hummingbird phenotype characterised by elongation and scattering of the cells (Higashi *et al.*, 2002; Olbermann *et al.*, 2010; Takahashi-Kanemitsu *et al.*, 2020). A study on transgenic mice found that over expression of phosphorylated-CagA resulted in gastric adenocarcinoma, myeloid leukaemia and B cell lymphoma, while the expression of phosphorylation-resistant CagA did not lead to such pathological abnormalities (Ohnishi *et al.*, 2008), further supporting that phosphorylation of CagA is a critical factor in the development of *H. pylori*-associated precancerous lesions and gastric adenocarcinoma. Furthermore, a study using a gerbil model of *H. pylori* infection demonstrated that a functional T4SS is necessary for the translocation of CagA into gastric epithelial cells. Inactivation of *cagE* which encodes for CagE, a component of the T4SS resulted in milder gastric disease without peptic ulcers compared to animals inoculated with the cagPAI-positive (Ogura *et al.*, 2000). Additionally, in a recent study conducted on 156 patients with *H. pylori* infection in Bulgaria, the presence of an intact *cag*PAI was detected in 88% (29) of *H. pylori* strains that were isolated from patients with peptic ulcer disease (Markovska *et al.*, 2018). These studies provide evidence that CagA-positive *H. pylori* strains, with a functional *cag*A PAI, are associated with severe pathological conditions, notably, multifocal atrophic gastritis, intestinal metaplasia,

peptic ulcers and gastric cancer (Fazeli *et al.*, 2017; Higashi *et al.*, 2002; Markovska *et al.*, 2018; Ogura *et al.*, 2000).

1.6.3 Vacuolating cytotoxin A

Vacuolating cytotoxin A (VacA) is another well-studied virulence factor that contributes to the pathogenicity of *H. pylori* and the development of gastric adenocarcinoma (Fischer *et al.*, 2001). VacA is a 140 kDa precursor autotransporter protein that is encoded by a chromosomal *vacA* gene. The protein is made up of three regions: a signal sequence, a passenger domain and an auto-transporter domain. In the bacterium, VacA under goes proteolytic processing which results in a type V autotransporter system (50 kDa), a 33 amino acid signal sequence, and a mature VacA (88 kDa) toxin (T. L. Cover *et al.*, 1994; Leyton *et al.*, 2012; Schmitt & Haas, 1994; Telford *et al.*, 1994). To deliver the mature VacA toxin to host cells, *H. pylori* inserts the type V autotransporter system into the outer membrane of the bacterium. This process forms a pore that allows the mature VacA to be translocated across the bacterium's outer membrane. Subsequent binding of the mature VacA toxin to host cells and internalisation, causes severe vacuolation (Palframan *et al.*, 2012; Papini *et al.*, 1994). VacA is a multifunctional virulence factor that exerts multiple roles on target cells including disruption of mitochondrial functions and induction of caspase-dependent apoptosis, thus increasing the risk of peptic ulcers and gastric cancer development (T. L. Cover & Blanke, 2005; Palframan *et al.*, 2012). Studies have shown that VacA is polyphormic and two types of allelic variants exist in the signal (s1/s2) and the middle (m1/m2) regions (Letley *et al.*, 2003). *H. pylori* strains with *s1/m1* VacA have been shown to display the highest level of virulence and greater vacuolation compared to the *s2/m2* allele combination which has been shown to form pores slowly and typically

shows no vacuolating activity. The risk of developing severe inflammation and hence gastric cancer is greater with the *s1/m1* allele combination (Atherton *et al*., 1995; McClain *et al*., 2001; Sugimoto, Zali, *et al*., 2009).

1.7 Control and prevention of *H. pylori*

1.7.1 Diagnosis

The diagnosis of *H. pylori* has traditionally relied on non-invasive diagnostic tests. These tests include serology, which measures the presence of antibodies to *H. pylori* in serum and the urea breath test (UBT). The use of UBT was first reported in 1954 and it continues to be widely available and used to this day. The UBT is a highly sensitive and specific diagnostic test that works by detecting isotopically labelled carbon dioxide (CO_2) that is exhaled in infected patients. Advantages of using UTB is that it is non-invasive, cost-effective and easy to use. Since the UBT only requires the patient to drink a small amount of ^{13}C-labelled urea, it is not associated with the discomfort and risks associated with invasive procedures, such as endoscopy. Moreover, UBT is highly accurate with reported sensitivity ranging from 91 to 100% (Mauro *et al*., 2006; Savarino *et al*., 1999). However, with younger children specificity is reduced since this test requires full cooperation from the patient (Hino *et al*., 2004; Imrie *et al*., 2001). Another disadvantage of UBT is that false positives are common, for instance, if the contact time between *H. pylori* and urea in the gastric mucosa is short, the hydrolysis of urea does not occur and hence the amount of (CO_2) is undetectable. Therefore it is recommended that a meal is consumed at the same time as the labelled urea to reduce gastric emptying (Savarino *et al*., 1999). Another non-invasive diagnostic method that is commonly used to diagnose *H. pylori* infection is

the stool antigen test (SAT), which detects the presence of the bacterium based on antigens present in the faeces. SAT is typically performed using an enzyme-linked immunosorbent assay (ELISA) based method (Qiu *et al.*, 2021) or rapid commercial kits such as the *H. pylori* quik chek™ and the *H. pylori* chek™ assays (Halland *et al*., 2021). The rapid commercial kits use an immunochromatographic technology, which detects *H. pylori* antigens in faeces rapidly, with high sensitivity and ease of use compared to an ELISA-based method which takes approximately 2 hours (Fang *et al.*, 2020; Halland *et al*., 2021). SAT can be used with ease in small labs or labs with limited resources and it can be used in younger children (Antos *et al*., 2005; Kuloğlu *et al*., 2008).

Serology testing is based on the detection of *H. pylori* antigen-specific antibodies in sera, using an ELISA-based method or Latex agglutination techniques (a rapid-kit based method) (Chey *et al*., 2007; Suzuki *et al.*, 2003). However, this method has some limitations. Firstly, serological tests cannot be used to monitor the effectiveness of *H. pylori* therapy, because patients carry *H. pylori* antigen-specific antibodies in their serum for several months, even after eradication of the infection. Secondly, the American College of Gastroenterology does not recommend the use of serological tests in populations with low-prevalence because false-positive results occur in a majority of serological assays evaluated in these populations (Saleem & Howden, 2020). The prevalence of *H. pylori* impacts the positive predictive value (PPV), for instance, a low prevalence of *H. pylori* infection decreases the PPV, as a result, a more reliable test such as histological examination and culture of biopsy samples or the UBT should be used to further confirm positive serological test results (Loy *et al*., 1996; Tenny & Hoffman, 2022).

Invasive procedures that are currently used to diagnose *H. pylori* infection are endoscopy and biopsy methods. Endoscopy is expensive, invasive, and there have been reported complication in some patients (Levy & Gralnek, 2016). Additionally, it is not recommended for children, pregnant women, and patients with pre-existing risks (such as anticoagulated patients). It is not recommended for use when monitoring the effectiveness of *H. pylori* therapy due to high cost, invasiveness, and reduced patient safety (Vaira *et al*., 2000). Therefore, non-invasive methods are preferred methods for the diagnosis of *H. pylori* infection and can also be used to measure the effectiveness of antibiotic-based therapy. Although several non-invasive methods have been established, the most preferred methods are UBT which is considered a gold standard for the diagnosis of *H. pylori*. However, no one test is better than the other because many factors have to be considered including clinical status, cost-based issues, geographic location and age (Kayali *et al*., 2018; Sabbagh *et al*., 2019).

1.7.2 Treatment

In the last two decades, the first line of treatment for *H. pylori* eradication is based on a standard triple therapy which consists of a combination of two antibiotics (clarithromycin and amoxicillin or metronidazole) and a proton pump inhibitor (lansoprazole, omeprazole or pantoprazole) (Chu *et al*., 2000; Misiewicz *et al*., 1997; Papastergiou *et al*., 2014). Triple therapy regimens had a high acceptance rate since this treatment showed a high eradication rate (above 90%) and had a very good safety profile (Malfertheiner *et al*., 2007). Since, then the efficacy of triple therapy regimens has decreased below 70%, due to the emergence of multidrug-resistant strains of *H. pylori* to key antibiotics, mainly clarithromycin and also metronidazole and levofloxacin (Chang *et al*., 2017; John Albert

et al., 2006; Liang *et al.*, 2014). Factors that are contributing to the emergence of resistant strains include poor adherence, with patients discontinuing a course of prescribed antibiotics due to side effects, and overuse of antibiotics in both medical healthcare settings and agriculture (Boyanova *et al*., 2019). Moreover, clarithromycin targets the peptidyltransferase region of 23S rRNA and inhibits protein synthesis. It has been shown that resistance in *H. pylori* is due to point mutations in the peptidyltransferase region of domain V of the 23S rRNA (De Francesco *et al*., 2009; Taylor *et al*., 1997). Clarithromycin-resistant *H. pylori* is currently recognised by WHO as a high priority for the development of new antibiotics or alternative treatment regimens (Alba *et al*., 2017; Tacconelli *et al.*, 2018). A meta-analysis comparing the prevalence of antibiotic-resistant strains of *H. pylori* in ASEAN countries showed that clarithromycin-resistant strains significantly reduced the eradication rate of triple therapy regimens containing clarithromycin, and they also highlighted that metronidazole-resistant strains of *H. pylori* were very common in these countries. In the same study, the prevalence of clarithromycin-resistant *H. pylori* was variable among different ASEAN countries, with Cambodia and Vietnam, showing the highest prevalence (43% and 30%, respectively), Singapore (17%) and the lowest prevalence was seen in Malaysia (6.8%), Philippine (2%) and Myanmar (0%) (Vilaichone *et al*., 2018). An antimicrobial resistance (AMR) surveillance study assessing 16 hospitals in Vietnam showed that the proportion of AMR was high among different pathogens isolated from clinical specimens and resistance had increased by 40% between 2009 and 2013 (McKinn *et al*., 2021). The same study reported that antibiotics are commonly used as a trusted remedy in Vietnam and together with a limited understanding of antibiotic resistance has resulted in the overuse of antibiotics in this country (McKinn *et al*., 2021). Additionally, some areas in Spain have reported that

the prevalence of clarithromycin-resistant *H. pylori* strain was 49%, compared to the Netherlands (only 1%) (Papastergiou *et al*., 2014). The observed variations in antibiotic resistance levels among different countries are suspected to be closely connected to distinct antibiotic usage policies implemented in each country. This highlights the importance of educating the public and healthcare professionals about proper antibiotic usage while also emphasising the need for new antibiotics or alternative treatment for infections caused by multi-drug resistant strains of *H. pylori*. Countries with a high prevalence of clarithromycin-resistant *H. pylori* should implement protocols for regular susceptibility testing of *H. pylori* to ensure that clinicians choose the most effective treatment regimens for patients with *H. pylori* antibiotic-resistant strains. Metronidazole is another key antibiotic that is widely used in triple therapy regimens and is also associated with a high level of resistance (Malfertheiner *et al.*, 2007). The prevalence of metronidazole-resistant strains of *H. pylori* has been estimated to be 48.9% in Asia (Shrestha *et al*., 2023), 90% in Africa (Falsafi *et al*., 2004), 17 to 44% in Europe (De Francesco *et al*., 2010) and America (Ogata *et al*., 2013). The prevalence is very high in Africa due to the use of metronidazole to treat parasitic and/or gynaecological infections (John Albert *et al*., 2006).

Alternative treatment regimens have been implemented to combat the increasing prevalence of *H. pylori* resistance to traditional antibiotics. One such regimen is bismuth quadruple therapy which consists of a combination of two antibiotics (typically tetracycline and metronidazole), bismuth and a proton pump inhibitor. Bismuth salts act as an adjuvant that enhances the performance of antibiotics (Gatta *et al*., 2009; McMahon *et al*., 2016). This treatment is particularly useful in areas where clarithromycin resistance

is high, and if metronidazole resistance is also high, amoxicillin or clarithromycin can be used instead (McMahon *et al*., 2016; Tacconelli *et al*., 2018). A multicenter study in Spain showed that bismuth-based therapy consisting of metronidazole and tetracycline resulted in an eradication rate of 65% in patients who have experienced two previous eradication failures with traditional triple-based therapies consisting of clarithromycin and levofloxacin. However, adverse events were reported in 22% of patients, nausea (12%), abdominal pain (11%) and metallic taste (8.5%) (Gisbert *et al*., 2014). Despite the development of alternative regimens, *H. pylori* treatment remains challenging because alternative regimens are still at risk of antibiotic resistance.

1.7.3 Probiotics

Probiotics are defined as living microorganisms that, when ingested in adequate amounts can confer positive health benefits to the host. Probiotics are used extensively in humans and can consist of yeast or bacteria. Currently, most probiotics are consumed in the form of tablets or capsules or as fermented dairy products. Among the different types of probiotics, lactic bacteria (*Lactobacillus*) and *Bifidobacterium* are the most commonly used (Fontana *et al*., 2013; Rodes *et al*., 2013). The latter has been extensively studied for their beneficial effects including, *a*) promotion of gut maturation and integrity, *b*) their ability to suppress or interfere with the natural growth of pathogens and (*c*) regulating the hosts innate and adaptive immune responses (Karimi *et al*., 2018; Sankarapandian *et al*., 2022; F. Yan & Polk, 2011). A study by Sugimura *et al*, reported that *L. gallinarum* culture supernatant significantly reduced intestinal tumour number and size by promoting apoptosis. Moreover, *L. gallinarum* secreted indole-3-lactic acid (ILA), which was shown to inhibit intestinal tumorigenesis *in vivo* (Sugimura *et al*., 2022). There has been a

growing interest in the use of probiotics in combination with antibiotics regimens since they possess properties that can enhance the effects of antibiotics and potentially improve *H. pylori* eradication, as well as reducing the related side effects. A multicenter study comparing a triple therapy regimen and triple therapy supplemented with fermented milk (Actimel) containing *L. casei* DN-114 001 showed that the use of probiotic supplementation improved the eradication rate of *H. pylori* in children (84.6%) compared to triple therapy alone (57.5%). Probiotic supplementation simultaneously alleviated the severity of side effects (Sýkora *et al*., 2005). Moreover, the use of probiotics can achieve a better therapeutic outcome, could be beneficial to patients with *H. pylori*-related precancerous lesions, and could potentially help to reduce tumour formation and metastasis by regulating gut microbiome (Goderska *et al*., 2018).

1.8 Coronavirus pandemic (COVID-19)

Coronaviruses (CoVs) are positive-sense, single-stranded ribonucleic acid (+ssRNA) viruses that belong to the family of *Coronaviridae* and the order *Nidovirales* (Paules *et al*., 2020). Under electron microscopy, these viruses show a characteristic appearance that resembles a crown, due to the presence of spike glycoprotein on the surface of the envelope (Ksiazek *et al*., 2003). The subfamily *Orthocoronavirinae* consists of four genera including, *Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus*. *Alphacoronaviruses* and *Betacoronaviruses* primarily infect mammalian species and are known to cause respiratory and gastrointestinal diseases in both humans and animals. On the other hand, *Gammacoronaviruses and Deltacoronaviruses* have a wide host range and can also infect avian species (Corman *et al*., 2018; Gorbalenya *et al*., 2020). Humans can be infected with CoVs such as HCoV-229E and HCoV-OC43, which have been circulating worldwide since the mid-1960s, and also the more recently identified CoVs such as HCoV-NL63 and HCoV-HKU1. These CoVs typically cause seasonal and self-limiting respiratory tract infections which often mimic the symptoms of the common cold (D. X. Liu *et al*., 2021). However, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) which emerged in the human population over the past 20 years, and the recently emerged SARS-CoV-2 are considered to be more severe and virulent compared to the previously mentioned CoVs (J. F. W. Chan *et al*., 2015; Cheng *et al*., 2007). SARS-CoV, MERS-CoV and SARS-CoV-2 can infect human bronchial epithelial cells, pneumocytes, and cells in the upper respiratory tract and can progress into severe life-threatening respiratory pathologies. Additionally, several extra-respiratory

manifestations with variable clinical severity can also occur including diarrhoea, lymphopenia, deranged liver and renal function tests, and multiorgan dysfunction syndrome. Epidemics caused by SARS-CoV and MERS-CoV in both immunocompetent and immunocompromised hosts have resulted in mortality rates of ~10% and ~35%, respectively (Gerges Harb *et al.*, 2020; Peiris *et al.*, 2003; Yeung *et al.*, 2016; H. Zhou *et al.*, 2021).

The first case of SARS-CoV-2 was reported in Wuhan city of China in December 2019 and was isolated from patients with atypical pneumonia (ECDC, 2020). Genomic characterisation revealed that SARS-CoV-2 has a high nucleotide similarity to a bat SARS-like-CoVZXC21 (89%) and that of human SARS-CoV (J. F.-W. Chan *et al*., 2020; Lu *et al*., 2020; Y. Wan *et al*., 2020; F. Wu *et al*., 2020). The earliest date of symptoms of onset was in December 2019, and the symptoms included fever, malaise, dry cough, and dyspnea. A total of 41 patients were admitted to a hospital in Wuhan with reports of pneumonia and chest computerised tomography scans showed abnormal findings. Additionally, 32% of those admitted required intensive care and 15% died (C. Huang *et al.*, 2020). According to the World Health Organisation (WHO), COVID-19 was declared a global pandemic. As of 29 March 2023, there has been 761,402,282 confirmed cases of COVID-19 and 6,887,000 deaths worldwide. The primary source for the global and regional spread of SARS-CoV-2 was closely associated with travel, and WHO reported that this virus spread to more than 200 countries (Q. Li *et al.*, 2020). The U.S. reported the highest number of COVID-19 cases as well as, the highest number of deaths as a result of SARS-CoV-2 infection, followed by India and Brazil. In the U.S., COVID-19 was the third leading cause of death in 2021, after heart disease and cancer,

reporting over 460,000 cases (Ahmad, 2022). The UK also reported that COVID-19 was the third leading cause of death in 2021, after cancer and heart disease, reporting over 67,350 cases (Raleigh, 2022). The case fatality rate (CFR, the ratio between the number of deaths and the number of confirmed cases) varies between countries and the contributing factors include age, underlying pre-existing conditions, the severity of illness, and economic and political variables. Countries that reported the highest values of disability-adjusted life years (DALYs; a measure of overall disease burden) as a result of cardiovascular, cancer and chronic respiratory diseases had the highest values of COVID-19 CFR (Sorci *et al.*, 2020). Although there is limited information regarding the CFR of COVID-19, some studies reported that CFR values varied from 0·048% in Singapore to 10·16% in Mexico as of October 2020 (Dong *et al*., 2020). Other studies also demonstrated that CFR values of COVID-19 can vary in an individual country at different stages of the disease outbreak, for example, studies in France reported a CFR value of 1.4% as of March 2020, which increased to 19.6% in May 2020, and drastically dropped to 2.3% in November 2020 (Sorci *et al*., 2020). Various factors can contribute to the regional CFR differences including (but not limited to), patient access to health care, COVID-19 testing capacity, age, race, vaccination status, compliance with government guidance and the emergence of SARS-COV-2 variants (Nishiura *et al.*, 2020; Oran & Topol, 2021; H. Wang *et al*., 2020; Yoshikura, 2012).

RNA viruses, including SARS-CoV-2, are prone to genetic evolution and rapidly mutate compared to DNA viruses. The emergence of new variants continues to raise widespread concern and represents a major burden to public health and the economy, especially when the mutation causes substantial changes in antigenicity, transmissibility, and virulence.

Moreover, multiple variants have been identified, of which a few are being classified as variants of concern (VOCs) because they have the potential to enhance transmissibility, reduce neutralisation antibodies that have been acquired through natural infection or vaccination and have the ability to reduce vaccination efficacy. SARS-CoV-2 variants including Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and Delta (B.1.617.2) were previously designated as VOCs, and each variant was shown to have transmission advantages over preceding variants. The recently emerged and currently circulating variant of concern (VOCs) Omicron (B.1.1.529) including sublineages such as BA.1, BA.2 and BA.5 are highly infectious and have spread globally (Chavda *et al.*, 2022; A. Choi *et al.*, 2021; Grubaugh *et al.*, 2021; Silva *et al.*, 2021; Tegally *et al.*, 2021; X. Xie *et al.*, 2021). The Omicron variant was first identified in South Africa, in November 2021, and was declared as VOCs because it acquired more than 30 amino acid changes in the spike protein (Callaway, 2021; H. Gu *et al.*, 2022; Vaughan, 2021). Artificial intelligence (AI) modelling suggests that Omicron shows a 13-fold increase in viral infectivity and is approximately twice as infectious as the Delta variant (J. Chen *et al.*, 2021).

1.9 SARS-CoV-2 infection

1.9.1 Transmission of SARS-CoV-2

The origin of SARS-CoV-2 is currently unknown, however, genomic comparison studies suggest that this virus might have evolved from a bat coronavirus (named, Bat-CoV-RaTG13), which was detected in *Rhinolophus affinis* from Yunnan province, China, implicating zoonotic transmission. The whole genome sequence of RaTG13 is 96.2% identical to SARS-CoV-2, which supports the hypothesis that SARS-CoV-2 likely

originated from bats (P. Zhou *et al*., 2020). In a recent study, molecular and phylogenetic analyses revealed that pangolin coronavirus (pangolin-CoV-2020) isolated from two sick Malayan pangolins is genetically related to SARS-CoV-2 and Bat-CoV-RaTG13 and has a sequence similarity of up to 90.32% and 90.24%, respectively. Despite this discovery, it is still unknown whether the emergence of SARS-CoV-2 in humans was directly associated with bats or through an intermediate host (pangolins and/or minks) (P. Liu *et al*., 2020; Xiao *et al*., 2020).

SARS-CoV-2 is transmitted primarily through exposure to respiratory droplets from close contact or droplet transmission with asymptomatic and symptomatic individuals harbouring the virus (Q. Li *et al*., 2020). Recently, fomite transmission was identified as another potential source of SARS-CoV-2 transmission by the CDC and stated that although the risk of transmission is low, individuals can still be infected through contact with inanimate surfaces (Kampf *et al*., 2020; van Doremalen *et al*., 2020). Many studies reported that SARS-CoV-2 remains viable and stable on inanimate surfaces including stainless steel, and plastic for up to 3 days and cardboard, for up to 1 day. Interestingly, viable SARS-CoV-2 was isolated from nonporous glass and stainless steel for up to 28 days, at 20°C (Z.-D. Guo *et al*., 2020; Riddell *et al*., 2020; van Doremalen *et al*., 2020).

1.9.2 Pathogenesis of SARS-CoV-2

SARS-CoV-2 is structurally and phylogenetically similar to SARS-CoV-1 and MERS-CoV and is composed of four main structural proteins including, spike (S) glycoprotein, envelope, nucleocapsid and the membrane protein, as well as 16 non-structural proteins and 5 to 8 accessory proteins (Naqvi *et al*., 2020). Among them, S glycoprotein is located

on the surface of SARS-CoV-2 and plays an important role in initiating viral attachment, fusion and entry. The S protein consists of two subunits, the S1 and S2 subunit. The S1 subunit is located in the N-terminal and it consists of the receptor binding domain (RBD) which initiates viral-mediated binding to human angiotensin-converting enzyme-2 (ACE2) receptors on the host cell. The S2 subunit is located in the C-terminal region and triggers virus-cell membrane fusion (L. Du *et al*., 2009; Y. Huang *et al*., 2020; Xia *et al*., 2020). The RBD recognises the ACE2 receptors, which are predominantly expressed by type II alveolar epithelial cells. Immunostaining of the lung showed that 83% of cells expressing ACE2 are type II alveolar epithelial cells. Other cells have been identified that express ACE2, including type I alveolar epithelial cells, airway epithelial cells, fibroblasts and macrophages (Zhao *et al*., 2020). Many extrapulmonary tissues including, the cornea, oesophagus, heart, kidney, endothelium and intestine also express ACE2 receptors. This distribution of ACE2 receptors is thought to play a role in the multi-organ involvement seen in severe COVID-19 cases. (Crackower *et al*., 2002; Danilczyk *et al*., 2006; Ding *et al*., 2004; J. Gu *et al*., 2005; Sungnak *et al*., 2020). Once SARS-CoV-2 binds to ACE2 receptors on the host cell, the furin cleavage motif activates the transmembrane protease serine protease 2 (TMPRSS2) in the host cell, which subsequently cleaves the S protein at the S2 subunit cleaving site. Cleavage exposes the S2 subunit, which is an important step in enabling the fusion of the viral and host membrane. This fusion allows the viral ribonucleoprotein complex to be released into the host cell cytoplasm, thereby facilitating viral entry and infection (for the life cycle see Figure 1.5**)** (Beumer *et al*., 2021; Hoffmann *et al*., 2020; W. Li *et al*., 2003). Another potential route of viral entry involves the endosome pathway. In this pathway, the cathepsin L enzyme cleaves the spike protein,

however, this is not the main route that is used by the primary epithelial cells (Beumer *et al*., 2021; Lamers *et al*., 2021; Mykytyn *et al*., 2021).

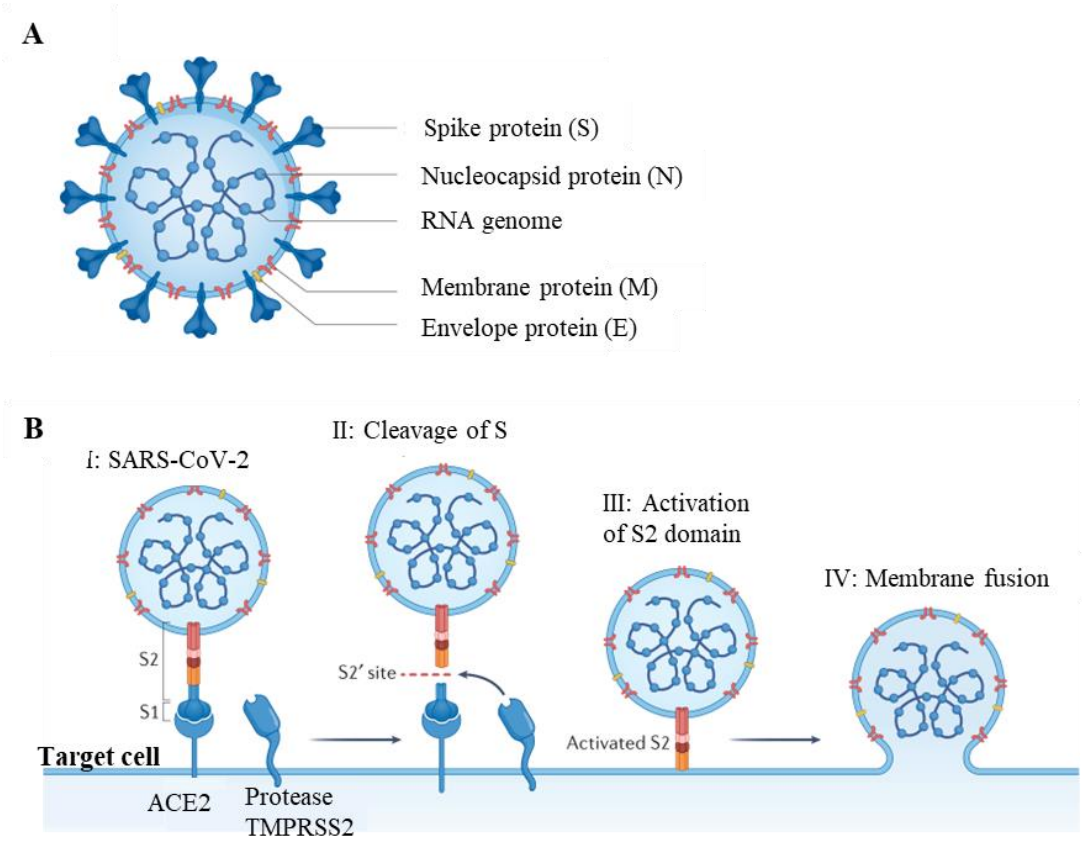

Figure 1.5. SARS-CoV-2 virion and life cycle. A) SARS-CoV-2 virion consists of structural proteins such as spike (S), nucleocapsid (N), membrane (M) and envelope (E). **B**) The S protein, precisely, the RBD which is located in the S1 domain attaches to the ACE2 receptor on the host target cell (Stage I). Followed by the cleavage of the S protein which is mediated by TMPRSS2 protease (Stage II), which results in the activation of the S2 domain (Stage III). The activated S2 domain drives the fusion of the viral and host lipid bilayers, followed by the release of the positive-sense, single-stranded ribonucleic acid into the hosts cell (Stage IV) for further processing and eventually, the synthesis of more virions using the host replication machinery. Modified from (Lamers & Haagmans, 2022).

It has been reported that during natural infection the first cells SARS-CoV-2 targets are likely to be multiciliated cells in the nasopharynx or trachea, or sustentacular cells in the nasal olfactory epithelium (Ahn *et al*., 2021; Hou *et al*., 2020; Khan *et al*., 2021). Upon entry into a host cell, the +ssRNA plays an important role in initiating the production of replicase proteins which trigger the formation of replication factories. These factories are double-membrane vesicles (or organelles) that are synthesised by virus-induced remodelling of the host endoplasmic reticulum membrane. All the necessary components required for the replication, assembly and maturation of infectious virions are present in these replication factories (Knoops *et al*., 2008; Ogando *et al*., 2020). RNA-dependent RNA polymerase facilitates the copying of the +ssRNA which results in the formation of double-stranded RNA (dsRNA) that is used to generate more +ssRNA, which can be translated to produce more viral proteins that are essential for the assembly and maturation of infectious virions. Replication factories are important for shielding the dsRNA from detection by the hosts innate immune responses. For example, when the dsRNA is exposed in the cytoplasm, cytoplasmic pattern recognition receptors (PRRs) such as NOD-like receptors (NLRs) recognise the dsRNA (C.-P. Chan & Jin, 2022; Kasuga *et al*., 2021; Manik & Singh, 2022; Robson *et al*., 2020; Romano *et al*., 2020). A recent study identified and showed that MDA5 is an additional cytoplasmic PRR capable of sensing dsRNA and other viral signals and in response regulates IFN induction in response to SARS-CoV-2 infection (Yin *et al*., 2021). Moreover, membrane-bound PRRs such as endosomal toll-like receptors (TLRs) can also detect dsRNA. In all cases, the detection of dsRNA by NLRs, TLRs and MDA5 initiates a signalling cascade which induces the transcription of type I and III interferons, which are important cytokines produced as the first line of antiviral defence. IFNs initiate the expression of IFN-

stimulated genes (ISG) that have various antiviral functions, among them, dsRNA-dependent protein kinase R (PKR) is an essential component of the innate immune system and can impair the translation of viral RNA through phosphorylation of elF2α, a eukaryotic initiation translation factor (Kayesh *et al*., 2021; Y. Liu *et al*., 2020; Thompson *et al*., 2011; Yin *et al*., 2021). Phosphorylated elF2α cannot form tRNAMet-GTP–eIF2 complex, subsequently inhibiting the synthesis of cellular and viral proteins (Y. Liu *et al*., 2020). Another ISG that has been characterised is the ISG Ly6E, which has been shown to inhibit viral-host membrane fusion, thus inhibiting viral entry. Moreover, it has been reported that a knockout mice model lacking the lymphocyte antigen 6 complex, locus E (Ly6e), were highly susceptible to murine-CoV (murine hepatitis virus) infection and displayed a higher splenic viral burden and loss of hepatic immune cells (Pfaender *et al*., 2020). IFNs are essential for initiating the recruitment of various innate immune cells including natural killer cells, dendritic cells and macrophages. Furthermore, IFNs are capable of activating the adaptive immune response (T and B cells). The activation of both the innate and adaptive immune responses contribute to viral clearance through the release of cytokines such as tumour necrosis factor-α (TNF α), granulocyte-macrophage colony-stimulating factor, IL-1, IL-6, IL-1β, IL-8, IL-12 and interferon (IFN)-γ (Y. Liu *et al*., 2020; Min *et al*., 2021). Intriguingly, a study identified circulating antibodies that target type I IFN in both blood and nasopharyngeal mucosa of patients with SARS-CoV-2 infection. This study reported that these patients have reduced IFN-mediated responses and are at a higher risk of developing severe COVID-19 infection and death (Lopez *et al*., 2021).

Failure to clear the virus in the upper respiratory tract either through the innate or adaptive immune responses can increase the risk of the virus spreading to the lower respiratory tract, which can lead to infection of the alveoli subsequently, causing inflammation and limited gas exchange (Sette & Crotty, 2021). SARS-CoV-2 has evolved various immune evasion strategies. First, is the formation of replication factories, using absolute quantification of viral RNA and replication factories it has been shown that SARS-CoV-2 genomic RNA persists in these factories after many weeks and is not degraded by nucleases (J. Y. Lee *et al*., 2022). This study highlights the importance of replication factories in immune evasion and it has been reported that these factories protect SARS-CoV-2 from innate immune sensors that recognise viral RNA (Kikkert, 2020; J. Y. Lee *et al*., 2022). Thorne *et al*, reported that the alpha variant showed increased levels of subgenomic RNA and certain proteins that are associated with the down regulating the innate immunity. This study specifically observed an increase in nucleocapsid, Orf9b and Orf6 proteins, which have all been shown to have an inhibitory effect on the innate immune response (Thorne *et al*., 2022). This study also reported that Orf9b protein alone could inhibit the antiviral innate immune functions of the mitochondria by interacting with the mitochondrial surface adaptor protein, TOM70, which is required for the activation of the mitochondrial antiviral-signaling protein (MAVS) (Kreimendahl & Rassow, 2020; Thorne *et al*., 2022). The MAVS is an important component of the innate immune response as it triggers the activation of NFκB and Interferon Regulatory Factor and induction of interferons in response to viral infection (Sun *et al*., 2006). Additionally, Orf9b and N protein can interact with MDA5 pathway, thus inhibiting the expression of INF-β and pro-inflammatory cytokines (Han *et al*., 2021; J.-Y. Li *et al*., 2020; Sui *et al*., 2021). Clinical studies have reported that

patients with mild and moderate COVID-19 have reduced levels of type I and III INF which results in reduced expression of IFN-stimulated genes (ISG), thereby, reducing viral clearance up to 37 days. Prolonged viral clearance in patients with COVID-19 increases the risk of severe disease and death. (Han *et al*., 2021; Sui *et al*., 2021; Xue *et al*., 2020; F. Zhou *et al*., 2020).

1.9.3 COVID-19 clinical findings

The median incubation period of SARS-COV-2 is 4 to 5 days before symptoms develop (Lauer *et al*., 2020; Q. Li *et al*., 2020). In some cases the infection is asymptomatic and a meta-analysis of 95 studies reported that the global prevalence of asymptomatic COVID-19 ranges between 20 to 40% (Diamond & Kanneganti, 2022; Q. Ma *et al*., 2021), and in other cases, infected individuals develop mild to moderate respiratory disease including, fever, cough, headache, myalgia and diarrhoea (Figure 1.6) (N. Chen *et al*., 2020; Guan *et al*., 2020; C. Huang *et al*., 2020). The global prevalence of patients with mild to moderate symptoms largely varies between countries, for instance, large cohort studies in Europe showed that 45% and 63.2 % of patients had fever and cough (Lechien *et al*., 2020), respectively, compared to a large cohort study conducted in China which showed that over 80% and up to 65.5% of patients showed signs of fever and cough, respectively (Feng *et al*., 2020). Prolonged viral clearance can result in severe disease, which typically begins 1 week after symptom onset. The most common symptoms of severe COVID-19 disease include shortness of breath as a result of hypoxaemia, which can progress into acute respiratory distress syndrome (ARDS). ARDS is defined as a form of severe respiratory failure, which results in lung injury due to severe inflammation, pulmonary vascular leakage and atelectasis (loss of aerated lung tissues). Patients with COVID-19

that have hypoxaemia and ARDS have been shown to have hyperinflammation evidenced by a rise in the release of proinflammatory cytokines including, IL-1, IL-6, IL-8 and TNF-α. It has also been reported that the levels of IL-6, IL-8 and TNF-α cytokines present in serum at the time of hospitalisation is a strong and independent predictor of potential disease severity and patient survival (Del Valle *et al*., 2020). According to a survey-based study conducted at the beginning of the COVID-19 pandemic until July 2020, it was reported that approximately 33% of hospitalised COVID-19 patients developed ARDS. Among those COVID-19 patients who were transferred to an intensive care unit, almost 63% received invasive mechanical ventilation. Moreover, the study stated that the estimated mortality rate in COVID-19-associated ARDS was approximately is 45% (Tzotzos *et al*., 2020).

Although, COVID-19 is primarily considered a respiratory disease, it can also result in extrapulmonary disease and can affect various organs. The most common extrapulmonary manifestations include, kidney and liver injury, acute myocardial injury, gastrointestinal symptoms and coagulopathy (Figure 1.6) (Berlin *et al*., 2020; Gupta *et al*., 2020). The severity of COVID-19 disease is influenced by various risk factors including, viral load, age, presence of comorbidities, and use of immunosuppressants. Moreover, genetic factors such as polymorphisms of the ACE2 gene may also play a role in the severity of COVID-19 (Aziz & Islam, 2022; Möhlendick *et al*., 2021). For instance, ACE2 rs2285666, the GG genotype was associated with a two-fold increase in SARS-CoV-2 infection risk and a three-fold increase in the risk of developing severe COVID-19 or fatality (Möhlendick *et al*., 2021).

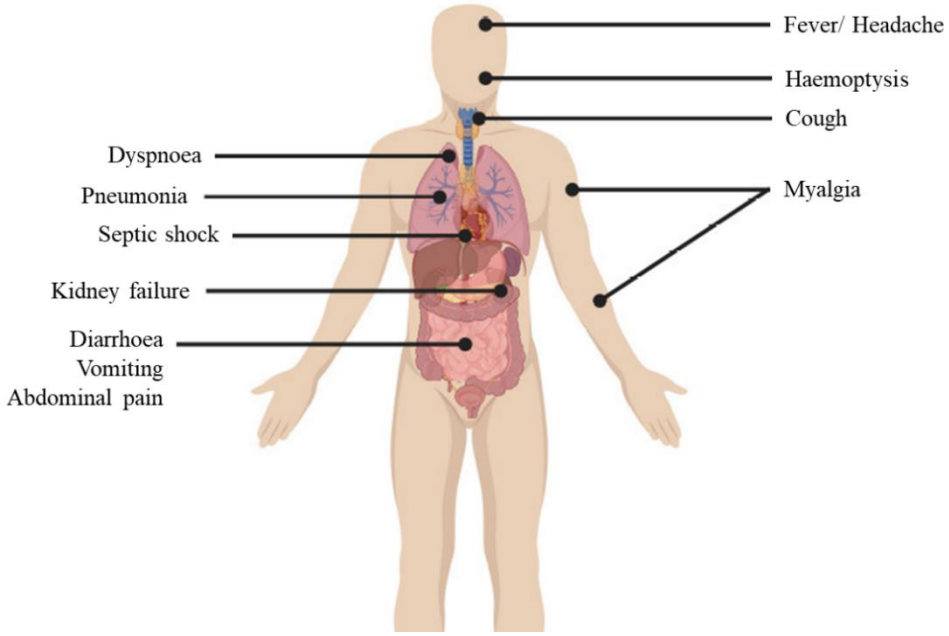

Figure 1.6. Clinical presentation of COVID-19. Modified from (Cascella *et al.*, 2023).

1.10 Treatment and management of COVID-19

1.10.1 Diagnosis

The primary method for diagnosing SARS-CoV-2 infection is the use of nasopharyngeal swabs to detect SARS-COV-2 nucleic acid using a real-time PCR assay. In addition, the Food and Drug Administration (FDA) approved commercial PCR assays (rapid antigen tests) with emergency use authorizations (EUAs) for the detection of SARS-CoV-2 antigens from samples obtained through nasopharyngeal and oropharyngeal swabs. Bronchoalveolar lavage samples can be used for testing, however, due to the invasive nature of sampling (a bronchoscopic procedure) it can only be performed in patients that are receiving invasive mechanical ventilation. The sensitivity of PCR testing can be affected by various factors including, time from exposure, specimen source, and technical

specimen collection. Moreover, these factors can also contribute to false-negative PCR results (Sethuraman *et al*., 2020; W. Wang *et al.*, 2020; Wiersinga *et al*., 2020; Y. Yang *et al*., 2020). A modelling study demonstrated that the sensitivity of RT-PCR testing is approximately 33% four days after exposure, but increased to 80% three days after symptoms onset (Kucirka *et al*., 2020). This highlights the importance of time of sample collection to achieve accurate results, particularly early in the course of infection. Another study found that RT-PCR positivity was highest in bronchoalveolar lavage specimens (93%), followed by sputum (72%), nasal swab (63%), and pharyngeal swab (32%) (W. Wang *et al*., 2020). False-negative results mainly occurred when the method of specimen collection was through nasopharyngeal swabs and the reason was due to inappropriate timing of specimen collection and/or poor specimen quality. Studies have shown that samples collected from the lower respiratory tract (bronchoalveolar lavage fluid), are more sensitive than samples collected from the upper respiratory tract. However, collecting samples from the lower respiratory is invasive and technically challenging and can increase the risk of exposure especially when collecting from patients that have not been intubated (Murphy, 2020; Y. Yang *et al*., 2020).

Several serological tests have been developed to evaluate the presence of SARS-CoV-2 specific antibodies (IgM, IgG or IgA) in serum, plasma or whole blood and they can also be used to measure immunity conferred from natural infection or COVID-19 vaccines. These tests include point-of-care assays (PoCT) and high-throughput enzyme immunoassays (Bond *et al*., 2020). However, serological tests may not be effective during the early stage of infection (incubation period) when antibodies are either undetectable or present at low levels. A study showed that SARS-CoV-2 antigen-specific IgM antibodies

are detectable five days after symptoms onset, with their levels significantly increasing between two to three weeks after infection. On the other hand, antigen-specific IgG antibodies are detectable approximately two weeks after symptoms onset (L. Guo *et al*., 2020). Another study showed that serological tests, specifically PoCT and a commercial ELISA kits, were more effective in detecting SARS-CoV-2 specific antibodies when samples were collected two weeks after the onset of symptoms, achieving sensitivities over 90% (Bond *et al*., 2020). These findings suggests that serological assays have limited diagnostic use during the acute stage of COVID-19 and RT-PCR is much more preferred and a gold standard for diagnosing acute COVID-19 (Bond *et al*., 2020; Lou *et al*., 2020; Sethuraman *et al*., 2020).

1.10.2 Oxygenation and ventilation management in COVID-19

Among patients that are hospitalised with COVID-19 and showing signs of acute lung injury, over 75% require supplemental oxygen therapy which is delivered via the nasal canula or Venturi mask to maintain oxygen saturation between 92 to 96% (Alhazzani *et al*., 2020). Those that are unresponsive to conventional oxygen therapy and/or worsening clinical symptoms can receive heated high-flow nasal canula oxygen. Patients that require invasive mechanical ventilation can receive lung protective ventilation with a low tidal volume (4-8 mL/kg, predicted body weight) and a plateau pressure of less than 30 mg Hg, which is recommended by the National Institutes of Health (Alhazzani *et al.*, 2020). Lung protective ventilation administered in patients with respiratory failure, with no evidence of ARDS was also beneficial and resulted in better clinical outcomes and short hospitalisation (Serpa Neto *et al*., 2012). In addition, prone position ventilation is a resource-intensive programme used to enhance oxygenation in patients with ARDS, and

when conventional oxygen supplementation methods and/or high-flow nasal cannula fails. However, invasive ventilation (i.e endotracheal intubation) are recommended for patients with severe ARDS and hypoxaemia. Invasive ventilation has been shown to reduce mortality rates, but they also carry risks such as pressure sores, endotracheal tube obstruction and ulcers (Alhazzani *et al*., 2020; Binda *et al*., 2021; Wiersinga *et al.*, 2020; X. Yang *et al*., 2020).

1.10.3 Management of COVID-19: pharmacologic therapies

The COVID-19 pandemic triggered a global demand for prophylactic vaccines and became a high priority among governments, as well as the pharmaceutical industry and academia. Less than a year after COVID-19 was declared a pandemic, vaccines were developed at remarkable speed and vaccination campaigns were initiated, followed by a rapid roll-out globally. Currently, three broad pharmacological approaches to COVID-19 have been developed and are available which include antiviral therapy, neutralising antibody-based therapy, and immunomodulatory (Coopersmith *et al*., 2021; Kalinke *et al*., 2022).

Antivirals that are currently approved by the FDA include remdesivir, which is a nucleoside analogue that has broad-spectrum antiviral activity and has been proven effective against Ebolavirus, MERS-CoV and SARS-CoV-1. Remdesivir has also shown promising antiviral activity against SARS-CoV-2 in in a cell-based assay (Ogando *et al*., 2020; M. Wang *et al.*, 2020). A randomised double-blind placebo-controlled trial demonstrated that a three day course of remdesivir, when administered in non-hospitalised symptomatic COVID-19 patients reduced the risk of hospitalisation and

mortality by 87%. In addition, the safety profile of remdesivir was found to be acceptable and comparable to the safety profile observed in the placebo group. Both groups experienced mild adverse events such as nausea, headache, and cough, with a similar incidence rate of 42.3% in the remdesivir group and 46.3% in the placebo group (Gottlieb *et al*., 2022). Ritonavir-boosted nirmatrelvir (Paxlovid) is the second FDA approved antiviral agent. It is an anti-SARS-CoV-2 protease inhibitor that exerts its antiviral activity by inhibiting proteases that are involved in the replication stage (Marzi *et al*., 2022). In a recent phase 2 and 3 clinical trial consisting of 1219 symptomatic patients, the administration of a three day course of paxlovoid within three days of symptoms onset resulted in a reduction in hospitalisation or mortality by 89% (Mahase, 2021a).

Severe COVID-19 is accompanied with inflammation-related lung injury and some cases can lead to multiple organ failure and in the worst case, death. Subsequently, immunomodulatory agents that can modulate the inflammatory response in symptomatic COVID-19 patients have been approved by the FDA and are currently recommended for use by the NIH. Dexamethasone is a steroid compound, (a corticosteroid) that is translocated into the host cell, where it can inhibit the production of proinflammatory cytokines and can also activate the production of IL-10, which is an anti-inflammatory cytokine (Chikanza, 2002; Croxtall *et al*., 2000). A Randomised Evaluation of COVID-19 Therapy (RECOVERY) trial studied 2104 COVID-19 patients who received a ten-day course of dexamethasone and compared them to 4321 COVID-19 patients receiving usual care. The study showed that patients who displayed symptoms for more than seven days and were on invasive mechanical ventilation had a reduced 28-day mortality following treatment with dexamethasone. However, dexamethasone was not beneficial in patients

that were not receiving oxygen supplementation therapy and those with a shorter symptom duration (Horby *et al.*, 2020). The NIH panel currently recommend the use of Dexamethasone in children and adults with severe COVID-19 and requiring oxygen supplementation therapy or mechanical ventilation (The RECOVERY Collaborative Group, 2021).

Neutralising monoclonal-based therapies that have received Emergency Use Authorisation from the FDA include, bebtelovimab and sotrovimab as a monotherapy, bamlanivimab with etesevimab or casirivimab with imdevimab as a combination therapy (Gottlieb *et al*., 2021; Hentzien *et al*., 2022; Mahase, 2021b; Weinreich *et al*., 2021). Neutralising monoclonal antibodies have the potential to bind to the S protein on the viral surface which masks the binding site and indirectly prevents the binding of ACE2-RBD. Furthermore, failure of ACE2-RBD binding inhibits viral entry into the host cell. Data from a clinical phase 3 trial using etesevimab or casirivimab (REGN-CoV-2) showed that hospitalisation or mortality was reduced by 70% in patients with COVID-19. However, studies have shown that REGN-CoV-2 is not effective against the currently circulating Omicron variant. Bamlanivimab with etesevimab also target the RBD domain and a phase 2 trial showed that this combination therapy reduced SARS-CoV-2 viral load (Gottlieb *et al*., 2021) and a phase 3 trial, reported that the risk of hospitalisation and death was reduced by 87% (Dougan *et al*., 2021). Sotrovimab targets S protein, and a multicenter, double-blind placebo-controlled phase 3 trial reported a reduction in hospitalisation and death by 85% in COVID-19 patients with mild to moderate COVID-19 (Gupta *et al*., 2021). Lastly, bebtelovimab targets the RBD domain and has been shown to have broad neutralising to SARS-CoV-2 variants and the currently circulating Omicron (VOCs)

(Hentzien *et al.*, 2022). In phase 2, BLAZE-4 trial it has been reported that low-risk patients with mild to moderate COVID-19 that received either bebtelovimab alone or in combination with bamlanivimab and etesevimab resulted in significant reductions in viral load (Dougan *et al*., 2022)

1.10.4 Prevention of COVID-19

Although many Public Health and Social Measures (PHSM) and infection control measures have been implemented globally to limit the risk of spreading SARS-CoV-2, and the development of novel therapeutics, the most important step to contain the COVID-19 pandemic is through vaccination. Extraordinary efforts by clinical, academic and industrial researchers worldwide have resulted in the development of novel COVID-19 vaccines at an unprecedented speed in an attempt to contain and prevent COVID-19 or to reduce the severity of the disease. As per 'Our World in Data', an international vaccination dashboard, 13.37 billion doses of COVID-19 vaccine have been administered globally (69.8% global population) and in low-income countries, approximately 28.7% of people received at least one dose (Mathieu *et al*., 2020).

Currently, eight COVID-19 vaccines have received Emergency Use Authorisation by WHO which includes *a*) adenoviral-vectored vaccines; ChAdOx1 nCoV-19 (University of Oxford/AstraZeneca), Ad26.CoV2.S (Janssen), Covishield, CrAdOxI, nCoV-19 (Serum Institute of India *b*) whole-inactivated coronavirus; Covilo/BBIBP-CorV (SinoPharm/Beijing Institute of Biological Products), CoronaVac (Sinovac) and Covaxin, BBV152 (Bharat Biotech) and *c*) messenger RNA (mRNA) vaccines; mRNA-1273 (Moderna) and BNT162b2 (Pfizer-BioNTech) (see Table 1.2 for more detail**)**. All

approved vaccines utilise the native SARS-CoV-2 S protein, specifically from the Wuhan-Hu-1 strain. However, the way in which the S antigen is presented to the immune system varies between different vaccine categories (WHO, 2021h). mRNA vaccines work by presenting the mRNA material of S proteins directly into the host cell, where the antigen is synthesised using the host protein translation machinery. Adenovirus-vectored vaccines, use human or primate adenovirus vectors, for instance, AstraZeneca used a replication-deficient chimpanzee adenovirus that codes for S protein. Adenovirus vectored vaccines work in a similar way to mRNA vaccines, but first, the virus infects the host cells, followed by the delivery of S genetic material into the nucleus for transcription and translation generating the spike protein vaccine (Heinz & Stiasny, 2021; Vanaparthy *et al*., 2021; Voysey *et al*., 2021). This chapter will focus more on the COVID-19 vaccines that have been approved for use in the UK, including, Moderna, Pfizer-BioNTech and AstraZeneca. In clinical trials, Pfizer-BioNTech and Morden reported higher efficacy against moderate to severe forms of SARS-CoV-2 infection (~95% and ~94%, respectively) than AstraZeneca (~70%). However, studies have reported that immunity induced by natural immunity or vaccination despite repeated booster vaccination significantly declined at 3 and 6 months after immunisation, reducing the effectiveness of the vaccines (Baden *et al*., 2021; Naaber *et al*., 2021; Polack *et al*., 2020; Voysey *et al*., 2021). Concerning the emergence of VOCs, the effectiveness of Pfizer-BioNTech against the Alpha variant was 87%, and for the Beta variant (75%) (Levine-Tiefenbrun *et al*., 2021). A test-negative study showed that the effectiveness of Pfizer-BioNTech against the Omicron variant after the third booster dose was 82.4% and dropped to 53.6% after 3 months (Stowe *et al*., 2022). A study conducted in Qatar showed that the effectiveness of the Moderna vaccine against severe or fatal COVID-19 disease

as a result of the Alpha variant and Beta variant was approximately 81.6% and 95.7% after the first and second booster doses, respectively, suggesting that Moderna vaccine was effective against these two variants (Chemaitelly *et al*., 2021). The efficacy of the Moderna vaccine against the Omicron variant after three booster doses has been shown to have lower effectiveness compared to the other variants, for instance, a test-negative case-control study reported that the vaccine efficacy against Omicron was 71.6% at 14 to 60 day and 47.4% after 60 days (Tseng *et al*., 2022). Currently, the UK COVID-19 vaccination programme does not recommend boosting with AstraZeneca. A small amount people received an AstraZeneca booster as compared to a Pfizer or Moderna booster. Those that received AstraZeneca booster were most likely those in clinical risk groups, vulnerable or severely immunosuppressed (Kirsebom *et al*., 2022; UK Health Security Agency, 2023).

Table 1.2. COVID-19 vaccines listed by WHO. Vaccine efficacy, safety profile and protection against SARS-CoV-2 from clinical and preliminary studies.

COVID-19 Vaccine	Technology platform	Country; date of WHO listing	Efficacy and safety profile	Reference
Pfizer (BNT162B2)	mRNA	USA, January 2020	92-100% against infection, 87% and 92% against hospitalisation and severe disease, respectively. Rare cases of myocarditis in young men. VE against Alpha (98.4%), Beta (75%), Gamma (88%), Delta (90.9%) and Omicron variant (65.5%).	(Andrews et al., 2021; WHO, 2021e)
Moderna (Spikevax, mRNA-1273)	mRNA	USA, April 2021	94-100% against infection, 100% against hospitalisation, severe COVID-19 and mortality. Rare cases of myocarditis reported in young men. VE against Alpha (98.4) Delta (94.5%) and Omicron (75.1%).	(Song *et al*., 2012) (Andrews et al., 2021; Bruxvoort et al., 2021; WHO, 2021d)
Janssen (Ad26.CoV-2)	Human adenovirus type 26	Netherlands, March 2021	66% against infection, 77% and 93% against severe COVID-19 and hospitalisation, respectively. Rare reports of thrombosis with thrombocytopenia (3 to 15 post immunisation).	(WHO, 2021a)
AstraZeneca (ChAd0x1, AZD1222)	Chimpanzee adenovirus	UK, February 2021	91% protection against hospitalisation with Delta, 74% against symptomatic COVID-19 caused by Alpha and 66.1% against symptomatic COVID-19 caused by Omicron.	(Kirsebom et al., 2022; G. Li et al., 2022; WHO, 2021b)

CoronaVac	Inactivated SARS-CoV-2	China, June 2021	50-84% against infection, 80-100% in preventing severe COVID-19 infections, hospitalisation and mortality. 51-83.5% in preventing symptomatic COVID-19 infection, 49.6% against infection (Gamma variant) and 50.7% (Brazillian P.2 variant). Safe and well tolerated in older adults, no reports of rare adverse events.	(E. Y. F. Wan *et al*., 2022; WHO, 2021f)
Covishield (ChAdOx1-S)	Chimpanzee adenovirus	India, February 2021	72-85% against infection, 92% protection against hospitalisation caused by Delta; 86% protection against hospitalisation with Alpha. No severe allergic reactions but thrombosis with thrombocytopenia syndrome was reported 3-30 days post-vaccination.	(WHO, 2021g)
BIBP/Sinopharma	Inactivated SARS-CoV-2	China, May 2021	79% against infection and hospitalisation. No severe allergic reactions reported.	(WHO, 2021c)
Covaxin (BBV152)	Inactivated SARS-CoV-2	India, November 2021	78% efficacy against infection and hospitalisation, 50% and 69% VE has been reported against symptomatic and severe COVID-19 caused by Delta. No reports of severe adverse events reported in clinical trials	(Bhatnagar *et al*., 2022; Ella *et al*., 2021; WHO, 2022)

As summarised in Table 1.2, current COVID-19 vaccines have shown considerable efficacy and protection against mild to severe COVID-19 cases with a low risk of adverse events. In clinical trials, subjects reported mild to moderate local response to all vaccines including, soreness, discomfort, redness and inflammation at the site of injection, and systemic responses included fatigue, headache, body and muscle ache, and fever (Falsey *et al*., 2021; Polack *et al*., 2020). However, rare cases of adverse

events following COVID-19 vaccinations have been reported. For example, a multi-national, placebo-controlled, efficacy clinical trial reported four serious adverse events following immunisations with Pfizer. These included, shoulder injury, right axillary lymphadenopathy, paroxysmal ventricular arrhythmia and right leg paresthesia (Polack *et al*., 2020). In March 2021, there were reports of thrombocytopenia and thrombosis which occurred after the administration of AstraZeneca. As of April 2021, approximately 34 million people were vaccinated in the European Economic Area (EEA) and the United Kingdom and among those vaccinated, 169 cases of cerebral venous sinus thrombosis (CVST) and 53 cases of internal vein thrombosis have been reported (Lavin *et al*., 2021). Despite this, Pfizer, Moderna and AstraZeneca have an acceptable short-term safety profile, and additional studies are currently being conducted to assess the long-term safety profile of these vaccines (Chirico *et al*., 2022; A. Choi *et al*., 2021; Falsey *et al*., 2021; Q. Wu *et al*., 2021). To conclude, clinical studies have shown that boosting with either Pfizer or Moderna after primary immunisation with either AstraZeneca or Pfizer increased protection against the currently circulating Omicron variant. However, protection drastically waned over time, further supporting the need for more booster doses or the development of new vaccines or different immunisation schedules (Andrews *et al*., 2022; Hermosilla *et al*., 2022; Kirsebom *et al*., 2022).

1.11 Aims

The primary objective of this project was to utilise thymineless death principles via the THY-X-CISE® cloning system to engineer thymine auxotrophic *B. subtilis* strains capable of expressing candidate vaccine antigens on the spore surface.

The first project aim was to assess the immunogenicity of these thymine auxotrophic *B. subtilis* spores, expressing the receptor binding domain (RBD), heptad repeat 1 and 2 (HR1-HR2), and nucleocapsid antigens, when administered as nasal boosts following systemic prime immunisation in mice. Additionally, this project seeks to evaluate the protective efficacy of these spores against SARS-CoV-2 infection in Syrian hamsters.

The second project aim was to investigate the mucosal and systemic immune responses evoked by thymine auxotrophic spores expressing Urease A and B subunits when delivered as an oral prophylactic vaccine in mice. Furthermore, this project aims to assess whether these spores can confer protection against *H. pylori* infection in mice.

1.11.1 Chapter 3 objectives

- Engineer thymine auxotrophic spores expressing the RBD, HR1-HR2 and nucleocapsid from SARS-CoV-2 and urease A, and urease B subunits from *H. pylori* via fusion to spore coat proteins (CotB or CotC) using the THY-X-CISE cloning system

- Assess the expression of chimeric proteins on the spore surface using Western blotting and Whole spore ELISA

- Characterise thymine auxotrophic mutants *in vitro* to evaluate their growth and sporulation abilities in the presence and absence of thymine

1.11.2 Chapter 4 objectives

- Evaluate the immunogenicity of thymine auxotrophic spore vaccine booster candidates, including PK120 (CotB-RBDWuh), PK122 (CotB-HR1-HR2), SporCoVax (a 1:1 mixture of PK120 and PK122 spores), and PK230 expressing the RBD fragment from the Omicron variant, following systemic prime immunisations with an S protein subunit vaccine or Oxford AZD1222 vaccine in BALB/c mice. Assess mucosal and systemic antibody titres and perform T cell cytokine analysis via indirect ELISA and flow cytometry, respectively

- Investigate the immunogenicity of PK128 spores displaying the nucleocapsid fragment from the Wuhan-Hu-1 strain as a monomer booster vaccine and PK-S+N (a 1:1:1 mixture of PK120, PK122, and PK128) as a trivalent spore booster vaccine, following systemic prime immunisations consisting of a 1:1 mixture of S and N protein subunits in BALB/c mice. Evaluate cytokine profiles and measure mucosal and systemic antibody titres through indirect ELISA

- Assess the efficacy of SporCoVax in a hamster SARS-CoV-2 model of infection following immunisation and challenge with SARS-CoV-2. Evaluate protective immune responses based on weight variation and viral load in nasal turbinates, oropharyngeal swabs, and lung samples

1.11.3 Chapter 5 objectives

- Evaluate the immunogenicity of thymine auxotrophic spore vaccine candidates, PK82 (CotB-UreA) and PK78 (CotB-UreB^{CT}), following oral immunisation in C57 BL/6 mice. Urease-specific mucosal and systemic immunoglobulin (Ig) titres in faecal (sIgA) and serum (IgG) samples were evaluated by indirect ELISA

- Assess the efficacy of the thymine auxotrophic spore vaccines by measuring the bacterial load in stomach samples following *H. pylori* challenge in Mlac: ICR mice

CHAPTER 2: Material and Methods

2.1 General methods

General methods for working with *B. subtilis* including preparation of spores and extraction of spore coat proteins are described elsewhere (Harwood & Cutting, 1990). Work with *Escherichia coli* including cloning are as described elsewhere (Sambrook & Russell, 2001).

2.2 Bacterial strains

B. subtilis strain PY79 is a prototrophic (Spo^+) laboratory strain. Thymine auxotrophic strains of PY79 constructed and used in Chapter 3, 4 and 5 are listed in Table 2.1. Thymine auxotrophic strains were constructed by inserting chimeric genes (*thyA::cotB*-gene of interest or *thyA::cotC*-gene of interest) into the *thyA* locus and an empty plasmid (*pThyB::*Δ) into the *thyB* locus, as described elsewhere (Hosseini et al., 2018) and detailed in Section 2.4. These strains include: PK78 (*thyA::cotB-ureB*$^{365\text{-}568}$ *thyB::*Δ), PK82 (*thyA::cotB-UreA*$^{1\text{-}237}$ *thyB:*Δ*:*), PK120 (*thyA::cotB*-$RBD^{Wuh(319\text{-}530)}$ *thyB::*Δ), PK122 (*thyA::cotC*-HR1-$HR2^{Wuh(820\text{-}1204)}$ *thyB::*Δ), PK128 (*thyA::cotB*-$N^{Wuh(1\text{-}422)}$ *thyB::*Δ) and PK230 (*thyA::cotB*-$RBD^{Omi(316\text{-}538)}$ *thyB::*Δ). PK118 (*thyA::*Δ *thyB::*Δ) is an isogenic thymine auxotrophic mutant strain, which carries no chimeric gene insertion and served as a control. Thymine auxotrophic strains have resistance to trimethoprim and are dependent on exogenous thymine supplementation during culturing.

Work conducted by our collaborators from Institut de Recerca i Tecnologia Agroalimentàries (Professor Joaquim Segalés) consisted of a SARS-CoV-2 Cat02 isolate

(variant D614G, ID EPI_ISL-47147) and was used for the challenge study. It was isolated from a patient in a laboratory-confirmed COVID-19 case in Barcelona, Spain, and propagated in Vero E6 cells (ATCC CRL-1586). Work conducted by our collaborators from the Institute of Vaccines and Biological Medicals (Dr Van Duy Nguyen), consisted of a clinical strain of *H. pylori*, strain HP34, obtained from the Hospital of the University of Medicine and Pharmacy, Hue University, Vietnam. HP34 was isolated (05/05/2020) from a 54-year-old female, peptic ulcer patient, with endoscopy displaying superficial duodenal ulceration, inflammation in the fundus, and antral erosions. The virulence genotypes were shown to be positive to *ureA*, *ureB*, *cagA* and *vacA* (Genbank accession number CP122516). Identity was confirmed by whole genome sequencing. The strain was resistant to clarithromycin but sensitive to tetracycline, metronidazole, amoxicillin, and levofloxacin. *H. pylori*, strain 26695, is a laboratory strain obtained from Dr. Amanda Rossiter.

Table 2.1. Strains used in this study.

Strain	Identity	Description	Source
E. coli	*E. coli* Turbo		NEB
PY79	*B. subtilis*		*Bacillus* Genetic Stock Centre
PK118	*B. subtilis*	pThyA::Δ pThyB::Δ	
PK78	*B. subtilis thyA::cotB-ureB thyB::*Δ	pThyA::CotB-UreB pThyB::Δ (Ala365-Phe568)	
PK82	*B. subtilis thyA::cotB-ureA thyB::*Δ	pThyA::CotB-UreA pThyB::Δ (Met1-Glu237)	
PK120	*B. subtilis thyA::cotB*-RBD^{wuh} *thyB::*Δ	pThyA::CotB-RBD^{wuh} pThyB::Δ (Arg319-Ser530)	
PK122	*B. subtilis thyA::cotC*-HR1-HR2 *thyB::*Δ	pThyA::CotC-HR1-HR2 pThyB::Δ (Gln920-Gln1202)	
PK128	*B. subtilis thyA::cotB*-NC *thyB::*Δ	pThya*::*CotB-N pThyB::Δ (M1-Asn422)	
PK230	*B. subtilis thyA::cotB*-RBD^{omi} *thyB::*Δ	pThyA*::*CotB-RBD^{omi} pThyB*::*Δ (Arg316-Phe538)	
26695	*H. pylori*		Provided by Dr. Amanda Rossiter (University of Birmingham)
HP34	*H. pylori*	*ureA*$^+$, *ureB*$^+$,*cagA*$^+$ *and vacA*$^+$	Patient (Vietnam)
D614G	SARS-CoV-2	ID EPI_ISL-47147	Patient (laboratory-confirmed, Barcelona)

Unless stated otherwise, *B. subtilis* was routinely cultured in modified Difco sporulation medium (DSM) and DSM agar (for 1 litre: nutrient broth containing 1% yeast extract 8 g (BD Bacto), KCl 1g (Sigma), 0.25 g $MgSO_4$:$7H_2O$ (Sigma) with 15 g agar (Sigma) or without agar and following autoclaving 1 ml of each of the following filter sterilised solutions were added: 1 M $Ca(NO_3)_2$, 10 mM $MnCl_2$ and 1 Mm $FeSO_4$. *H. pylori* was cultured in selective horse blood agar (HBA), which was prepared using 4% (w/v) blood

agar base (Oxoid) and following autoclaving the following supplements were added: 8% (w/v) defibrinated horse blood (Oxoid), 1% (v/v) sodium lactate (Sigma) and Skirrow's antibiotic selective supplement (consisting of vancomycin (Sigma), 10 μg/mL; polymyxin B (Sigma), 25 ng/mL; trimethoprim (Sigma), 5 μg/mL; amphotericin B (Sigma), 2.5 μg/mL). *H. pylori* inoculated from agar plates were cultured in brain heart infusion (BHI) medium (Oxoid) supplemented with 5% (v/v) fetal bovine serum (FBS, Thermofisher Scientific). Incubation was made in a microaerophilic chamber using an Oxoid CampyGen 2.5 L sachet (5-7% O_2, 5-10% CO_2, and 85% N_2) at 37°C, with passaging every 48.

To preserve and store bacterial strains a single colony of *B. subtilis* and large loop of *H. pylori* was used to inoculate 10ml of the appropriate media. *B. subtilis* strains were incubated overnight at 37°C, under aerobic conditions. *H. pylori* was cultured for 3 to 4 days, passaging every 48 hours, and incubated in a microaerophilic chamber using an Oxoid CampyGen 2.5 L sachet (5-7% O_2, 5-10% CO_2, and 85% N_2) at 37°C. 1ml of the culture was added to 1ml of autoclaved 50% glycerol and stored at -80°C.

2.3 pThyA::CotB and pThyA::CotC plasmid construction

To design and construct pThyA::CotB and pThyA::CotC modular plasmids to permit directional sub-cloning of heterologous genes, pThyA (4, 274 bp) plasmid was used as a template plasmid. pThyA plasmid carries a segment consisting of the left and right homology arms of *B. subtilis thyA* gene surrounding the multiple cloning site (MCS) and the ampicillin resistance gene *(bla*) (Hosseini *et al*., 2018). pThyA::CotB and pThyA::CotC modular plasmids were constructed by inserting c*otB* (1, 299 bp) or *cotC* (497 bp) genes including the promoter region into the NdeI-BamHI restriction sites

located in the MCS of pThyA vector. pThyA::CotB and pThyA::CotC sequences are given in Appendix A and shown schematically in Figure 2.1.

The heterologous DNA used in this study includes, the receptor binding domain (RBD), two heptad repeat regions, (HR1-HR2) and nucleocapsid (NC) open reading frames (ORFs) from SARS-CoV-2, Wuhan-Hu-1 strain and was codon optimised for codon usage in *B. subtilis* (Azenta Life Sciences, UK). The RBD^{omi} ORFs from SARS-CoV-2, Omicron variant BA.1 strain was codon optimised for codon usage in *B. subtilis* (Azenta Life Sciences, UK). The entire coding ORF of urease-A (*ureA*) and the C-terminal region of urease-B (*ureB*) were amplified (not codon optimised for usage) from *H. pylori* 26695 strain (Supplied by Dr. Amanda Rossiter). Heterologous genes were subcloned into the pThyA::CotB and pThyA::CotC plasmid vector by fusing the heterologous DNA to the 3'-end of the *cotB* or *cotC* gene of *B. subtilis* (including the promoter). All genes were cut at the 5' and 3' end using BamHI and EcoRI, respectively and fused in-frame to the 3'-end of *cotB* or *cotC*, by ligating, with *thyA::cotB* or *thyA::cotC* (cut using BamHI and EcoRI at the 5' and 3' end, respectively, (Appendix B). The pThyB (4, 973 bp) vector carries a segment consisting of the left and right homology arms of *B. subtilis thyB* gene surrounding the MCS and the ampicillin resistance gene *(bla*) (Hosseini *et al*., 2018). Table 2.2 shows the primers used to amplify the genes above.

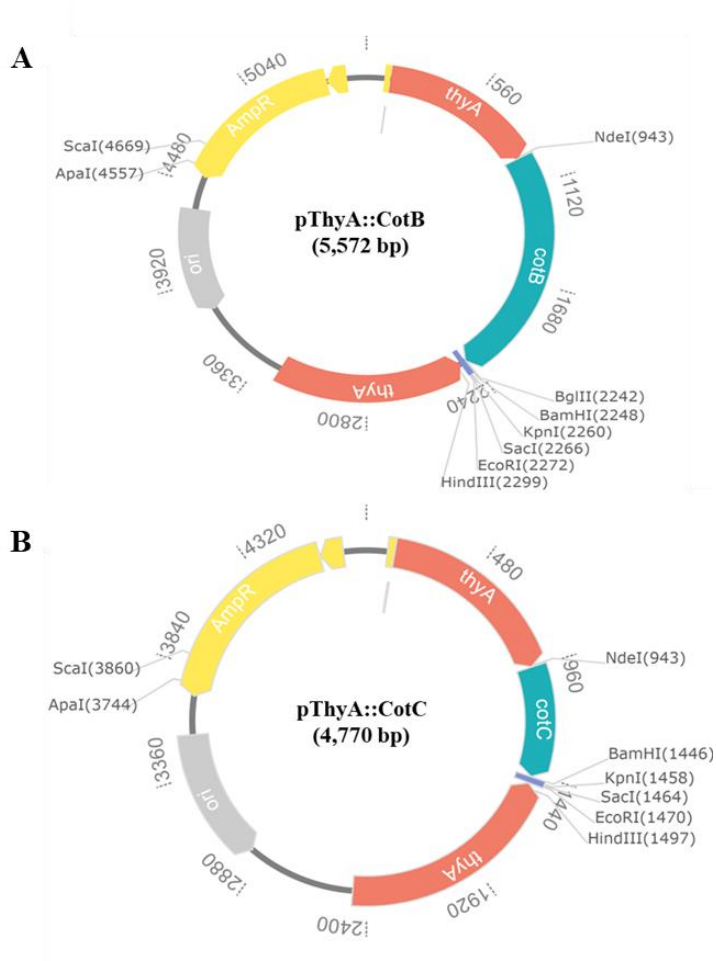

Figure 2.1. Schematic representation of pThyA::CotB and pThyA::CotC plasmid vector. The plasmid pThyA::CotB **(A)** and pThyA::CotC **(B)** were generated by cloning the anchor proteins of *B. subtilis* (*cotB* and *cotC*, respectively) into the Ndel and BamHI restriction sites of the MCS of *thyA*, located in the middle of the left and right homology arm of the *B. subtilis thyA* gene. The *bla* (β-lactamase) represents the ampicillin resistance gene. pThyA::CotB and pThyA::CotC have a total of 5, 572 bp and 4, 770 bp, respectively.

Table 2.2. PCR primers for the amplification of different genes

Primer	Direction	Sequence	Restriction site
CotB			
CotB_F	Forward	AAA***CATATG***CGAAAATCATGGCGATGTAT	NdeI
CotB_R	Reverse	AAA***GGATCC***AGATCTTGGTGATCGTTTAG	BamHI
CotC			
CotC_F	Forward	AAA***CATATG***ATAAACTGCCGGCGCTT	NdeI
CotC_R	Reverse	CCC***GGATCC***GTAGTGTTTTTTATGCTTTTTATACTC	BamHI
RBD^{wuhan}			
RBD^{wuh}_F	Forward	AAA***GGATCC***ATGCGCGTTCAGC	BamHI
RBD^{wuh}_R	Reverse	AAA***GAATTC***TTATTAGCTTTTCTTCGGTCCGCAAAC	EcoRI
RBD^{omicron}			
RBD^{omi}_F	Forward	AAA***GGATCC***AGAGTTCAACCGAC	BamHI
RBD^{omi}_R	Reverse	CCC***GAATTC***TTATTAAAAATTAACGCA	EcoRI
HR1-HR2^{wuhan}			
HR1-HR2	Forward	AAA***GGATCC***CAGAAGCTTATCGCTAAC	BamHI
HR1-HR2	Reverse	CCC***GAATTC***TTATTATTCTTGAAGATCGA	EcoRI
ureA			
UreA_F	Forward	AAA***GGATCC***AAACTCACCCCAAAAGAGTT	BamHI
UreA_R	Reverse	AAA***GAATTC***TTACTCCTTAATTGTTTTTACATAGTTGTCA	EcoRI
ureB			
UreB_F	Forward	AAA***GGATCC***GCTATGGGTCGTGTGGGT	BamHI
UreB_R	Reverse	AAA***GAATTC***CTAGAAAATGCTAAAGAGTTGCGCCAA	EcoRI

2.4 Construction of recombinant *B. subtilis* vaccine candidate strains using THY-X-CISE® cloning system

A cloning method referred to as the THY-X-CISE® system, which consists of a two-step, ectopic cloning system was used to introduce heterologous genes into the chromosome of *B. subtilis* (Figure 2.2) (Hosseini *et al*., 2018). In the first stage, cells of a wild-type recipient strain (in the work described here the prototrophic strain PY79 was used) were made competent using a two-step transformation procedure described by Dubnau and Davidoff-Abelson and in common use in *Bacillus* labs (Cutting, 1990; Dubnau & Davidoff-Abelson, 1971). Linearisation of the resulting pThyA::CotB or pThyA::CotC insertion plasmid and introduction into *B. subtilis thyA* locus (encodes thymidylate synthase A) by a double crossover recombination event generates trimethoprim-resistant transformants that will starve in the absence of thymine (thymine-dependent). Cells were plated on SMM agar (Harwood & Cutting, 1990) supplemented with thymine (50 μg/ml) and trimethoprim (3 μg/ml). After 72 to 96 hours of growth, single colonies were colony purified and assessed for growth at 37°C and 46°C on SMM agar with or without thymine (50 μg/ml) and trimethoprim (3 μg/ml). Cells carrying an insertion at the *thyA* locus could grow at 37°C with or without thymine but were unable to grow at 46°C unless supplemented with thymine (50 μg/ml). The selection of positive transformants was based on trimethoprim resistance on plates supplemented with trimethoprim (3 μg/ml) and thymine 50 μg/ml) at 37°C (Hosseini *et al*., 2018). Further verification was to amplify, by PCR, the presence of a heterologous fusion gene from transformants using primers annealing to the *thyA* sequences (Table 2.2). The resulting *thyA::insertion* strain was made chemically competent using the same procedure as above (Cutting, 1990; Dubnau

& Davidoff-Abelson, 1971) and was then used as a recipient in a second transformation where an empty linearised pThyB vector is introduced into the *thyB* locus (encodes thymidylate synthase B). Cells were plated on SMM containing 0.2% (wt/vol) CAA (SMM-CAA), thymine (50 µg/ml) and trimethoprim (~0.4 mg/ml) and incubated at 37°C for 72 to 96 hours. To confirm the presence of both *thyA* and *thyB* insertions, colonies were plated on SMM-CAA agar with or without thymine (50 µg/ml) and grown at 37°C and 46°C. Cells carrying both *thyA* and *thyB* insertions were unable to grow at 37°C and 46°C unless supplemented with thymine. The resulting strain (*thyA::insertion thyB::*Δ) is dependent on exogenous thymine, but resistant to a higher concentration of trimethoprim (Hosseini *et al*., 2018). The diagnostic primers used for confirmation of insertion in the *thyA* and *thyB* locus are shown in Table 2.2. Strains constructed are listed in Table 2.1

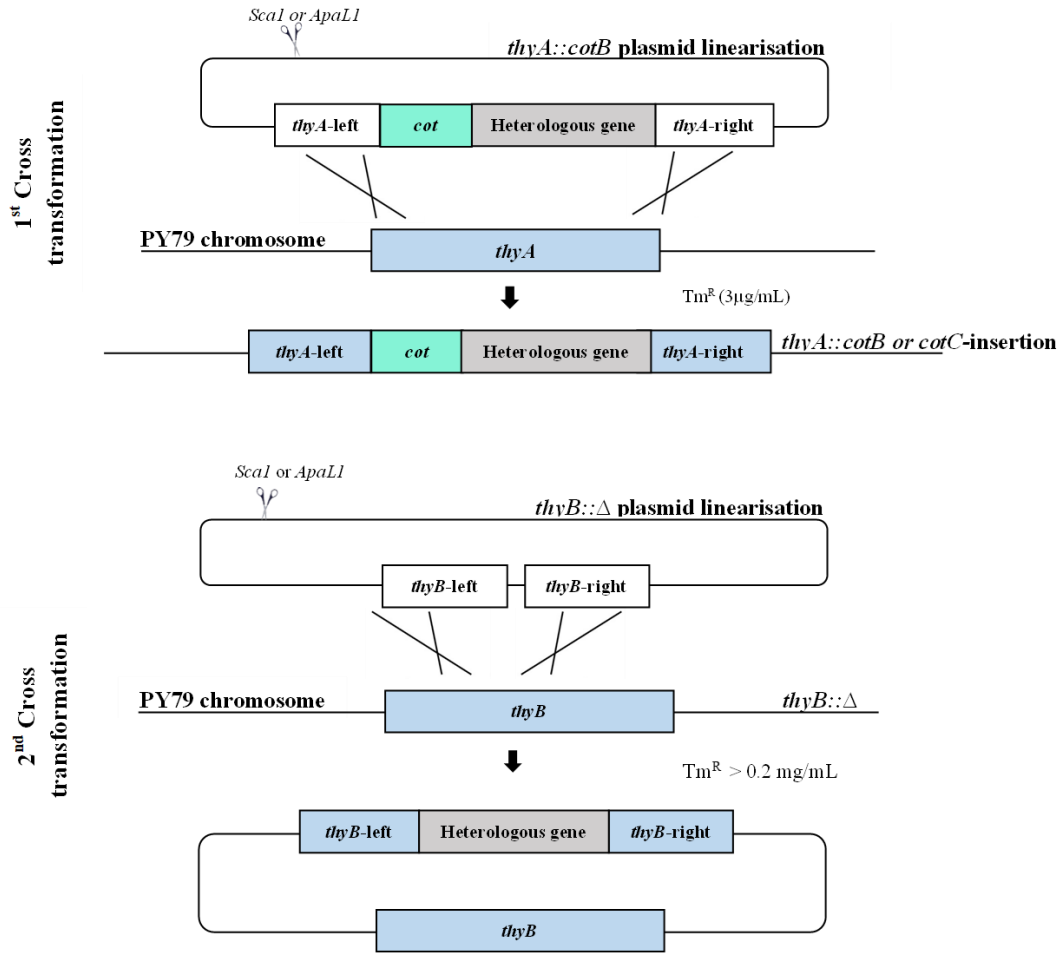

Figure 2.2. Schematic diagram of *thyA thyB* strain construction. To construct ectopic insertions at the *thyA* and *thyB* loci of *B. subtilis*, a two-step transformation is required. First, a pThyA::CotB or pThyA::CotC plasmid carrying a heterologous gene is linearised (ScaI digestion) and introduced into cells of a wild-type *B. subtilis* strain by DNA-mediated transformation. Trimethoprim-resistant (Tm^r) transformants are selected on SMM agar containing trimethoprim (3 µg/ml) and thymine (50 µg/ml) and carry an insertion of homologous *thyA* DNA together with the chimeric gene, by marker replacement, as shown. Next, a linearised pThyB sector carrying an empty *thyB* gene (no insertion) is introduced into cells of the *thyA* insertion mutant strain created in the first step. Selection for Tm^r is made on SMM-CAA agar containing trimethoprim (>200 µg/ml) and thymine (50 µg/ml).

Table 2.2: Diagnostic primers for confirmation of insertion into *thyA* and *thyB* genes

Primer	Direction	Sequence
thyA		
ThyA_F	Forward	AAACAAGAAGACGGAACCAT
ThyA_R	Reverse	AGAATTCTTACTCCTTAATTGTTTTTACATAGTTGTCA
thyB		
ThyB_F	Forward	GATATTAAAACAAATCCGAACTC
ThyB_R	Reverse	GTCAGACACATAGAATTG

2.5 Sporulation and spore purification

Sporulation of thymine auxotrophic *B. subtilis* strains was induced by the exhaustion method in DSM (Difco-sporulation) medium supplemented with thymine (50 μg/ml). Sporulating cultures were harvested 24 h after the initiation of sporulation and purified using lysozyme treatment to break residual sporangial cells followed by washing steps in 1 M NaCl, 1 M KCl and water three times, as described elsewhere (Nicholson *et al*., 2000). Then spores were treated at 65°C for 1 h to kill residual cells. The spore suspension was titrated immediately for CFU/ml before freezing at -20°C.

2.6 Spore coat extraction

Spores pellets (2×10^9) were resuspended in spore coat extraction buffer and heated at 65°C for 1 h, as previously described (Monroe & Setlow, 2006). After centrifugation, the supernatant was removed and mixed with 4X SDS sample loading buffer and incubated for 10 min at 95° C before SDS-PAGE analysis.

2.7 Western Blotting of spore coat extracts

Western blotting was carried out as described previously (Permpoonpattana *et al.*, 2013). Spore coat proteins were fractionated by size on 12% SDS-PAGE gels using approximately 10 mg of protein per lane. The gels were transferred onto an immobilon-P PVDF Membrane (Merck) using a Trans-Blot® Turbo™ Transfer System (Bio-Rad). Membranes were dehydrated in 100% MeOH and allowed to dry for 1 hour at RT. Followed by blocking for 1h at 37°C with PBS (containing 0.05% (v/v) Tween 20 and 3% (w/v) skimmed milk). The blot was probed with primary antibodies to the relevant SARS-CoV-2 domains: anti-S^{Wuh} PAbs raised against the entire S^{Wuh} polypeptide (SinoBiological, 40589-T62), anti-S^{Omi} MAbs raised against the RBD of the S^{Omi} (SinoBiological, 40592-MM117) and anti-N PAbs raised against the entire N polypeptide (PA5-32168) and primary antibodies relevant to the relevant *H. pylori* domains: anti-*ureA* raised against the entire UreA polypeptide (Thermofisher, PA5-117505) and anti-*ureB* raised against the entire UreB polypeptide (Thermofisher, PA5-32168) were diluted in conjugate buffer (1:2, 000 in 0.01M PBS, 3% (w/v) skimmed milk, 1% (v/v) and 0.05% (v/v) Tween 20) and incubated overnight at 4°C. The blots were incubated in the appropriate horseradish peroxidase-conjugated anti-rabbit IgG (Sigma Cat No. 12-348) or anti-mouse IgG (Dako Cat No. P0447) diluted in conjugate buffer (1:3,000 in 0.01M PBS, 3% (w/v) skimmed milk, 1% (v/v) and 0.05% (v/v) Tween 20) for 1h at RT and developed using Amersham ECL Western blotting substrate (GE Healthcare) on Amersham hyper-film ECL (GE Healthcare).

2.8 Whole-spore ELISA

An ELISA method was used to detect surface-exposed proteins as described previously (Permpoonpattana *et al*., 2013). Briefly, spores were diluted 2 x 10^8 spores/ml in PBS and 50µl of suspension was used to coat microplate wells (Greiner, high binding) overnight at 4°C. Followed by blocking for 1h at 37°C with PBS (containing 0.05% (v/v) Tween 20 and 2% (w/v) bovine serum albumin (BSA). Primary antibodies to the relevant SARS-CoV-2 domains: anti-S^{Wuh} PAbs raised against the entire S^{Wuh} polypeptide (SinoBiological, 40589-T62), anti-S^{Omi} MAbs raised against the RBD of the S^{Omi} (SinoBiological, 40592-MM117) and anti-N PAbs raised against the entire N polypeptide (PA5-32168) and primary antibodies relevant to the relevant *H. pylori* domains: anti-*ureA* raised against the entire UreA polypeptide (Thermofisher, PA5-117505) and anti-*ureB* raised against the entire UreB polypeptide (Thermofisher, PA5-32168) were diluted in conjugate buffer (1:2, 000 in 0.01M PBS, 1% (w/v) BSA, 1% (v/v) and 0.05% (v/v) Tween 20) and incubated for 2h at 30°C. The appropriate horseradish peroxidase-conjugated anti-rabbit IgG (Sigma Cat No. 12-348) or anti-mouse IgG (Dako Cat No. P0447) was diluted in conjugate buffer (1:3,000 in 0.01M PBS, 1% (w/v) BSA, 1% (v/v) and 0.05% (v/v) Tween 20) and used as a secondary antibody. Plates were incubated for 1 h at RT and then developed using tetramethyl benzidine (TMB) substrate (0.1 mg/mL 3.3',5.5'-tetramethylbenzidine in 0.1 M sodium acetate buffer (pH 5.5). Reactions were stopped using 2 M H_2SO_4, and ODs were read at 450 nm.

2.9 Growth curves

To assess the fitness of the constructed thymine auxotrophic strains, growth curves for each of the *thyA::insertion* strains including the isogenic strain PK118 (*thyA::*Δ *thyB::*Δ) were plotted. Strains were grown overnight and sub-cultured into DSM with or without thymine (50μg/ml) with a starting optical density of 600nm (OD_{600}) of 0.04 and grown for 8 hours at 37°C. OD_{600} was measured every hour.

2.10 Stability of double *thy*-insertion

To investigate the stability of the double *thy*-insertion mutant strains three strains were used for this assay including PK78 (*thyA::cotB-ureB*^{CT} *thyB::*Δ), PK120 (*thyA::cotB*-RBD^{wuh} *thyB::*Δ) and PK122 (*thyA::cotC*-HR1-HR2 *thyB::*Δ). A single colony of double *thy*-insertion mutants, PK78, PK120, and PK122 was used to inoculate 25 ml of DSM supplemented with thymine (50 μg/ml), grown for 24h at 37°C. After 24 h, a sample of the culture was removed, plated on DSM supplemented with thymine (50 μg/ml) and DSM only (no thymine) and CFU counts were obtained. 1 ml of the same culture was used to subculture into fresh DSM medium, one of which was supplemented with thymine and the other with no thymine and CFU counts were obtained. The process was repeated four times (2nd to 5th subculture rounds).

2.11 Immunogens

Recombinant Spike protein rS^{Wuh} and rS^{Omi} (baculovirus expressed) were obtained from SinoBiological (Cat Nos. 40589-V08B1 and 40589-V08B33, respectively). Prior to intramuscular injection, 5 μg of protein for mice immunisations or 10 μg of protein for hamster immunisations was suspended in PBS and complexed (1:1) with the adjuvant AddaVax (Invivogen, vac-adx-10). The COVID-19 vaccine ChAdOx1 nCoV-19

(AZD1222) with a concentration of 3.8 X 10^9 infectious units (IU)/mL (3.7 × 10^{11} virus particles/mL) was provided by Prof. Sarah Gilbert (Jenner Institute, Oxford University) and referred to henceforth as AZD1222. Prior to use, AZD1222 was diluted in PBS to give a working concentration of 2 X 10^9 IU/mL.

2.12 Heterologous prime-boost immunisations in mice

BALB/c female mice were obtained from Charles River, UK (aged 8-10 weeks before the study started) and allocated into groups (n=5-6) for recombinant prime and n=6 for AZD1222 prime).

2.12.1 Recombinant spike prime-boost vaccination

Group 1 received no immunisations and served as a naive, control group. Groups 2-6 each received an intramuscular injection (i.m.) of 50 µL (5 µg) of formulated rS^{Wuh} protein in each hind quadriceps muscle. Group 2 was primed (i.m.) with rS^{Wuh} on day 1 and culled on day 48. Groups 3-6 were primed (i.m.) with rS^{Wuh} on day 1 and then intranasally (i.n.) boosted three and five weeks later with spores (1 X 10^9 CFU) of either PK118 (*thyA::*Δ *thyB::*Δ) spores (Group 3) or PK120 (CotB-RBD^{Wuh}) (Group 4) or PK122 (CotC-HR1-$HR2^{Wuh}$) (Group 5) or 1:1 mixture of PK120 (CotB-RBD^{Wuh} and PK122 (CotC-HR1-$HR2^{Wuh}$) referred to as SporCoVax (Group 6). Each intranasal administration (10µl total/animal, 5 µL/nare, no anaesthesia) consisted of three administrations given on consecutive days (Boost 1, days 21, 22, 27 and Boost 2, days 35, 36, 37). The dosing regimen is shown in Figure 2.3A.

2.12.2 AZD1222 prime-boost vaccination

Group 1 was a naïve group and received no immunisations. Groups 2-5 each received in the right hind quadriceps muscle 50 µL of AZD1222 (1.0 X 10^8 IU) in phosphate-buffered saline (PBS). Group 2 was culled on day 48. Groups 3-5 were boosted (intranasal; 5 mL/nare, no anaesthesia) three and five weeks later with spores (1 X 10^9 CFU) of PK118 (*thyA::*Δ *thyB::*Δ; Group 3), or SporCoVax (a 1:1 mixture of CotB-RBD^{Wuh} and CotC-HR1-HR2^{Wuh} spores; Group 4) or PK230 (CotB-RBD^{Omi}; Group 5). Each intranasal administration (10µl total/animal, 5 µL/nare, no anaesthesia) consisted of three administrations given on consecutive days (Boost 1, days 21, 22, 23 and Boost 2, days 35, 36, 37). The dosing regimen is shown in Figure 2.3A.

2.12.3 Recombinant nucleocapsid prime-boost vaccination

Group 1 received no immunisations and served as a naive, control group. Groups 2-4 each received an intramuscular injection (i.m.) of 50 µL (5 µg) of formulated rN^{Wuh} protein in each hind quadriceps muscle. Group 2 was primed (i.m.) with rN on day 1 and culled on day 48. Groups 3-4 were primed (i.m.) with rN on day 1 and then intranasally (i.n.) boosted three and five weeks later with spores (1 X 10^9 CFU) of either PK118 (*thyA::*Δ *thyB::*Δ, no antigen expression) spores (Group 3) or PK128 (CotB-NC) spores (Group 4). Each intranasal administration (10µl total/animal, 5 µL/nare, no anaesthesia) consisted of three administrations given on consecutive days (Boost 1, days 21, 22, 27 and Boost 2, days 35, 36, 37). The dosing regimen is shown in Figure 2.3B.

2.12.4 Recombinant spike and nucleocapsid prime-boost vaccination

Group 1 received no immunisations and served as a naive, control group. Groups 2-4 each received an intramuscular injection (i.m.) of 50 μL of formulated rN and rS (5 μg of each protein in a single formulation, referred henceforth as rS+N) in each hind quadriceps muscle. Group 2 was primed (i.m.) with PK-S+N on day 1 and culled on day 48. Groups 3-4 were primed (i.m.) with rS+N on day 1 and then intranasally (i.n.) boosted three and five weeks later with spores (1 X 10^9 CFU) of either PK118 (*thyA::*Δ *thyB::*Δ, no antigen expression) spores (Group 3) or a 1:1:1 mixture of PK120 (CotB-RBD), PK122 (CotC-HR1-HR2), PK128 (CotB-RBD) spores (referred henceforth as PK-S+N) (Group 4). Each intranasal administration (10μl total/animal, 5 μL/nare, no anaesthesia) consisted of three administrations given on consecutive days (Boost 1, days 21, 22, 27 and Boost 2, days 35, 36, 37).

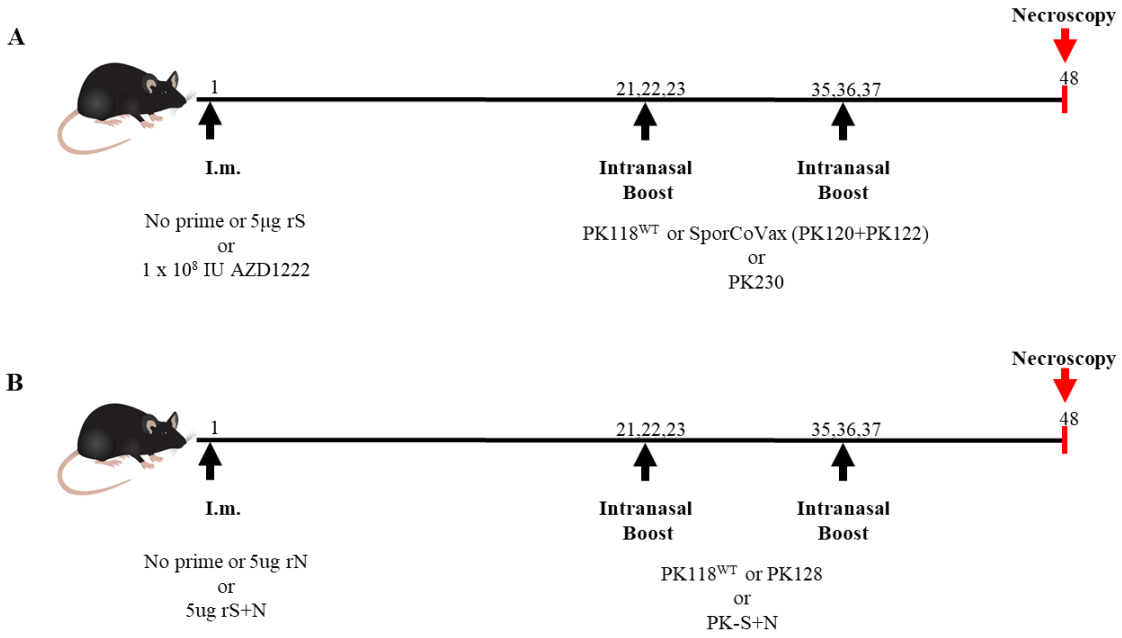

Figure 2.3. Schematic representation of the prime-boost experimental design. (A) BALB/c female mice (age 8-10 weeks old), were allocated into groups n=5-6 for recombinant spike prime and n=6 for AZD1222). Mice were primed with rS protein intramuscularly (i.m.) or AZD12222 followed by two intranasal boosts with 1 x 10^9 spores of PK118 (no antigen expression), SporCoVax (1:1 mixture of PK120 (CotB-RBD^{wuh}) and PK122(CotC-HR1-HR2^{wuh}) or PK230 (CotB-RBD^{omi}) three and five weeks post-prime. **(B)** BALB/c female mice (age 8-10 weeks old), were allocated into groups n=5-6. Mice were primed with recombinant nucleocapsid (rN) protein or rN+S (i.m). Followed by two intranasal boosts with 1 x 10^9 spores of PK118 (no antigen expression) or PK128 (CotB-NC) or PK-S+N (a 1:1:1 mixture of PK120, PK122 and PK128) spores three and five weeks post-prime. For all immunisation studies saliva and serum samples were collected (days 0, 20, 34 and 49) and antigen-specific antibodies were determined by ELISA. All mice were sacrificed 48 days post-prime and spleens were harvested for cytokine analysis.

2.13 Homologous immunisations to *H. pylori* infection using spores

C57 BL/6 female mice obtained from Charles River, UK (9 weeks of age) were housed in groups (n = 6) and were used for the immunity studies. The intragastric (i.g.; 0.2 mL) dosing regimen is shown in Figure 2.4. Groups included group 1 (naive), dosed with PBS; group 2, dosed with PK118 (*thyA::*Δ *thyB::*Δ, no antigen expression) spores; group 3,

dosed with PK78 (CotB-UreB^{CT}) spores and group 4, dosed with PK82 (CotB-UreA) spores. The dosing regimen consisted of 4 doses with each dose corresponding to three daily administrations (i.g.) of 0.2 ml (PBS or spore vaccine). For Groups 2-4 each i.g. administration consisted of 1 × 10^{10} spore CFU and daily administrations were used to reduce the viscosity of the i.g. inoculation. Samples of faeces were taken on days -1, 15, 31, 46 and 61 and serum was taken on day 62.

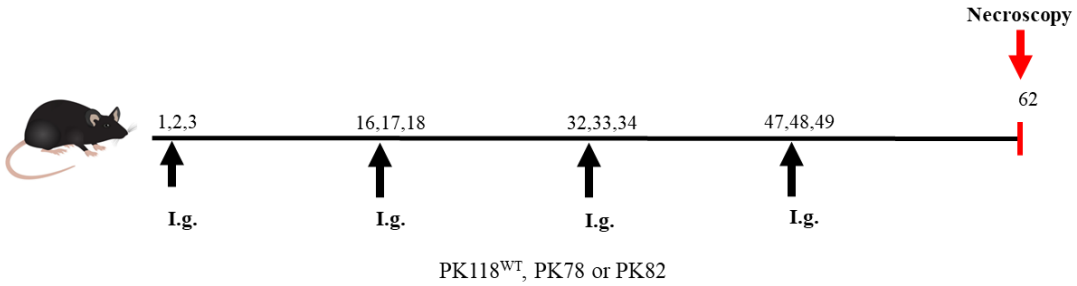

Figure 2.4. Schematic representation of the intragastric dosing experimental design. C57 BL/6 female mice (age 9 weeks old), were allocated into groups n= 6. Mice were immunised by oral route with suspensions of either spores expressing CotB-UreB (PK78) or CotC-UreA (PK82 or control spore (PK118 *thyA::*Δ *thyB::*Δ). Oral immunisations were performed with 1.0 × 10^{10} spores in a volume of 0.2 ml of water administered by intragastric lavage on days 1, 2, 3, 16, 17, 18, 32, 33, 34, 47, 48 and 49. Animals were sacrificed on day 62, and serum samples were collected. Faeces were collected throughout.

2.14 Determination of mouse antibody titers by indirect ELISA

For analysis of immunological responses, saliva was collected on days -1, 20, 34 and 48, serum was taken on day 48 and lungs were collected on day 48 and stored at -80°C. Faecal samples were collected on days -1, 15, 31, 46 and 61. For lungs and faeces, sample extractions were made at a one-fifth (w/v) dilution in extraction buffer (2% (v/v) fetal calf serum) containing protease inhibitors, EDTA (0.05 mg/mL), as previously described (Smeekens *et al.*, 2020). Saliva extractions were made at a one-tenth (w/v) dilution in

PBS. Samples were gently shaken for 2 h at 4°C to disrupt solid material, centrifuged (8000 x g, 15 min.) and the supernatant was used for analysis. Antibody levels in saliva (sIgA), lungs (IgA), faeces (sIgA) and serum (IgG) were quantified by indirect enzyme-linked immunosorbent assay (ELISA). Greiner 96-well plates (MaxiSorp) were coated with 2 µg/mL (50 µl/well) of either rS^{Wuh} or rS^{Omi} or rN or 8 µg/mL of either rUreA (residues 1-237) or rUreB (residues 365-568) (supplied by Professor Graham Christies, Cambridge) in PBS overnight at 4°C, followed by blocking for 1h at RT with PBS containing 2% (w/v) bovine serum albumin (BSA).

Saliva and faecal samples were diluted 1:20 in PBS. Lung and serum samples were diluted 1:10 and 1:1,000, respectively, in diluent buffer (0.01M PBS, 1% (w/v) BSA, 2% (v/v) FBS, 0.1% (v/v) Triton X-100, 0.05% (v/v) Tween 20). Samples were added to plates and 2-fold serially diluted. Plates containing saliva, lung and faecal samples were incubated for 2 h at 30°C and those containing serum samples were incubated for 2 h at RT. Levels of IgA and IgG were detected using the appropriate horseradish peroxidase-conjugated anti-mouse IgA (Sigma Cat No. A4789-1) or anti-mouse IgG (Dako Cat No. P0447) in conjugate buffer (2% (v/v) FBS, 1% (v/v) BSA, 0.05% (v/v) Tween 20 in 0.01 PBS). Plates were incubated for 1 h at RT and then developed using tetramethyl benzidine (TMB) substrate (0.1 mg/mL 3.3',5.5'-tetramethylbenzidine in 0.1 M sodium acetate buffer (pH 5.5)). Reactions were stopped using 2 M H_2SO_4, and ODs were read at 450 nm. Dilution curves were created for each sample and endpoint titers were estimated as the maximum dilution that gave an absorbance reading above the average naïve sample.

2.15 Isolation of mouse splenocytes and cytokine quantification

Mouse spleens were harvested, homogenised, and treated with ACK lysis buffer (Sigma Cat. No 11814389001) to remove erythrocyte contamination. Splenocytes were cultured at 1×10^6/well in 96-well plates. Cells were stimulated with 2.5 μg/mL of rS^{Wuh} or rN^{wuh} for 72h. IL-2, IL-5, IL-6 and TNF-α in culture supernatants were measured using an 8-plex multiplex immunoassay (Biolegend Cat No. 741053) according to the manufacturer's instructions. Data was acquired using a BD FACS Canto, and analysis was performed using Legendplex software (Biolegend).

2.16 Heterologous prime-boost immunisations in hamsters and SARS-CoV-2 challenge

Hamster studies were conducted by our collaborators at the Institut de Recerca i Tecnologia Agroalimentàries (Professor Joaquim Segales). Golden Syrian hamsters (age 6-8 weeks, 80-120g) were allocated into groups (50% male, 50% female). Group 1 (n=6) was a naive, 'unchallenged', control group. Animals were dosed with PBS buffer on day 1 (i.m.), day 22 (i.n.) and day 35 (i.n.) and culled on day 49. Group 2 (n=12) was a negative control group, 'unimmunised' but challenged with SARS-CoV-2 on day 42 and culled on day 49. Group 3 (n=12) was primed (i.m.) on day 1 with rS^{Wuh} (10 μg, formulated 1:1 with the AddaVax adjuvant) and then boosted (intranasal) on days 22 and 35 with SporCoVax (a 1:1 mixture of CotB-RBD^{Wuh} and CotC-$HR1\text{-}HR2^{Wuh}$ spores at 2.5 X 10^9 CFU) after which animals were challenged with SARS-CoV-2 on day 42 and culled on day 49. Group 4 (n=12) was primed (i.m.) on day 1 with rS+rN (10 μg each, formulated 1:1 with the AddaVax adjuvant) and then boosted (intranasal) on days 22 and

35 with PK-S+N (a 1:1:1 mixture of PK120 (CotB-RBD), PK122 (CotC-HR1-HR2), PK128 (CotB-RBD)) spores at 2.5 X 10^9 CFU after which animals were challenged with SARS-CoV-2 on day 42 and culled on day 49. For intranasal administrations, a total volume of 0.1mL/dose (50 μL/nare) was used and procedures were conducted under anaesthesia (isofluorane).

SARS-CoV-2 Cat02 isolate (variant D614G, ID EPI_ISL-47147) was used for the challenge. It was isolated from a patient in a laboratory-confirmed COVID-19 case in Barcelona, Spain and propagated in Vero E6 cells (ATCC CRL-1586). The inoculum was prepared on the same day of the challenge at a concentration of 10^5 $TCID_{50}$/mL and kept refrigerated until use. 0.1 mL was administered per challenge dose (50 μL/nare under isofluorane anaesthesia) giving a challenge dose of 10^4 $TCID_{50}$. At 2 and 4 days post-challenge, 4 animals/group were sacrificed for necropsy and at day-7 all remaining animals were culled. Experiments with SARS-CoV-2 were performed at the Biosafety Level-3 (BSL-3) facilities of the Biocontainment Unit of IRTA-CReSA (Barcelona, Spain). The dosing and challenge regimen is shown in Figure 2.5.

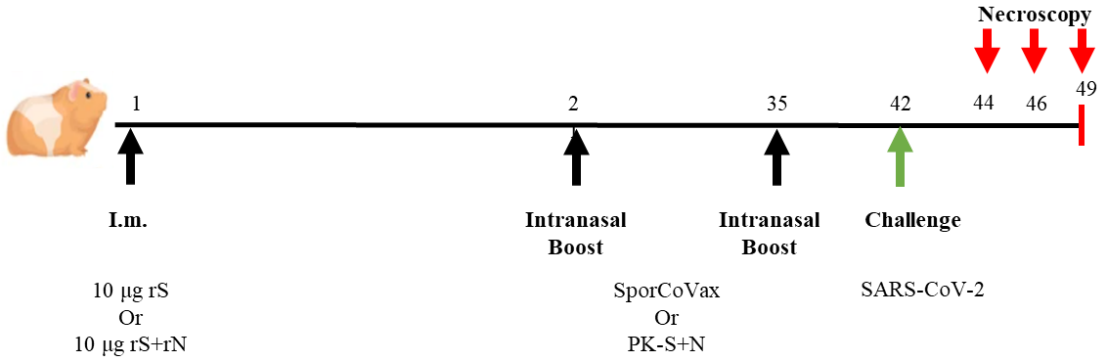

Figure 2.5. Schematic representation of the experimental design and challenge with SARS-CoV-2. Golden Syrian Hamsters (age 6-8 weeks, 80-120g) were allocated into groups (50% male, and 50% females) n=6-12 mice). Hamsters were primed with rS or a combination of rS+N subunit protein (i.m.) followed by two intranasal boosts with 2.5 x 10^9 spores of SporCoVax (1:1 mixture of PK120 (CotB-RBD^{wuh}) and PK122(CotC-HR1-HR2^{wuh}) or PK-S+N (a 1:1:1 mixture of PK120, PK122 and PK128 (CotB-N) three and five weeks post-prime. Seven days after the final boost animals were intranasally challenged with (SARS-CoV-2 (D164G variant). Animals were euthanised (2, 4 and 7 days post-challenge), necropsies were performed and viral load of SARS-CoV-2 were determined by qPCR in nasal, oropharyngeal and lungs.

2.17 Homologous immunisations in mice and *H. pylori* challenge

Challenge studies were conducted by our collaborators at the Institute of Vaccines and Biological Medicals (Dr Van Duy Nguyen). Mice (Mlac: ICR, males, 5-6 weeks of age, 18-20 g) were used for this study. Dosing schedules are shown in Figure 2.6 and consisted of 4 oral (i.g.) doses on days 0, 14, 28 and 53. Four groups (Group; n=6) were used; Group 1, naïve receiving sterile PBS, Gp2, PK118 (WT) spores, Group 3, PK82 (*cotB-ureA*) and Group 4, PK78 (*cotB-ureB*^{CT}). i.g. dosing consisted of 0.2 mL of either PBS (Group 1) or spores (1×10^{10} CFU) (Groups 2-4). On days 7-9 following the last dose animals were challenged daily with 0.2 mL/day of freshly grown *H. pylori* HP34 culture qualified by

OD_{600} measurements to contain ~ 10^8 *H. pylori* CFU. Stomach samples were taken on day 83 to enumerate *H. pylori* CFU by plating on HBA.

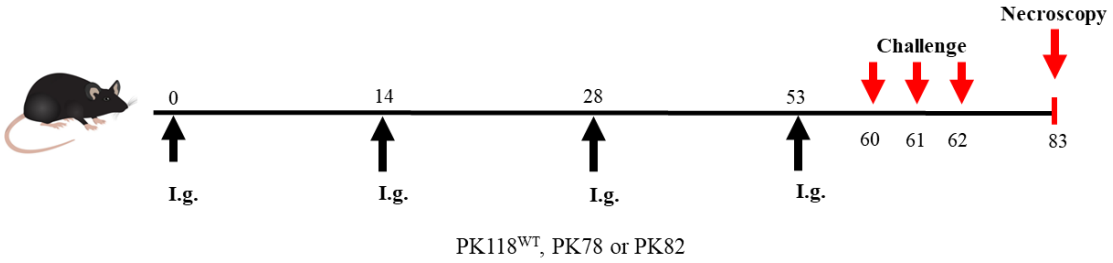

Figure 2.6. *Helicobacter pylori* challenge study: Mice (Mlac: ICR) were immunised by the oral route (i.g.) with suspensions of either spores expressing PK82 (CotB-UreA), PK78 (CotB-$UreB^{CT}$) or PK118 (control spores). A naive, non-immunised control group was also included. Oral immunisations were performed with a volume of 0.2 ml of 1.0×10^{10} spores on days 0, 14, 28, and 53. Mice were then challenged with *H. pylori* HP34 on days 60, 61 and 62. Samples of stomach were taken on day 83 to enumerate *H. pylori* CFU.

2.18 Ethical approval

Murine studies were conducted with approval from the Royal Holloway University of London Ethics Committee and an approved UK Home Office animal project license PB9FA6ABB.

Hamster studies were approved by the Institutional Animal Welfare Committee of the *Institut de Recerca i Tecnologia Agroalimentàries* (CEEA-IRTA, registration number CEEA 262/2021) and by the Ethical Commission of Animal Experimentation of the Autonomous Government of Catalonia (registration number CEA-OH/11579/2) and conducted by certified staff. Experiments with SARS-CoV-2 were performed at the Biosafety Level-3 (BSL-3) facilities of the Biocontainment Unit of IRTA-CReSA (Barcelona, Spain).

Challenge studies were conducted with approval from the Research and Ethics Committee of the Institute of Vaccines and Biological Medicals (IVAC) (decision no. 241/QD-VXSPYT 29/07/2022.

2.19 Statistical analysis

Statistical significance was assessed by the Mann-Whitney U test or the Dunnett's test using Prism (GraphPad or Dotmatics). Flow cytometry data were analysed using LEGENDplex™ Data Analysis Software.

CHAPTER 3: Construction of spore vaccines using THY-X-CISE® biocontainment system

3.1 Introduction

Bacillus subtilis is a widely recognised and intensively studied bacterium for its exceptional use in the industrial production of enzymes, specifically proteases and amylase (Mukhtar & Ikram-ul-Haq, 2012). It has also been explored as a platform for spore-based vaccines and as a live-cell host for the delivery of foreign proteins (Harwood, 1992; Potot *et al*., 2010; Wong, 1995). Three aspects of *B. subtilis* are notable. The first is that *B. subtilis* has a good safety record and is considered safe for human and animal consumption in the form of a probiotic (de Souza *et al*., 2014; Oggioni *et al*., 2003). The second is that *B. subtilis* is readily adaptable to genetic modification, and stable constructs can be integrated into the bacterial chromosome, making *Bacillus* a suitable host for vaccine engineering. Furthermore, the development of appropriate genetic tools has bought a further interest in the utilisation of GM *Bacillus* as a host for the expression and surface display of recombinant proteins with pharmacological and immunological activities (Isticato *et al*., 2001; Iwanicki *et al*., 2014; Ning *et al*., 2011; Potot *et al*., 2010). The third is the production of spores, sporulation is a cell differentiation process that occurs in *B. subtilis* following exposure to environmental stresses such as starvation, resulting in a metabolically dormant cell type. Several studies have demonstrated and confirmed that the utilisation of spores as a live-cell host for the expression and surface display of recombinant immunogenic antigens fused to outer coat proteins can elicit an antigen-specific immune response, which is needed for long-lasting immunity (Ciabattini

et al., 2004; Hoang *et al.*, 2008; L. Li *et al*., 2009; Ning *et al*., 2011; Permpoonpattana *et al*., 2013; Sung *et al*., 2022).

Despite the potential use of recombinant *Bacillus* spores for industrial and biotechnological applications, there are several risks associated with the release of GM *Bacillus* spores into the environment. First, is the use of antibiotic resistance (Ab^R) genes as selectable markers for the selection of positive transformants. To date, the majority of genome-editing techniques available for inserting heterologous DNA into the chromosome of *B. subtilis* consist of plasmid vectors that carry antibiotic-resistance genes as positive selection markers, thereby increasing the risk of spreading antibiotic resistance genes into the environment (Fels *et al*., 2020; A.-M. Guérout-Fleury *et al*., 1996; Mignon *et al*., 2015; Shimotsu & Henner, 1986; Vandermeulen *et al*., 2011). Second, is spore formation, *Bacillus* generates a cell type that is dormant and once released can survive indefinitely in the environment and their resilience to environmental assaults increases their likelihood of persisting after deliberate release (Cano & Borucki, 1995; Nicholson *et al*., 2000).

The THY-X-CISE cloning system was developed by Hosseini *et al* as a strategy to prevent the environmental dissemination of engineered *B. subtilis* spores (Hosseini *et al*., 2018). This concept of biological containment is similar to methods described for *Lactococcus lactis* (Steidler *et al*., 2003) that rely on indigenous suicide resulting from thymineless death, and has been used to enable clinical evaluation of *L. lactis* IL-10 delivery system. Neuhard *et al*., (1978) highlighted the absolute requirement for thymine in *B. subtilis*, the expression of two thymidylate synthase enzymes (TSaseA and TSaseB) is dependent on two functional unlinked *thyA* and *thyB* genes present in the chromosome

of *B. subtilis* (Neuhard *et al*., 1978). Thymidylate synthases play an essential role in the folate biosynthetic pathway which is important for the synthesis of thymine (a pyrimidine) and is required for DNA synthesis and repair (S.-W. Choi & Mason, 2000). Briefly, tetrahydrofolate (THF) is an essential cofactor, a product that is formed from the conversion of dihydrofolate (DHF) by dihydrofolate reductase enzyme (DHFR) (for more detail see Figure 3.1). It acts as the carrier for one-carbon groups, necessary for many biosynthetic pathways such as amino acid and nucleic acid metabolism (Chatterjee *et al*., 2008; Neuhard *et al*., 1978; O'Donovan & Neuhard, 1970). Thymidylate synthases are responsible for catalysing the tetrahydrofolate-dependant methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP; a pyrimidine nucleotide composed of thymine, deoxyribose and a phosphate group), which is an important rate-limiting step in DNA synthesis. THY-X-CISE® cloning system involves the insertion of fusion genes into two thymidylate synthase genes, *thyA* and *thyB* of *B. subtilis*, using a two-step transformation process (Hosseini *et al*., 2018). This system has been used to engineer thymine auxotrophic *Bacillus* strains that display chimeric proteins (passenger protein fused to *B. subtilis* spore coat protein) (Hosseini *et al*., 2018). Examples of proteins that have been successfully displayed on the surface of thymine auxotrophic strains include VP26 and VP28 which are envelope proteins of the shrimp virus WSSV (A. T. V. Nguyen *et al*., 2014; Valdez *et al*., 2014), as well as subtilisin E (AprE) and and alpha-amylase (AmyE), which are enzymes of industrial importance and commonly incorporated in animal feed (Hosseini *et al*., 2018; Tran *et al*., 2010). The resulting strain is unable to produce thymidylate synthase and the cell becomes dependent on exogenous thymine, and its absence initiates cell death (Hosseini *et al*., 2018; Rolfe, 1967). Most importantly, thymine-requiring mutants readily develop resistance to the

folate antagonist trimethoprim in the presence of exogenous thymine (Hosseini *et al*., 2018; Neuhard *et al*., 1978). Trimethoprim (TMP) is a potent antimicrobial agent that targets the folate pathway by acting as a competitive inhibitor of the dihydrofolate reductase (DHFR) enzyme which catalyses the formation of tetrahydrofolate (THF), as previously mentioned (Figure 3.1). This naturally occurring trimethoprim-resistant phenotype following the inactivation of thymidylate synthase genes enables the selection of *thyA thyB* insertion mutants based on higher levels of trimethoprim in the presence of exogenous thymine, but without introducing antibiotic resistance genes into the genome (Hosseini *et al*., 2018; Neuhard *et al*., 1978; O'Donovan & Neuhard, 1970; Sasaki *et al*., 2004). This phenomenon has been observed in many thymine auxotrophic bacteria including (but not limited to) *L. acidophilus* (Fu & Xu, 2000), *E. coli* (Okada *et al*., 1960; Rengarajan *et al*., 2004) and *Streptococcus thermophilus* (Sasaki *et al*., 2004).

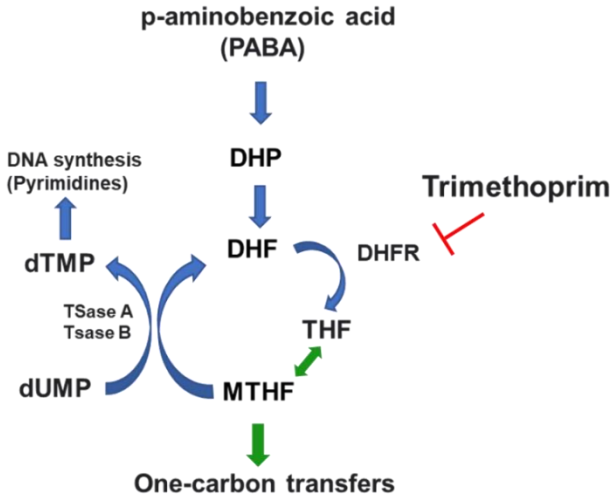

Figure 3.1: Folate biosynthesis pathway and site of action of trimethoprim. Thymidylate synthase catalyses the conversion of dUMP to dTMP, which is required for DNA synthesis. This methylation reaction uses 5,10-methyleneTHF and generates DHF. DHF is converted back to THF by DHFR. Subsequently, the intracellular stores of THF derivatives are replenished and play an important role as the source of one-carbon donors require for the synthesis of thymidylate, purines and other amino acids. Trimethoprim selectively inhibits DHF which blocks the biosynthesis pathways of thymidylate, purine and several other amino acids. PABA, para-aminobenzoic acid; DHP, dihydropteroate; DHF, dihydrofolate; DHFR, dihydrofolate reductase; THF, tetrahydrofolate; MTHF, 5,10-methylene tetrahydrofolate; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate. Modified from (Rengarajan *et al*., 2004).

3.2 Results

3.2.1 Construction of pThyA::CotB and pThyA::CotC plasmid

The pThyA and pThyB plasmids were designed by Hosseini *et al.* (2018). Briefly, both plasmids carry the relevant left and right homology arms of *B. subtilis thyA* or *thyB* which are interrupted at the midpoint by a multiple cloning site (MCS) to allow for the insertion of heterologous DNA. Most importantly, both plasmids when linearised can be integrated into the *B. subtilis* genome via a double crossover recombination event, and this is made possible by the presence of the left and right homology arms of *B. subtilis thyA* or *thyB*

(Hosseini *et al*., 2018). In this chapter, pThyA underwent further modification and two new plasmids were constructed referred to as pThyA::CotB and pThyA::CotC. Both plasmids were synthesised by inserting *cotB* and *cotC* (encoding the spore coat anchor proteins required for the display of heterologous protein) into the MCS of the pThyA plasmid (for more detail see Section 2.3, Figure 2.1). The modified pThyA::CotB or pThyA::CotC plasmid allows in-frame fusions to be conducted directly in the plasmid through the fusion of the *cotB* or *cotC* to the open reading frame (ORF) of the relevant antigens.

3.2.2 Construction of recombinant thymine auxotrophic *B. subtilis* strains

Hosseini *et al*., (2018) proof of principle studies address the biological containment of GMO *B. subtilis* by utilising the concept of thymineless death. The results from this study highlight the potential utility of the THY-X-CISE cloning system to generate thymine auxotrophic *B. subtilis* strains for application as live vaccine vectors.

The THY-X-CISE cloning system was employed to construct multiple thymine auxotrophic *B. subtilis* strains. This was achieved by inserting chimeric genes in the two thymidylate synthase genes (*thyA* and *thyB*) using a two-step transformation process. In each case, the expression had been achieved by C-terminal fusion to a *B. subtilis* gene encoding a surface-expressed spore coat protein (either CotB or CotC). The immunogenic antigens selected in this chapter were based on a wide range of data available from bioinformatic, immunological and clinical studies and are mentioned below. I) The receptor binding domain (RBD^{Wuh}, amino acids 319-350, MW 25.64 kDa) of Spike protein from SARS-CoV-2 Wuhan-Hu-1 and RBD^{Omi} (amino acid 316-538, MW 24.6

kDa) from SARS-COV-2 Omicron variant. Previous studies have shown that when administered intranasally, RBD elicits a robust local and systemic humoral immunity as well as a strong potent neutralising antibody response (Y. Du *et al.*, 2021; Jearanaiwitayakul *et al.*, 2021). II) heptad repeat 1 (HR1) and heptad repeat 2 (HR2) (HR1-HR2^{Wuh}, amino acids 920-1201, MW 31.2 kDa) of spike protein from SARS-CoV-2 Wuhan-Hu-1 variant. HR1-HR2 when administered via the parental route has been shown to elicit potent cross-neutralising antibodies against SARS-CoV-2 and its variants of concerns (VOCs) (H. Liu *et al.*, 2021; Pang *et al.*, 2022). III) Nucleocapsid (NC, amino acid 1-422, MW 46 kDa) from SARS-CoV-2 Wuhan-Hu-1 variant. (Hajnik *et al.*, 2022) Hajnik *et al.*, 2022 showed that intramuscular delivery of an mRNA vaccine expressing the N protein of SARS-CoV-2 conferred protection in a hamster SARS-CoV-2 model of infection. IV) Urease subunit A (UreA, amino acids 1-237 MW 26.4 kDa)); V) carboxy-terminus of urease subunit B (UreB^{CT}, amino acids 365-568, MW 22.9 kDa) from *H. pylori*. Both UreA and UreB have been shown to confer protection when delivered orally (Corthésy-Theulaz *et al.*, 1998; Gómez-Duarte *et al.*, 1998a; Smythies *et al.*, 2005; Z. Zhou *et al.*, 2017a). In this chapter, a truncated urease B lacking the amino-terminal enzymatic domain was used (Jabri *et al.*, 1995; Shin *et al.*, 2018). Using pThyA::CotB or pThyA::CotB vectors, in-frame fusions of the *cotB* and *cotC* genes to the open reading frame (ORF) of RBD^{Wuh}, RBD^{Omi}, HR1-HR2^{Wuh}, NC, UreA, UreB^{CT} (Appendix B) were performed directly in the vectors. The pThyA insertion gene cassettes were next linearised and used to transform competent cells of the wild-type prototrophic *B. subtilis* strain (PY79) with selection for trimethoprim resistance on plates supplemented with trimethoprim (3 μg/ml) and thymine at 37°C. Transformants carrying *thyA* insertions could be recognised by their failure to grow at 46°C without thymine (data not shown) as

seen in *thyA* mutant strains constructed by Hosseini *et al*. In the second step, classical DNA-mediated transformation of competent cells from step one (strains lacking thyA) was used to introduce an empty pThyB plasmid (without a chimeric gene) at the *thyB* locus using selection with a higher concentration of trimethoprim (~200 μg/ml) (see method Section 2.4 for more detail). Strains carrying insertions at *thyA* and *thyB* were verified by their failure to grow at both 37°C and 46°C in the absence of thymine (Table 3.1). Using the two-step ectopic cloning system, several strains carrying insertions at the *thyA* and *thyB loci* were constructed.

The resulting strains include, PK78 (*thyA::cotB-ureB*^{CT} *thyB*Δ), PK82 (*thyA::cotB-ureA thyB*Δ), PK120 (*thyA::cotB-RBD*^{Wuh} *thyB*Δ), PK122 (*thyA::cotC-HR1-HR2*^{Wuh} *thyB::*Δ), PK128(*thyA::cotB-N thyB*Δ) and PK230 (*thyA::cotB-RBD*^{Omi} *thyB::*Δ). An isogenic strain, PK118 (*thyA::*Δ *thyB::*Δ), which carries no gene insertions was made using the same procedure by introducing empty pThyA and pThyB vectors into *thyA* and *thyB* loci (Table 3.1). After constructing each strain as listed in Table 3.1, the integrity of the *thyA* and *thyB* insertion was further confirmed by polymerase chain reaction analysis (data not shown).

Table 3.1: Phenotype of *B. subtilis* recombinant strains

Strain	Genotype	37°C[1]		46°C[1]	
		+ thy	- thy	+ thy	- thy
PY79	*thyA*$^{+}$ *thyB*$^{+}$	+	+	+	+
PK118	*thyA::*Δ *thyB::*Δ	+	-	+	-
PK78	*thyA::cotB-ureB* *thyB::*Δ	+	-	+	-
PK82	*thyA::cotB-ureA* *thyB::*Δ	+	-	+	-
PK120	*thyA::cotB-RBD*^{wuh} *thyB::*Δ	+	-	+	-
PK122	*thyA::cotC-HR1-HR2* *thyB::*Δ	+	-	+	-
PK128	*thyA::cotB-NC* *thyB::*Δ	+	-	+	-
PK230	*thyA::cotB-RBD*^{omi} *thyB::*Δ	+	-	+	-

[1]Growth (+) or no growth (-) on SMM agar with or without thymine (50 μg/mL)

3.2.3 Expression of heterologous genes on the surface of *B. subtilis* spores

The expression and display of chimeric proteins on the spore surface was confirmed by two immunological methods. First, Western blotting of proteins from recombinant spore coat extracts using polyclonal (PAbs) or monoclonal (MAbs) antibodies that recognise RBD$^{(wuh)}$, RDB$^{(omi)}$, HR1-HR2, NC antigens of SARS-CoV-2 and UreA and UreB antigens of *H. pylori* was performed.

Using SARS-CoV-2 Wuhan-specific anti-S^{Wuh} PAb on protein extracted from PK120 (CotB-RBD^{wuh}, Figure 3.2A**)** and the use of SARS-CoV-2 Omicron-specific anti-S^{Omi} Mab on protein extracted from PK230 (CotB-RBD^{Omi}, Figure 3.2B), it was observed that

the fusion proteins were rather degraded with the blot revealing several bands corresponding to ~40, (diffuse band), ~50 and ~60 kDa that were absent in protein extracted from the isogenic WT strain referred henceforth as PK118 spores, which served as the control. These bands appear to be the result of adsorption of aggregated and or degraded fusion protein on the spore surface (Rabilloud, 1996). One of these bands was in close agreement with the predicted size (62 kDa) of the CotB-RBD^{wuh} chimaera (PK120) and (63 kDa) of the CotB-RBD^{Omi} chimaera (PK230). PK122 (CotC-HR1-HR2) spore coat extracts when probed with Wuhan-specific anti-S^{Wuh} PAb revealed a single band (absent in PK118 spores) with the predicted size (40 kDa) of the CotC-HR1-HR2 chimaera (Figure 3.2A). PK122 is the only strain which carries the HR1-HR2 domain on the C-terminus of CotC. A strain called PK123 (CotB-HR1-HR2) was constructed, in which the HR1-HR2 domain was fused to the C-terminus of CotB. However, upon perfoming Western blot analysis of the spore coat extracts from PK123, no observable bands were detected that corresponded to CotB-HR1-HR2 chimaera (blot not shown). According to the literature, it is known that CotB-based fusions can show genetic instability (Isticato *et al*., 2004); and this is possibly due to the structure and folding of the heterologous protein (passenger protein) and the properties of the fusion protein on the spore surface (Negri *et al*., 2013). Negri *et al*., (2013), showed that the use of a helical peptide linker (GGGEAAAKGGG) in between the passenger protein (FliD) and CotB protein resulted in higher levels of expression of the CotB–linker–FliD fusion protein in the spore coat compared to CotB-FliD fusion protein (no peptide linker). In our study, it is possible that the insertion of a peptide linker between CotB and HR1-HR2 could have resulted in expression or higher levels of expression, however, this was not investigated further. Western blotting of PK128 (CotB-N) showed that the whole N protein was

expressed on the spore surface with the predicted size (84 kDa) of the CotB-N chimaera. Proteins extracted from spores of the isogenic WT strain (PK118, served as the control) devoid of SARS-CoV-2 antigens exhibited no cross-reaction (Figure 3.2A-C).

Western blotting of PK82 (*cotB-ureA*) revealed three bands (~40, (diffuse), 65 and 70 kDa.) that were absent in PK118 spores when probed with anti-UreA-specific PAb. One of these bands was in close agreement with the predicted size (63.4 kDa.) of the CotB-UreA chimaera (Figure 3.2D). It is possible that the larger band observed (70 kDa) corresponding to the higher molecular weight, is most likely representing oligomerised forms of CotB itself. Western blotting of PK78 (*cotB-ureB*^{CT}) was less clean with cross-reacting bands in PK118 spores when probed with anti-UreB-specific PAb. However, one abundant band of the correct size for CotB-UreB (~61 kDa) was present in PK78 and absent in PK118 spores (Figure 3.2E). Since the cross-reacting bands (observed when probed with anti-UreB PAb) are associated with the spore coat the most likely explanation is that of cross-recognition with spore coat proteins. However, using several bioinformatic tools including Basic Local Alignment Search Tool (BLAST) and FASTA, no spore coat proteins were found to display any degree of amino acid homology with either urease A or B. It should be noted that a tricistronic urease operon (*ureABC*) is present in most strains of *B. subtilis* with *ureC* corresponding to the enzymatic subunit in *H. pylori* named *ureB* (Cruz-Ramos *et al*., 1997). *B. subtilis* UreA shares homology with *H. pylori* UreA (~31%) and with UreC with about 75% homology with *H. pylori* UreB. The operon is transcribed during ordinary vegetative cell growth but only at high levels during nitrogen-limited growth (Cruz-Ramos *et al*., 1997; Wray *et al*., 1997). It has been confirmed that PK118, PK78 and PK82 do not produce a functional urease using a

standard agar-based biochemical method termed Christensen's slant agar (data not shown) (Christensen, 1946). The possibility of low levels of of *B. subtilis* -produced urease being present, possibly adsorbed to spores cannot be ruled out, which could potentially account for this cross-reaction.

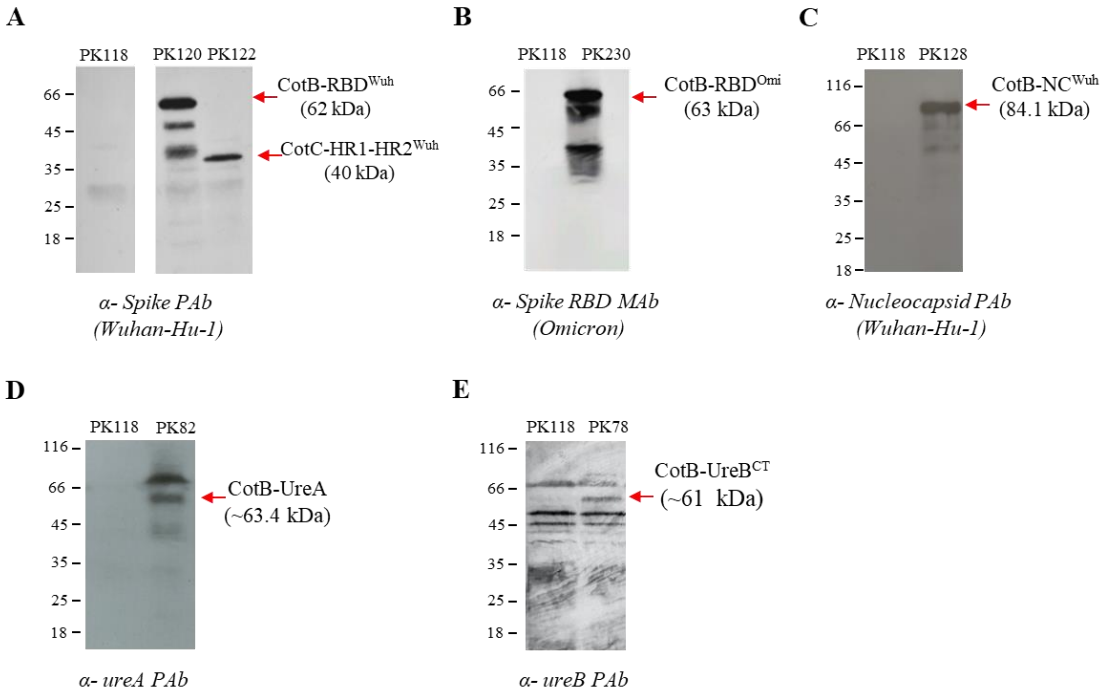

Figure 3.2. Spore coat expression of SARS-CoV-2 and *H. pylori* proteins. *B. subtilis* vaccine strains carrying insertions at the *thyA* and *thyB* loci were examined by Western blotting of SDS-PAGE size-fractionated spore coat proteins extracted from preparations of pure spores (approx. 2 X 10^9 spores/extraction). Panels show bands obtained in extracts of isogenic WT spores (PK118) and spores carrying *thyA* and *thyB* insertions, with no antigens. ***Panel A*** shows analysis of PK120 (CotB-RBD^{Wuh}) and PK122 (CotC-HR1-HR2^{Wuh}) probed with anti-S (Wuhan-Hu-1) PAbs. ***Panel B*** shows analysis of PK230 (CotB-RBD^{Omi}) probed with anti-S (Omicron) MAbs. ***Panel C*** shows analysis of PK128 (*C*otB-NC^{wuh}) probed with anti-N PAbs. ***Panel D*** shows analysis of PK82 (CotB-UreA) probed with anti-UreA PAbs. ***Panel E*** shows analysis of PK78 (CotB-UreB^{CT}) probed with anti-UreB PAbs.

The second immunological method used to further verify the expression and display of chimeric proteins on the spore surface was whole spore enzyme-linked immunosorbent assay (ELISA). Purified whole spores were subjected to ELISA. Whole spore ELISA clearly demonstrated recognition of the heterologous antigen on spores from PK120 (CotB-RBD^{Wuh}) and PK122 (CotC-HR1-HR2) in Figure 3.3A, PK230 (CotB-RBD^{Omi}, Figure 3.3B), PK128 (CotB-NC, Figure 3.3C), PK82 (CotB-UreA, Figure 3.3D) and PK78 (CotB-UreB^{CT}, Figure 3.3E). In each case, significant levels of detection were found showing that each heterologous antigen was expressed.

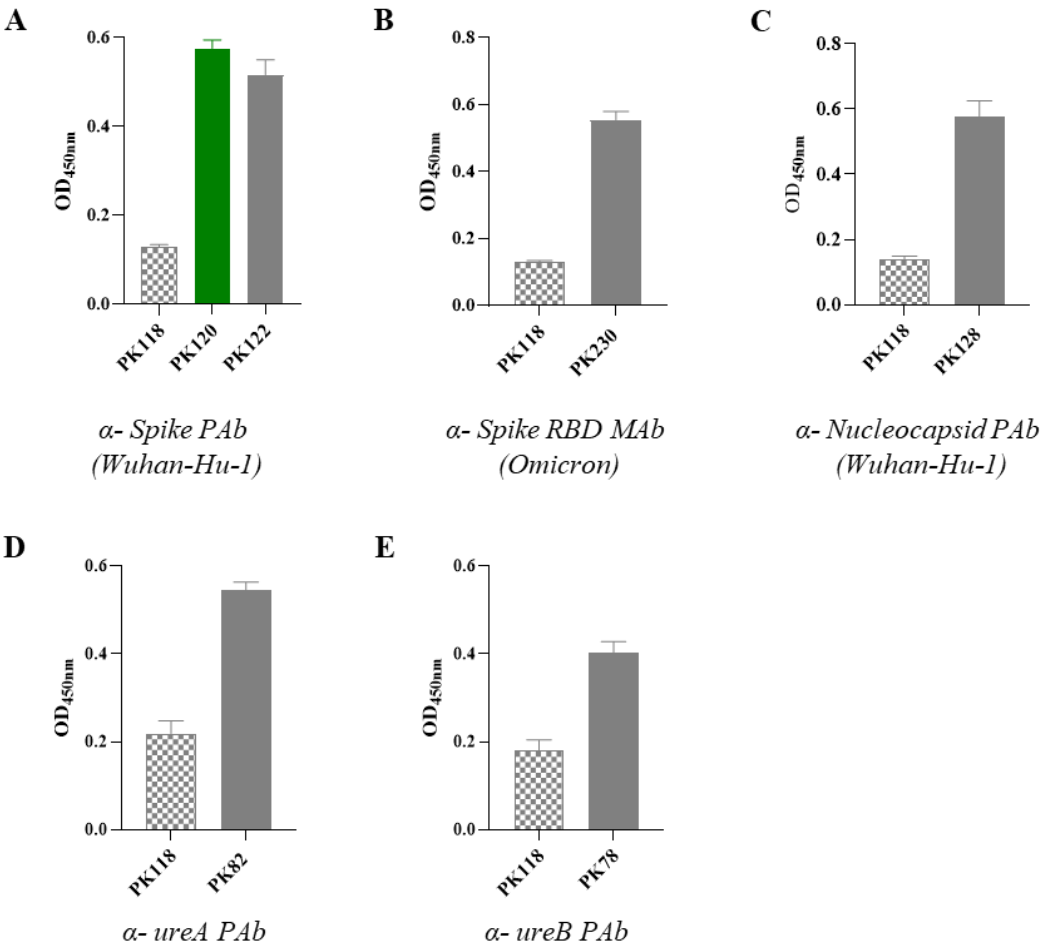

Figure 3.3 Surface expression determined by "Whole Spore ELISA". Microtiter plates were coated with spores at 1 x 10^8 CFU/well. ***Panel A***, PK118 (isogenic WT strain), PK120 (CotB-RBD^{Wuh}) and PK122 (CotC-HR1-HR2^{Wuh}) were labelled with anti-S (Wuhan-Hu-1) PAbs (1:2000) followed by anti-rabbit IgG-HRP secondary antibody (1:3000). ***Panel B***, PK118 and PK230 (CotB-RBD^{omi}) spores were labelled with anti-S (Omicron) PAbs (1:2000) followed by anti-mouse IgG-HRP secondary antibody (1:3000). ***Panel C***, PK118 and PK128 (CotB-N) spored were probed with anti-N (1:2000) polyclonal antibody followed by anti-mouse IgG-HRP secondary antibody (1:3000). ***Panel D***, PK118 and PK82 (CotB-UreA) spore probed with anti-UreA polyclonal (1:1000) antibody followed by anti-rabbit IgG-HRP secondary antibody (1:3000). ***Panel E,*** PK118 and PK78 (CotB-UreB^{CT}) and then probed with anti-UreB polyclonal (1:2000) antibody followed by anti-rabbit IgG-HRP secondary antibody (1:3000). This experiment was replicated twice.

3.2.4 Confirmation of thymineless death in sporulation media

To verify whether the *thyA thyB* insertional mutants are completely thymine dependent, growth assays of the insertional mutants were conducted at 37°C in DSM in the presence or absence of exogenous thymine. The *thyA* and *thyB* insertional mutant strains showed no growth in the absence of thymine (Figure 3.4)**.** Additionally, with thymine supplementation, all strains grew to similar OD, which was also observed in the *thyA thyB* insertional mutants constructed by Hosseini *et al.,* (2018).

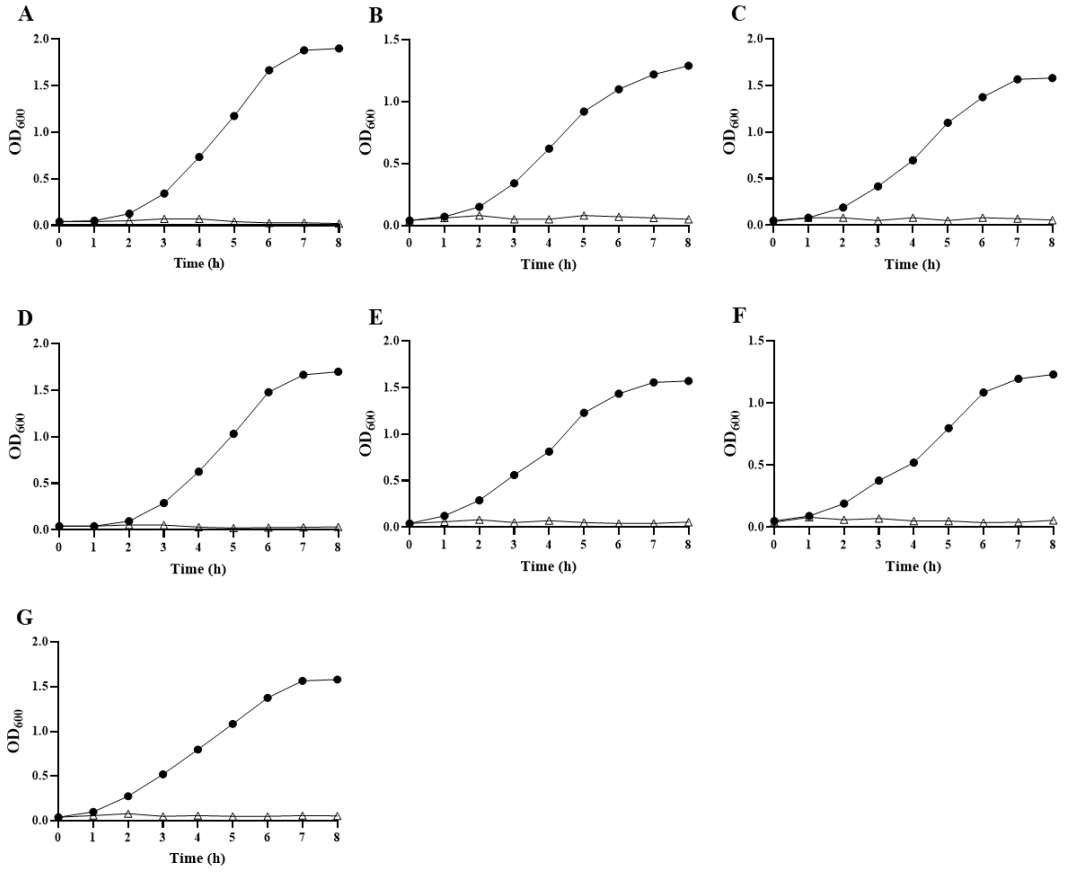

Figure 3.4. Growth of *thyA thyB* insertion mutant strains in sporulation media. **(*Panel A*)** Growth of PK118 (isogenic WT strain) **(*Panel B*)** PK120 (CotB-RBD^{wuh}), **(*Panel C*)** PK122 (CotB-HR1-HR2^{Wuh}), **(*Panel D*)** PK230 (CotB-RBD^{Omi}), **(*Panel E*)** PK128 (CotB-NC), **(*Panel F*)** PK78 (CotB-UreB^{CT}) **(*Panel G*)** PK82 (CotB-UreA) was grown in DSM media at 37°C with or without thymine supplementation (50 µg/ml). The starting OD_{600} of each culture was 0.04. Circles represent strains grown in the presence of thymine, triangles correspond to strains grown in the absense of thymine. This experiment was replicated twice.

3.2.5 Percentage sporulation of *thyA thyB* insertional mutants

Sporulation was induced by the exhaustion method in the DSM as described elsewhere (Harwood & Cutting, 1990). The sporulation efficiency of *thyA thyB* insertional mutant strains was assessed relative to the isogenic PK118 strain. After 24 h of growth in DSM, sporulation occurred, and the number of heat-resistant spores was essentially equivalent to those of PK118 (Table 3.2). The *thyA thyB* insertional mutants and their isogenic PK118 strain showed comparable efficiency, indicating that the presence of the fusion

protein did not significantly affect sporulation efficiency, which is consistent with the findings observed by Hosseini *et al.*, (2018).

Table 3.2: Percentage sporulation of *thyA thyB* insertion mutants

Strain[1]	Genotype	Total Count[2]	Heat count[3]	Sporulation %
PK118	*thyA::*Δ *thyB::*Δ	3.1 x 10^8	2.8 x 10^8	90.3
PK78	*thyA::cotB-ureB thyB::*Δ	2.8 x 10^8	2.4 x 10^8	85.7
PK82	*thyA::cotB-ureA thyB::*Δ	1.76 x 10^8	1.51 x 10^8	85.8
PK120	*thyA::cotB-RBD^{wuh} thyB::*Δ	3.1 x 10^8	2.5 x 10^8	80.6
PK122	*thyA::cotC-HR1-HR2 thyB::*Δ	3.9 x 10^8	3.4 x 10^8	87.2
PK128	*thyA::cotB-NC thyB::*Δ	2 x 10^8	1.6 x 10^8	80
PK230	*thyA::cotB-RBD^{omi} thyB::*Δ	1.8 x 10^8	1.6 x 10^8	89.8

[1] Different strains were grown for 24h in DSM supplemented with thymine (50 µg/ml); after 24h, untreated and heated (65°C, 1h) cultures were plated on appropriate plates. This experiment was replicated twice.
[2] Untreated CFU/ml
[3] Heat-treated CFU/ml

3.2.6 Reversion

Considerable evidence has shown that the integration of heterologous genes into *the* chromosome by a double crossover recombination event is considered more stable than integration based on a single crossover recombination event (Aranda *et al.*, 2010; Heap *et al.*, 2012; Kuzminov, 2011). Additionally, heterologous genes integrated into the chromosome are maintained in a single copy number even in the absence of selection markers (Middleton & Hofmeister, 2004; Vázquez-Cruz *et al.*, 1996). To demonstrate the stability of the integrations at the *thyA* and *thyB* loci, PK82 (CotB-UreB^{CT}), PK120

(CotB-RBD^{Wuh}) and PK122 (CotC-HR1-HR2) strains were selected for further analysis. To assess whether repeated culture using these strains in the absence of thymine would lead to a loss of thymine dependency. It was observed that repeated cultures of PK82, PK120 AND PK122 in the absence of thymine yielded no loss of thymine dependency, and no growth was observed in the subcultures and on DSM plates without thymine supplementation (Table 3.3). The data showed a similar trend as reported by Hosseini *et al*. Therefore, these results suggest that *thy* mutations are stable and there is no evidence to indicate reversion or suppression despite repeated passage (Hosseini *et al*., 2018).

Table 3.3: Reversion of double *thy* insertional mutants

Subculture	**PK82 (CFU/ml)[1]**		**PK120 (CFU/ml)[1]**		**PK122 (CFU/ml)[1]**	
	DSM + Thymine	**DSM**	**DSM + Thymine**	**DSM**	**DSM + Thymine**	**DSM**
1	2.12×10^8	0	1.95×10^8	0	3.6×10^8	0
2	2.50×10^8	0	2.06×10^8	0	3.1×10^8	0
3	2.08×10^8	0	3.38×10^8	0	2.25×10^8	0
4	3.08×10^8	0	1.75×10^8	0	2.04×10^8	0
5	2×10^8	0	2.42×10^8	0	2.43×10^8	0
[1]Number of bacteria (CFU/ml) every 24 h in DSM supplement with thymine and DSM only (no thymine)						

3.3 Discussion

This chapter highlights the potential utility of the newly developed THY-X-CISE cloning system (Hosseini *et al.*, 2018) to generate thymine auxotrophic *B. subtilis* strains that can be used as live-cell hosts for the expression and surface display of immunogenic proteins. Hosseini *et al* successfully adopted the principles of a thymineless death to exploit *B. subtilis* by introducing heterologous genes without the introduction of antibiotic resistance genes.

This chapter focused on the application of the principles of thymineless death and illustrated how the THY-X-CISE cloning system was used to genetically engineer *B. subtilis* to express immunogenic antigens derived from SARS-CoV-2 and *H. pylori.* The antigens were fused with anchor proteins to facilitate their display on the spore surface. The successful display of the antigens on the spore surface was deemed noteworthy, as it could potentially aid in the development of mucosal spore vaccines against SARS-CoV-2 and *H. pylori* infection. Some of the immunogenic antigens selected have been previously expressed on the spore surface using conventional cloning methods that require antibiotic-resistance gene markers for selection and do not offer biological containment. In each case, expression was achieved by fusion of the heterologous protein to *B. subtilis* surface-expressed spore coat protein. For example, Sung *et al*, constructed recombinant spores which anchored the RBD of SARS-CoV-2 to the outer spore coat proteins, CotA, CotB, and CotC. This study showed that the expression of the CotC-RBD chimaera in *B. subtilis* cells was much higher, while the CotA-RBD and CotB-RBD were not detectable. A significant increase in the level of antibodies against sRBD in both mice and humans was reported after the oral administration of spores that displayed the CotC-

RBD chimaera (Sung *et al*., 2022). Hinc *et al*, exploited *B. subtilis* to express UreA protein (produced by *H. pylori*) on the spore surface through the fusion of UreA to spore coat proteins, CotB, CotC, or CotG. This study highlighted that CotB was the most appropriate spore coat protein to display heterologous protein on the spore surface (Hinc *et al*., 2010). Urease B, when displayed on the surface of recombinant spores, has been shown to confer protection against *H. pylori* infection in mice dosed orally with these recombinant spores (Z. Zhou *et al*., 2015b, 2017a). The HR1-HR2 domain of the spike S2 subunit and nucleocapsid domain of SARS-CoV-2 have not been previously expressed in *B. subtilis* but were selected for their immunogenicity (Dangi *et al*., 2022; Hajnik *et al*., 2022; Pang *et al*., 2022). Systems such as the ones described above offer fascinating possibilities for the future use of recombinant *B. subtilis* spores for application as live vaccine vectors in humans. However, before they can be applied as live vaccines, a biocontainment strategy which prevents the spreading of GM *Bacillus* spores into the environment needs to be considered.

THY-X-CISE® system can be used to generate thymine auxotrophic *B. subtilis* strains, spores, that are bactericidal in the absence of exogenous thymine and if they germinate they are not able to replicate in the environment since thymine is not readily available. The thymineless death principle ensures biological containment and prevents the spread of a transgene by horizontal gene transfer as well as the survival of the GM spores in the environment (Hosseini *et al*., 2018). The inactivation of *thy* genes represents a mechanism of developing resistance to trimethoprim, a drug targeting folate metabolism which is essential for DNA synthesis (Neuhard *et al*., 1978). There are two possible explanations, In the first scenario, loss of thymidylate synthase activity prevents the completion of

folate pathway, possibly leading to the accumulation of DHF and other intermediates. This accumulation in turn, might lead to an increase in the activity of DHFR, which catalyses the conversion of DHF to THF (see Figure 3.1). It is possible that the increased activity of DHFR can lead to a reduction in the binding affinity of trimethoprim to DHFR, thereby resulting in increased resistance. It is worth noting that, THF is consumed largely by thymidylate synthase activity, and the loss of thymidylate synthase activity disrupts the thymine-biosynthesis stage of the folate biosynthesis pathway and the utilisation of THF derivatives in this step is abolished (Tjong *et al*., 2022). In the second scenario, loss of thymidylate synthase might lead to the accumulation of DHF, which might act as competitive inhibitor of DHFR and hinder the binding of trimethoprim to DHFR. Therefore, the cell can afford to have a relatively large fraction of its DHFR inactivated by TMP, which in turn, increases the level of resistance to trimethoprim (aminopterin) (Huovinen *et al*., 1995). It is possible that both scenarios might lead to resistance to trimethoprim, however, the exact mechanism through which loss of thymidylate synthase activity confers resistance to trimethoprim is not entirely clear. In this study, the attribute of differential resistance to trimethoprim was utilised to construct double *thyA thyB* insertion mutants using a two-step, ectopic cloning system (Hosseini *et al*., 2018). Inactivation of the *thyA* locus produced a cell with a lower level of resistance to trimethoprim (~3 µg/ml), whereas, double *thyA thyB* mutants displayed a higher level of resistance (~200 µg/ml). Furthermore, TsaseA is not temperature-sensitive and retains 90-95% of the cell activity when grown at 46°C, and inactivation of the *thyB locus* allows cells to grow at an elevated temperature (46°C). Contrary, TsaseB is temperature-sensitive, only retaining ~5-8% activity at a restrictive temperature of 46°C, and inactivation of the *thyA* locus requires supplementation with thymine for growth at 46°C.

The temperature-sensitive phenotype of *thyA thyB* insertion mutants provides an additional method that enables both the selection and screening of mutants. Inactivation of both *thyA* and *thyB* resulted in a cell with an absolute requirement for thymine for growth at both 37°C and 46°C and in the presence of trimethoprim.

Interestingly, Hosseini *et al*, stated that the introduction of a pThyB insertion cassette (containing a chimeric gene) at the *thyB* locus using the classical DNA-mediated transformation of competent *thyA* mutant cells proved inefficient and the integration frequency was low. They found that using an electroporation method to insert a pThyB insertion cassette into the *thyB* locus yielded higher frequencies of integration. In this chapter, the classical DNA-mediated transformation method was successful in introducing an empty pThyB plasmid (no chimeric gene) at the *thyB locus* in strains carrying chimeric insertions at the *thyA* locus. The introduction of an empty pThyB plasmid (smaller insertion cassette) did not affect the transformation efficiency. It is worth noting that the size difference of the pThyB plasmids used might have influenced the results. It is possible that using an empty pThyB plasmid (no chimeric gene) which is likely smaller in size, may facilate the uptake of the plasmid via classical DNA-mediated transformation, making it easier to integrate into the *thyB* locus. Various kinds of literature have shown that bacterial cells transformed with larger-sized plasmid vectors show lower transformation efficiency and maintenance of the foreign DNA (McDonald *et al*., 1995; Ohse *et al*., 1995; Szostková & Horáková, 1998).

3.3.2 Comparison of the THY-X-CISE® system to existing markerless genetic manipulation techniques

The THY-X-CISE cloning system is not the first report of a system that allows the integration of chimeric genes without introducing antibiotic resistance gene markers. Three examples are described here. First, Iwanicki *et al*., (2014) constructed suicide plasmids to display chimeric proteins on the surface of *B. subtilis*. These suicide plasmids did not contain antibiotic resistance gene markers but instead included the *thrC, trpC,* and *lysA* genes as markers for the selection of positive transformants. The integration of chimeric genes at these gene sites resulted in strains that where auxotrophic for threonine, tryptophan and lysine, respectively. As a result, recombinant strains were be selected on minimal media with or without the relevant amino acid supplementation, serving as a strong selection marker without introducing antibiotic-resistance genes in the genome (Iwanicki *et al*., 2014).

Second, site-specific recombination (SSR) is another widely used system for gene insertions into bacterial chromosomes without introducing antibiotic resistance genes. SSR is a specialised system that catalyses DNA exchange between two defined DNA recombination sites. It serves a crucial role in the resolution of chromosome and plasmid dimerisation by converting multimeric forms to monomeric forms (Bloor & Cranenburgh, 2006; F. Castillo *et al*., 2017; Sciochetti *et al*., 2001; X. Yan *et al*., 2008). Bloor and Cranenburgh., (2006) designed an effective SSR method in *E. coli* and *B. subtilis*, allowing for the chromosomal integration of genes without introducing selectable marker genes into the genome. This system utilises an insertion cassette consisting of a chloramphenicol-resistance gene marker flanked by recombination target *dif* sites and

regions homologous to the chromosomal target locus. *dif* site is located in the chromosome's replication terminus region, which is recognised by chromosomally encoded tyrosine recombinases: XerC/XerD in *Escherichia coli* and RipX/CodV in *Bacillus subtilis.* The integration of an insertion cassette into the chromosomal target locus by homologous recombination can produce chromosome and plasmid dimers, and the native XerC/XerD (*E. coli*) or RipX/CodV (*B. subtilis*) recombinases can resolve the two dif sites flanking the selectable marker gene to a single site, in turn, excising the antibiotic resistance gene (Bloor & Cranenburgh, 2006; Sciochetti *et al*., 2001).

Third, Watzlawick and Altenbuchner., (2019) adopted the Clustered, Regular Interspaced Short Palindromic Repeat (CRISPR) system with Cas9 as a targeted nuclease to integrate five copies of the *ganA* expression cassette that contains the left and right homologous arms of multiple *B. subtilis* chromosomal target loci without affecting bacterial growth. The selection of recombinant strains that have successfully integrated a gene cassette into the target loci can be achieved by Cas9-mediated counterselection, without introducing antibiotic-resistance gene markers into the genome. Cas9 nucleases are lethal to non-recombinant strains with unedited target genes because of the introduction of double-strand breaks, serving as a strong counterselection (Shuman & Glickman, 2007; Watzlawick & Altenbuchner, 2019; Westbrook *et al*., 2016).

Although several genome-editing methods have been developed that do not require antibiotic-resistance gene markers for selection, the recently developed THY-X-CISE system addresses several safety and environmental issues associated with the wide-scale dissemination of engineered *B. subtilis* spores. First, the avoidance of antibiotic resistance genes as a selection marker. This system allows heterologous genes to be integrated into

B. subtilis thyA thyB loci without introducing antibiotic resistance genes. Second, thymine auxotrophy is bactericidal when exogenous thymine is depleted, thus ensuring biological containment of GM *B. subtilis* as evidenced by Hosseini *et al*. (2018). Biological containment prevents the spread of genetic modifications to other hosts. Lastly, biological containment prevents the unintended proliferation and spreading of GM *B. subtilis* in the environment since thymine is not readily available, as a result, thymine auxotrophic strains undergo thymineless death in response to thymine starvation. (Fu & Xu, 2000; Hosseini *et al*., 2018; Steidler *et al*., 2003).

3.4 Conclusion

A THY-X-CISE biocontainment strategy was established, wherein the essential genes *thyA* and *thyB*, present in the genome of *B. subtilis*, were inactivated by inserting a chimeric gene containing an immunogenic antigen fused to the *cotB* or *cotC* into the *thyA* locus. The immunogenic antigens included RBD, HR1-HR2, and N derived from SARS-COV-2 and *ureA* and *ureB* from *H. pylori*. The THY-X-CISE system, is a two-steps transformation process. Firstly, a pThyA insertion cassette is introduced into the *thyA* locus of *B. subtilis* (PY79). Secondly, an empty pThyB insertion cassette is introduced into the *thyB* locus of the *thyA* mutant. This system allows integration by a double-crossover recombination event at the *thyA* and *thyB* locus of *B. subtilis* without introducing antibiotic resistance gene markers for positive selection, but, instead, failure to produce thymidylate synthases disrupts the folate pathway. As a result, *thyA thyB* insertion mutants readily develop increasing levels of resistance to trimethoprim. Furthermore, the temperature-sensitive phenotype of *thyA thyB* insertion mutants, together with the trimethoprim-resistant phenotype enables the selection and screening of

thyA thyB insertion mutants. Using this system, thymine auxotrophic strains were successfully constructed and shown to express various SARS-CoV-2 and *H. pylori* immunogenic antigens on the spore surface. The resulting strains include; PK78 (CotB-UreB^{CT}), PK82 (CotB-UreA), PK120 (CotB-RBD^{Wuh}), PK122 (CotC-HR1-HR2^{Wuh}), PK128 (CotB-NC) and PK230 (CotB-RBD^{Omi}). Similar to the findings of Hosseini *et al*, the data presented in this chapter demonstrates the bactericidal effects of thymine auxtrophy upon depletion of exogenous thymine. The absence of any indication of reversion and suppression strengthens the realibity of this approach. Furthermore, the cloning system employed here offers an efficient, simple and robust mechanism for biological containment.

When considering whether thymine auxotrophic spores displaying the CotB-UreB^{CT} (PK78) and CotB-UreA (PK82) would be suitable as oral vaccine candidates against *H. pylori* infection, and whether spores displaying CotB-RBD^{Wuh} (PK120), CotC-HR1-HR2 (PK122), CotB-N (PK128) and CotB-RBD^{Omi} (PK230) would be suitable as a mucosal (intranasal) booster vaccine against SARS-COV-2 infection, several factors speak in their favour. First, *B. subtilis* has a good safety record and is also used as a probiotic supplement in humans and animals. Second, spores of *B. subtilis* have been used extensively as mucosal vaccine vehicles, and oral (intra-gastric or sublingual) or nasal administration has been shown to induce strong mucosal immunity (typically sIgA) as well as a Th1 bias (Y. Du *et al.*, 2021; Hoang *et al*., 2008; Jearanaiwitayakul *et al.*, 2021; Permpoonpattana *et al*., 2011; Y. Yang *et al.*, 2018). Third, spore vaccines can be engineered and constructed rapidly, they are heat stable and can be stored indefinitely in solution or desiccated form. However, this may not necessarily apply to heterologous

proteins displayed on the spore surface. Other spore vaccines that have been generated in the Cutting lab (Duc *et al.*, 2004, 2007; Permpoonpattana *et al*., 2011) do indeed show stability, but this would need to be addressed by an extensive program of formulation and stability studies. Considered together, the use of bacterial spores as oral or intranasal vaccine vehicles are compelling.

Further experiments were conducted using (PK78) and (PK82) as a prophylactic oral vaccine against *H. pylori* infection (Chapter 5) and PK120, PK122, PK230 and PK128 (Chapter 4) as an intranasal mucosal booster following a heterologous systemic prime. The purpose was to determine whether thymine auxotrophic *B. subtilis* spores can stimulate antigen-specific systemic and mucosal immunity and whether the immune responses are sufficient enough to provide protection against SARS-CoV-2 and *H. pylori* infection. This will be addressed and discussed in more detail in the relevant chapters.

CHAPTER 4: Inducing mucosal immunity to SARS-COV-2 using a heterologous systemic prime-intranasal boost vaccination

4.1 Introduction

The coronavirus infectious disease 2019 (COVID-19) pandemic will be remembered both for its impact on global health and society and, also for the remarkable speed at which prophylactic vaccines were implemented. The spike (S) glycoprotein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the main target for vaccine design and development with the goal of producing potent neutralising antibodies (Tregoning *et al*., 2021; Zost *et al*., 2020). S is a heterotrimeric protein that plays an important role in cell entry and is composed of the S1 subunit which harbours the receptor binding domain (RBD) and the S2 membrane fusion subunit which consists of the heptad repeat 1 and 2 (HR1-HR2) (Bj *et al*., 2004; F. Li, 2016). Currently, several S-vaccines including mRNA, adenovirus vectored and recombinant protein subunit vaccines have all shown high levels of protection (>50% protection efficacy) against the most severe forms of the disease, reducing morbidity and mortality (Mouro & Fischer, 2022; WHO, 2023). On the other hand, it has become apparent that current COVID-19 vaccines are unable to prevent person-to-person transmission of its causal agent, SARS-CoV-2, with breakthrough infections observed in fully vaccinated individuals supporting this (Brosh-Nissimov *et al*., 2021; Farinholt *et al*., 2021). The dissemination of the virus will undoubtedly continue

as a) immunity wanes and b) the reality that a significant portion of the world's population (~40–45%) has yet to receive their first vaccine dose (Mouro & Fischer, 2022).

The rapid evolution of SARS-CoV-2, as demonstrated by the continuous emergence of new variants of concern (VOCs), including the highly transmissible Omicron (B.1.529) variant continues to represent a major burden to public health, and the economy (CDC, 2020; Chavda *et al.*, 2022). SARS-CoV-2 VOCs have acquired mutations in the S glycoprotein, with multiple mutations clustering in the RBD. These mutations have been shown to reduce the neutralising activity of antibodies induced by COVID-19 vaccines based on Wuhan-Hu-1 S sequences, as well as antibodies elicited from previous infection with earlier strains such as Wuhan, D614G and Alpha variant (Edara *et al.*, 2021; Emary *et al.*, 2021; Faulkner *et al.*, 2021; Planas *et al.*, 2021; D. Zhou *et al.*, 2021). Moreover, the high frequency of mutation in the S protein could alter interactions with the host angiotensin-converting enzyme-2 (ACE2) receptor, thereby a) increasing the infection rate, b) increasing replication efficacy and, c) compromising the potency of neutralising antibodies, consequently, affecting the efficacy of vaccines and antibody-based therapies (Alaofi & Shahid, 2021; R. E. Chen *et al.*, 2021; Mohammadi *et al.*, 2021; F. Ye *et al.*, 2021). Recent studies have highlighted that the current COVID-19 vaccines have poor durability evidenced by a decline in antibody responses and clinical effectiveness after three to six months following primary and booster immunisations (Ferdinands *et al.*, 2022; L. Liu *et al.*, 2022; Pajon *et al.*, 2022; C. S. Tan *et al.*, 2022). Additionally, clinical studies have also demonstrated that the current COVID-19 vaccines have reduced efficacy against the Delta and Omicron variants and this may necessitate repeated booster campaigns and new vaccine designs that incorporate less variable and mutable viral

proteins, in addition to the S protein (Andrews *et al.*, 2021; Cai *et al*., 2021; Mannar *et al*., 2021; Tang *et al*., 2021; P. Wang *et al*., 2021). Among the SARS-CoV-2 viral protein, nucleocapsid (N) is an important conserved structural protein that plays a major role in the viral life cycle, including genome packaging, replication and transcription (Bai *et al*., 2021; Dutta *et al*., 2020). Dangi *et al*., (2022) showed that mice that received sera from mice immunised with an N-based vaccine displayed N-specific humoral responses which could mediate antibody-dependent cellular cytotoxicity (ADCC), thereby, providing protection against SARS-CoV-2 infection. Thus, the N protein is a promising immunogen. The incorporation of N protein in next-generation vaccines could confer an additional immunological benefit by potentially eliciting a more robust and protective immunity to current and future SARS-CoV-2 variants, as well as other coronaviruses (Chiuppesi *et al*., 2020; Dangi *et al*., 2021, 2022).

SARS-CoV-2 transmits person-to-person by respiratory droplets and infects the upper respiratory tract (URT). Mucosal immunity is required to neutralise SARS-CoV-2 at the mucosal surfaces of the URT, either by tissue-resident memory T cells (TRMs) or a secretory IgA (sIgA) that can neutralise or prevent virus attachment within the epithelial cells of the mucosa (Mantis *et al.*, 2011; Okuya *et al*., 2020). Vaccines administered via a parenteral route (noting that all COVID-19 vaccines so far are delivered by intramuscular injection) fail to evoke a significant mucosal immune response, probably accounting for their failure to prevent the transmission and dissemination of the virus (Moldoveanu *et al*., 1995). Accordingly, there is now a focus on developing mucosal COVID-19 vaccines of which intranasal is the optimal route of administration (Alu *et al*., 2022; Tiboni *et al*., 2021). Mucosal vaccination has the potential to provide robust

protective immunity at the mucosal site of pathogen entry by recruiting antigen-presenting cells (APCs) and/or engaging other innate immune cells. This requires safe and effective mucosal vaccination strategies that utilise both mucosal adjuvants and appropriate delivery systems (Alu *et al*., 2022). Sengupta *et al*., (2022) demonstrated that intranasal administration of a combination of S and N protein together with cationic (N3)/or anionic (L3) lipids as the adjuvant has been shown to elicit strong systemic and mucosal immune responses in a conventional mouse model (Sengupta *et al*., 2022). Additionally, intranasal vaccination with recombinant adenovirus type-5 expressing SARS-CoV-2 N protein has been shown to elicit systemic and mucosal T-cell responses sufficient to provide protection against SARS-CoV-2 infection in a transgenic mouse model (He *et al*., 2021). Early clinical data have suggested that without pre-existing immunity to SARS-CoV-2, immunity resulting from intranasal vaccination is suboptimal (Alu *et al*., 2022; S. Wu *et al*., 2021). One promising approach might be that of boosting a current COVID-19 vaccine with an intranasal vaccine. Adenoviral 5 and 19a vectored vaccines administered intranasally after a systemic mRNA vaccine prime was shown to induce high levels of both mucosal IgA and T_{RM}, was fully protective in a mouse model of SARS-CoV-2 infection and in neutralising VOCs (Lapuente *et al*., 2021). Additionally, a heterologous prime-boost regimen to deliver a systemic immunisation consisting of a cocktail vaccine that combines S and N followed by an intranasal boost consisting of S and N to generate a more robust and broadly protective immunity against VOCs seems promising (Hajnik *et al*., 2022; Lapuente *et al*., 2021; Ong *et al*., 2020).

4.2 Results

4.2.1 Pilot study evaluating the immunogenicity of SARS-COV-2 spore vaccines displaying RBD and HR1-HR2 immunogens following a systemic prime with spike

Preliminary reports from SARS-CoV-2 vaccine candidates suggest that the RBD fragment is immunogenic in human subjects. It has been proven that RBD is composed of several conformational epitopes recognised by serum IgG-neutralising antibodies that block the interaction between the RBD and ACE-2 receptor (Abayasingam *et al.*, 2021; Y. Li *et al.*, 2021). Additionally, RBD-specific secretory immunoglobulin A (sIgA) at mucosal surfaces have been reported in individuals with SARS-CoV-2 infection (Sterlin *et al.*, 2021; Z. Wang *et al.*, 2021). Thus, RBD was chosen as the target for spore vaccine design. Numerous literatures have reported that the HR1-HR2 fragment is highly conserved among different SARS-CoV-2 variants and coronaviruses (D *et al.*, 2021; X. Ma *et al.*, 2020; S *et al.*, 2020) and is composed of cross-reactive SARS-CoV-2 CD4+ and CD8+ T cell epitopes and could potentially elicit neutralising antibodies (nAbs) capable of neutralising a broad spectrum of SARS-CoV-2 variants (Elshabrawy *et al.*, 2012; Grifoni, Sidney, *et al.*, 2020; Grifoni, Weiskopf, *et al.*, 2020; Mateus *et al.*, 2020; Ng *et al.*, 2021). To improve immunogenicity the HR1-HR2 fragment was chosen as the second candidate to design a universal spore vaccine that could potentially have a more broadly neutralisation activity against different SARS-CoV-2 variants and other coronaviruses.

A mice pilot test was conducted to test the antigen specific immunogenicity of thymine auxotrophic *B. subtilis* spores displaying the RBD (PK120; CotB-RBD^{Wuh}) and HR1-HR2 (PK122; CotC-HR1-HR2^{Wuh}) fragment as a monomer booster vaccine and as a dual booster vaccine consisting of a 1:1 mixture of PK120 (CotB-RBD^{Wuh}) and PK122 (CotC-

HR1-HR2^{Wuh}), referred to henceforth as SporCoVax. Female BALB/c were first dosed by intra-muscular injection (i.m.) with the recombinant spike (rS^{Wuh}) protein as the prime and then, 21 and 35 days later, administered spore vaccines as an intranasal boost. For murine studies, a single dose consisted of three daily intranasal administrations (e.g., days 21, 22 and 23) and resulted from the regulatory dosing limitations required for mice. Following a prime of rS^{Wuh}, SporCoVax (a 1:1 mixture of PK120 and PK122) boosted mice displayed the highest spike (rS^{Wuh})-specific sIgA endpoint titers in the saliva and serum samples (Figure 4.1). The sIgA levels in mice primed and boosted with SporCoVax were significantly greater ($p < 0.001$) than in mice receiving rS^{Wuh} prime only (Figure 4.1A). sIgA levels in mice primed and boosted with SporCoVax were significantly higher ($P \leq 0.05$, $P \leq 0.05$ and $p < 0.01$, respectively) than in mice boosted with PK120 (CotB-RBD^{Wuh}), PK122 (CotC-HR1-HR2) monomer spores and isogenic PK118 spores (WT, no antigen expression), respectively. Although, SporCoVax elicited the highest rS^{Wuh}-specific sIgA endpoint titers, the monomer spore vaccine candidates (PK120 and PK122) also displayed S-specific sIgA levels that were significantly higher than the isogenic PK118 spores (WT, no antigen expression) (Figure 4.1A). rS^{Wuh}-specific IgG levels were measured at day 48 (Figure 4.1B) and showed significantly higher levels in animals primed and then boosted with SporCoVax spores compared to animals primed only ($p < 0.01$) or primed and then boosted with isogenic PK118 (WT) or PK120 or PK122 ($p < 0.01$, $p \leq 0.05$, $p < 0.01$, respectively). On the basis of these results, SporCoVax (a 1:1 mixture of PK120 and PK122) was chosen for further testing and moved into the next stages of development.

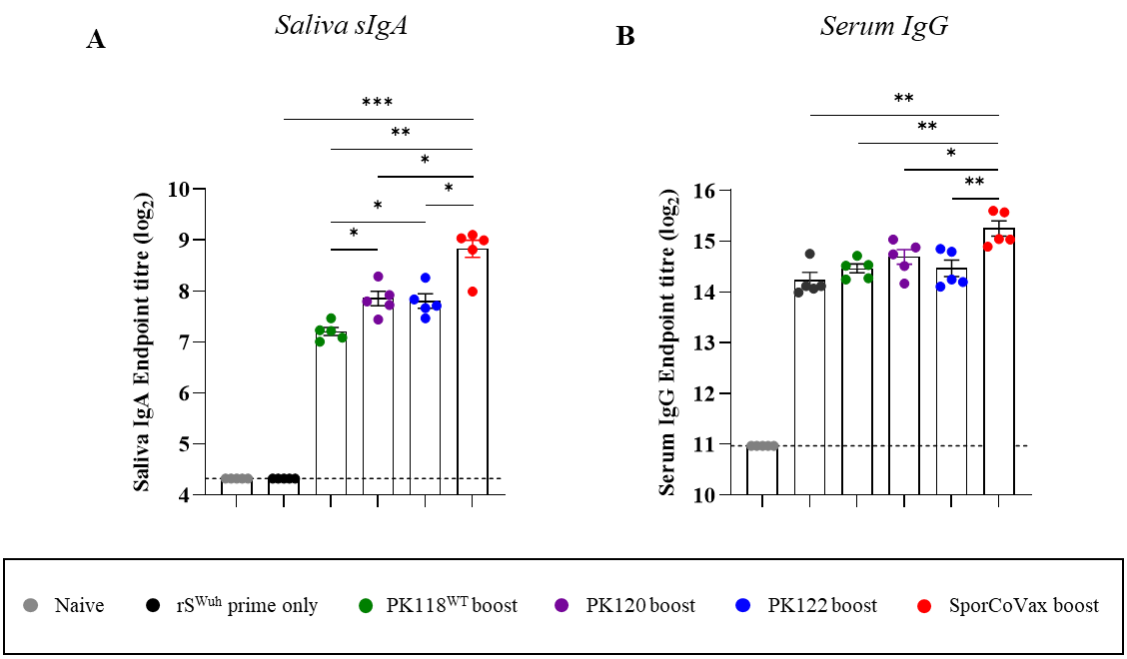

Figure 4.1. Intranasal boosting of a spike subunit prime. Female BALB/c mice were primed (i.m.) with recombinant Spike (rS^{Wuh}) followed by two intranasal boosts with 1×10^9 spores of either isogenic PK118 spore (WT, no antigen expression), PK120 (CotB-RBD^{Wuh}), PK122 (CotC-HR1-HR2) or SporCoVax (1:1 mixture of CotB-RBD^{Wuh} and CotC-$HR1\text{-}HR2^{Wuh}$ spores) three and five weeks post-prime. Panels show sIgA rS^{Wuh}-specific responses determined by ELISA ($OD_{450\,nm}$) 48 days post-immunisation. ***Panel A,*** rS^{Wuh}-specific sIgA in saliva samples, ***Panel B***, rS^{Wuh}-specific IgG in serum. Mann–Whitney, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$. This experiment was replicated twice.

4.2.2 Heterologous systemic prime–intranasal boosting using SporCoVax spores evokes mucosal immunity in mice

To further the examine the immunogenicity of SporCoVax, a 1:1 mixture of CotB-RBD^{Wuh} (PK120) and CotC-$HR1\text{-}HR2^{Wuh}$ (PK122) spores, mice were first dosed by intra-muscular injection (i.m.) with either the rS^{Wuh} protein or the AZD1222 vaccine and then, 21 and 35 days later, administered spore vaccines as an intranasal boost. Following a prime of rS^{Wuh} or AZD1222, animals intranasally boosted with SporCoVax spores exhibited rS^{Wuh}-specific sIgA in the saliva together with seroconversion (Figure 4.2A, B).

sIgA levels were significantly ($p < 0.001$) greater than the group receiving only a rS^{Wuh} or AZD1222 prime, each of which yielded essentially no detectable sIgA (Figure 4.2A, B). Interestingly, isogenic PK118 spores (WT) carrying no SARS-CoV-2 antigens also evoked rS^{Wuh}-specific sIgA but at levels significantly lower ($p < 0.01$) than in animals dosed with SporCoVax (Figure 4.2A, B). S^{Wuh}-specific IgA was also detectable in lung extracts (day 48) of animals primed only with rS^{Wuh} or AZD1222, as well as those primed and then boosted with WT spores, but levels were significantly higher ($p < 0.01$) in mice boosted with SporCoVax (Figure 4.2C, D). The presence of IgA in lung extracts of mice dosed only with rS^{Wuh} or AZD1222 (i.m.) most probably results from contamination with serum IgA and should be considered here as a baseline. Serum IgG levels measured (Figure 4.2E, F) at day 48 showed significantly ($p < 0.01$) higher levels of rS^{Wuh}-specific IgG in animals primed and then boosted with SporCoVax spores compared to animals primed only or primed and then boosted with WT spores ($p < 0.01$).

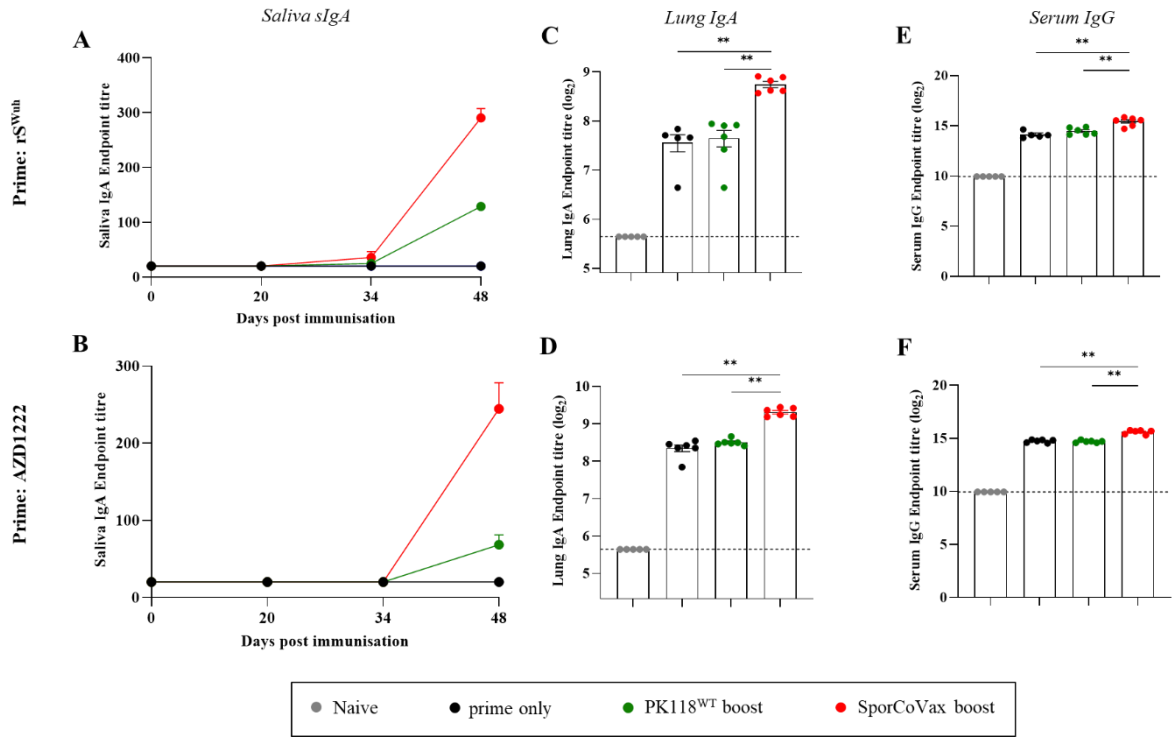

Figure 4.2. SporCoVax intranasal boosting of a spike subunit prime or AZD1222. Female BALB/C mice were primed (i.m.) with recombinant Spike (rS^{Wuh}) or AZD1222 (1.0×10^8 IU) followed by two intranasal boosts with 1×10^9 spores of either WT (naked spores, no antigen expression) or SporCoVax (1:1 mixture of CotB-RBD^{Wuh} and CotC-$HR1\text{-}HR2^{Wuh}$ spores) three and five weeks post-prime. Panels show S^{Wuh}-specific responses determined by ELISA ($OD_{450\ nm}$) 48 days post-immunisation. ***Panel A, B***) rS^{Wuh}-specific sIgA in longitudinal saliva samples, ***Panel C, D***) rS^{Wuh}-specific IgA in the lungs, ***Panel E, F***) rS^{Wuh}-specific IgG in serum. Mann–Whitney, ** $p < 0.01$. This experiment was replicated twice.

To evaluate the immunogenicity of PK230, a spore vaccine expressing CotB-RBD^{Omi}, mice were primed (i.m.) with the AZD1222 vaccine and boosted with either WT spores (no antigens) or PK230. As reported above for boosting with spores displaying rS^{Wuh}-specific antigens, boosting with PK230 (CotB-RBD^{Omi}) spores also evoked rS^{Omi}-specific sIgA in saliva and serum which was at significantly higher levels than in groups dosed with WT spores ($p < 0.001$ and $p < 0.01$, respectively) or in animals primed only ($p < 0.01$) (Figure 4.3). Together, these results show that intranasal boosting with spores

expressing SARS-CoV-2 antigens fused to CotB or CotC anchor proteins following a systemic prime evokes mucosal immunity.

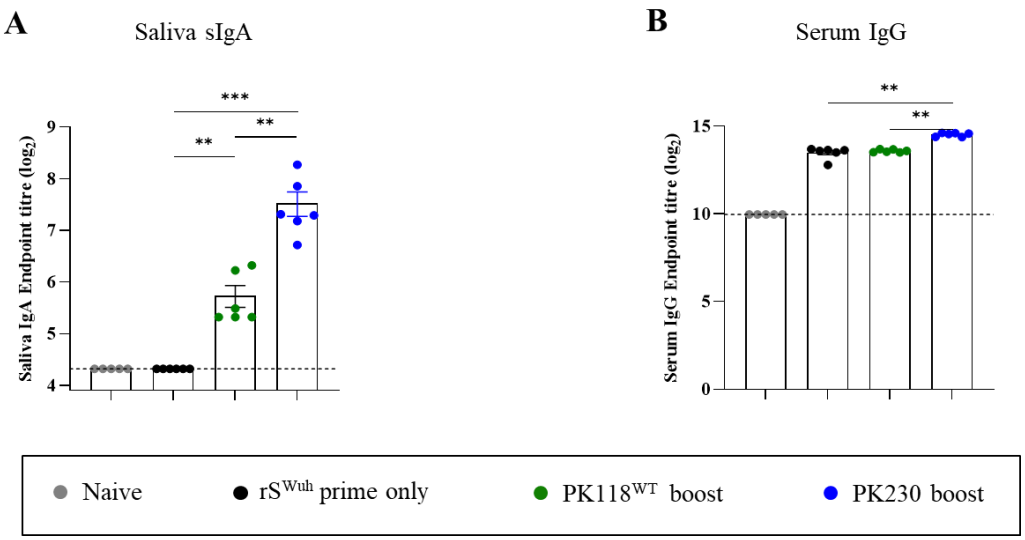

Figure 4.3: Intranasal boost of the AZD1222 vaccine with spores expressing Omicron spike specific antigens. Three groups (n=6) of mice (female BALB/c) were primed (i.m.) with the AZD1222 vaccine. Two groups then received two intranasal boosts with spores (1×10^9 CFU) of either WT (naked spores, no antigen expression) or PK230 (CotB-RBD^{Omi}) at 21 and 35-days post-prime. Panels show rS^{Omi}-specific responses determined by ELISA (OD_{450nm}) 48 days post-immunisation. ***Panel A***, rS^{Omi}-specific sIgA in saliva samples, ***Panel B***, rS^{Omi}-specific IgG in serum. Significance was tested using a two-tailed Mann-Whitney U test, $^{**}p < 0.01$, $^{***}p < 0.001$. This experiment was replicated twice.

4.2.3 Boosting with SporCoVax spores evokes cross-reactive antibodies in mice

From the study above in which animals had been primed with AZD1222 and then boosted with SporCoVax (1:1 mixture of CotB-RBD^{Wuh} and CotC-HR1-HR2^{Wuh}) spores showed that saliva samples from these mice carried rS^{Wuh}-specific sIgA antibodies that cross react with Wuhan and Omicron variant. Using ELISA plates coated with rS^{Omi}, SporCoVax-boosted animals exhibited antigen (rS^{omi})-specific sIgA in saliva samples which was

significantly greater ($p < 0.01$ and $p < 0.001$, respectively) than in animals dosed with WT spores or rS^{Wuh} only (Figure 4.4). As shown in the previous section, WT spores used alone as a boost also enhanced antigen-specific sIgA.

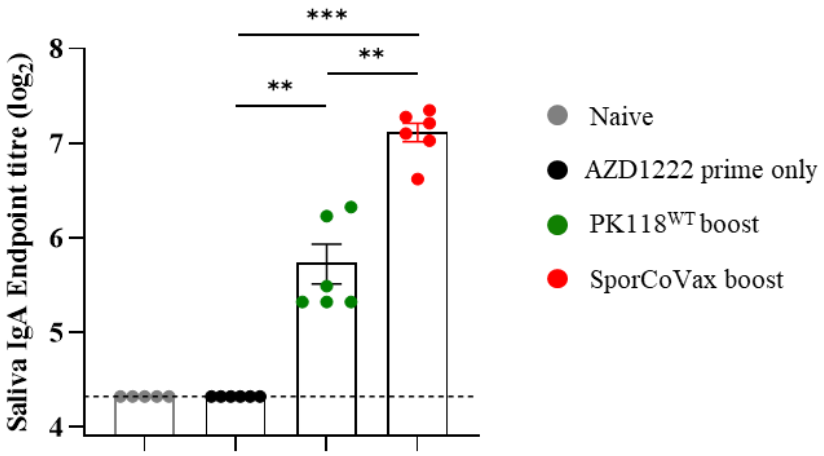

Figure 4.4. Spore-induced mucosal spike-specific sIgA cross-reacts with Wuhan and Omicron variants. Mice were primed (i.m.) with 1.0×10^8 IU of AZD1222 followed by two intranasal boosts with spores (1×10^9 CFU) of WT (naked) or SporCoVax (1:1 mixture of CotB-RBD^{Wuh} and CotC-$HR1\text{-}HR2^{Wuh}$ spores) at three and five weeks post-prime. rS^{Omi}-specific sIgA in the saliva 48 days post-immunisation is shown. Mann–Whitney, ** $p < 0.01$, *** $p < 0.001$. This experiment was replicated three times.

4.2.4 Intranasal boosting with SporCoVax spores results in a mixed T-cell cytokine profile

Next, the subsets of T helper (Th) cells and cytotoxic T cells that were responding to the rS^{Wuh} protein were examined. Animals (n = 3/group) were either primed with AZD1222 or primed (AZD1222) and then boosted with WT or SporCoVax spores. Splenocytes from immunised animals were cultured with rS^{Wuh} and assessed for the production of IL-2, TNF-α and IL-5, which are induced in Th1 (IL-2, TNF-α) and Th2 (IL-5) T-cell responses (Figure 4.5). A previous study on SARS-CoV-2 reported that a single dose of BNT162b2

or AZD1222 has been shown to induce increased levels of spike protein-specific effector T cells which were dominated by type 1 helper T cell responses, and this was evidenced in this chapter. SporCoVax-boosted animals exhibited marked increases in the expression of rS^{Wuh}-specific, polyfunctional T cells expressing IL-2, and TNF-α, as well as elevated levels of IL-5 than the other groups. Together, this shows that intranasal spore boosting confers both Th1 and Th2 responses.

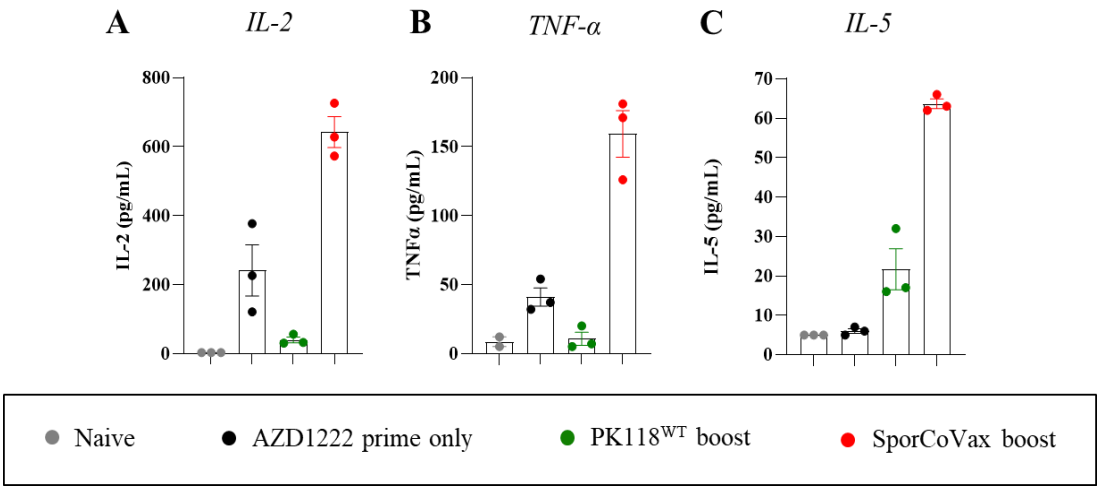

Figure 4.5. Spike specific cytokine profiles. BALB/c mice (female, aged 8 weeks; n = 3/gp) were immunised with AZD1222 (i.m.; 1.0×10^8 IU) followed by two intranasal boosts with spores (1×10^9 CFU/dose) of WT or SporCoVax (a 1:1 mixture of CotB-RBD^{Wuh} and CotC-$HR1\text{-}HR2^{Wuh}$) three and five weeks post prime immunisation. Control groups included untreated animals (naïve) and animals primed only (AZD1222). All mice were sacrificed 48 days post-prime, and their spleens were dissected and stimulated with 2.5 µg/mL rS^{Wuh} protein for 72 h, and levels of the cytokines IL-2 (***Panel* A**), TNF-α (***Panel B***) and IL-5 (***Panel C)*** determined by flow cytometry. This experiment was replicated twice.

4.2.5 Intranasal boosting with SporCoVax spores is protective in the hamster SARS-CoV-2 model of infection

The Golden Syrian hamster model is currently considered one of the best models to study the effects of SARS-CoV-2 infection because it permits high levels of SARS-CoV-2 replication and generates significant clinical signs (weight loss) and moderate to severe pneumonia (Muñoz-Fontela *et al*., 2020; Sia *et al*., 2020). To determine whether intranasal boosting with spores expressing SARS-CoV-2 antigens conferred protection, hamsters were primed (n = 12; Group 3) with rS^{Wuh} protein (i.m.) and then administered two intranasal boosts with 2.5×10^9 spores of SporCoVax (1:1 mixture of CotB-RBD^{Wuh} and CotC-HR1-$HR2^{Wuh}$ spores). Two additional groups, Group 1, a naïve, unchallenged group (n = 6), and Group 2, a negative control group (n = 12). Group 1 and group 2 both received PBS in place of immunogens (i.m. prime and two i.n. boosts). Seven days after the final boost, animals in Groups 2 and 3 were intranasally challenged with SARS-CoV-2 (D614G variant, 10^4 $TCID_{50}$/animal). Weight loss post-challenge was shown (panel A). Animals were sequentially euthanised (2, 4 and 7 dpi), necropsies were performed, and viral loads of SARS-CoV-2 were determined by qPCR in nasal turbinate (panel B), OP swabs (panel C) and lungs (panel D). Mann–Whitney, * $p < 0.05$, ** $p < 0.01$.

Weight loss was used to track the progression of the disease (Figure 4.6A**)**. Naïve (Group 1) animals showed no reduction in weight, whereas non-vaccinated animals (Group 2) challenged with SARS-CoV-2 showed progressive reductions in weight 2–7 dpi. By contrast, animals from Group 3 (SporCoVax boost) lost weight at 2 dpi and started to recover at 4 dpi, and finally regained 100% of their initial weight at 7 dpi ($M = -7.4$, $SE =$

1.73, 95%. *CI* [−11.65, −3.2], $p < 0.01$). Due to operational reasons, it was not possible to assign animals to a prime-only group and it has been acknowledged that this is a major flaw in the experimental design and interpretation. On the other hand, it has been extensively reported that a single systemic dose of S protein fails to induce protective antibodies (Johnson *et al*., 2022; Meseda *et al*., 2021; Tabynov *et al*., 2022).

To assess the impact of intranasal boosting on viral replication, SARS-CoV-2 RNA (gRNA) in nasal turbinates (NT) (Figure 4.6B**)**, oropharyngeal (OP) swabs (Figure 4.6C) and lungs (Figure 4.6D) was examined at 2, 4 and 7 days post infection (dpi). After SARS-CoV-2 inoculation (Groups 2 and 3), viral gRNA was detected at 2 dpi in NT, OP swabs and lungs in both groups. The viral load decreased progressively in OP swabs and both tissues in animals receiving intranasal boosts with SporCoVax (PK120:PK122 boost; Group 3), compared to the unimmunised–infected control group (Group 2), which was statistically significant at 7 dpi ($p < 0.01$). This data demonstrates that the heterologous systemic prime–intranasal boost regimen ameliorates the clinical and virological outcome of the SARS-CoV-2 challenge.

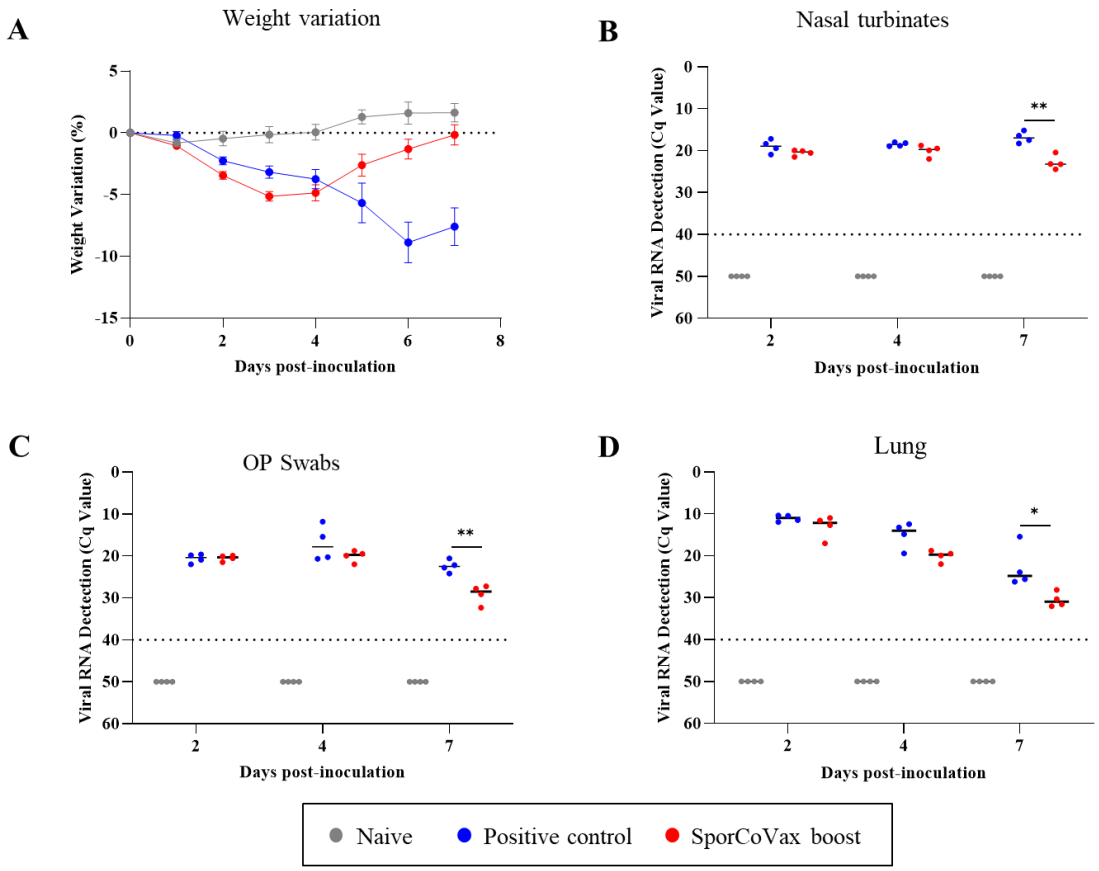

Figure 4.6. Protection in Golden Syrian hamsters following intranasal boosting with SporCoVax spores. Golden Syrian Hamsters were primed (i.m.) with recombinant spike (rS^{Wuh}) protein followed by two intranasal boosts with 2.5×10^9 spores of SporCoVax (1:1 mixture of CotB-RBD^{Wuh} and CotC-$HR1\text{-}HR2^{Wuh}$ spores) (Group 3). Negative, unvaccinated, (Group 2) and naive (Group 1) control groups received PBS in place of immunogens. 7-days after the final boost animals in Groups 2 and 3 were intranasally challenged with SARS-CoV-2 (D614G variant, 10^4 $TCID_{50}$/animal). Weight loss post-challenge is shown (***Panel A***). Animals were sequentially euthanised (2, 4, and 7 dpi), necropsies were performed, and viral load of SARS-CoV-2 were determined by qPCR in NT (***Panel B***), OP swabs (***Panel C***) and lungs (***Panel D***). Mann-Whitney, * $p < 0.05$, ** $p < 0.01$.

4.2.6 Inducing mucosal immunity in mice using a next generation SARS-CoV-2 nucleocapsid spored based booster vaccine

The coronavirus nucleocapsid (N) protein is an important immunogenic structural viral antigen. To determine whether a second-generation spore booster vaccine expressing the full-length N protein of SARS-CoV-2 (Wuhan-Hu-1 strain; Appendix B) would evoke mucosal immunity, a similar heterologous systemic prime-mucosal boost regimen was conducted. Mice were first vaccinated with recombinant N protein (rN) using intra-muscular injection (i.m. prime), followed 21 and 35 days later with i.n. administration of a spore vaccine expressing N protein (PK128; CotB-N) as an intranasal boost. As previously mentioned, a single dose consisted of three daily intranasal administrations (e.g., days 21, 22 and 23). Following a prime of rN and intranasal administration of PK128 (CotB-N; boost), spores exhibited rN-specific sIgA in the saliva together with seroconversion (Figure 4.7A**)**. sIgA levels were significantly ($p < 0.001$) greater than the group receiving only a rN prime, each of which yielded essentially no detectable sIgA (Figure 4.7A**)**. Isogenic spores (referred to as PK118) carrying no SARS-CoV-2 antigens also evoked antigen-specific sIgA but at levels significantly lower ($p < 0.01$) than in animals dosed with PK128 (Figure 4.7A). N-specific IgA was also detectable in lung extracts (day 48) of animals primed only with rN, as well as those primed and then boosted with PK118 spores, but levels were significantly higher ($p < 0.01$) in mice boosted with PK128 (Figure 4.7B). As previously mentioned, the presence of IgA in lung extracts of mice dosed only with rN (i.m.) most probably results from contamination with serum IgA and should also be considered here as a baseline. Serum IgG levels measured (Figure 4.7C) at day 48 showed significantly higher levels of rN-specific IgG in animals

primed and then boosted with PK128 (CotB-N) spores compared to animals primed only ($p < 0.01$) or primed and then boosted with the isogenic PK118 spores ($p < 0.05$). The isogenic PK118 spores carrying no SARS-CoV-2 antigens also evoked rN-specific sIgG but at levels significantly higher ($p < 0.05$) than in animals primed only.

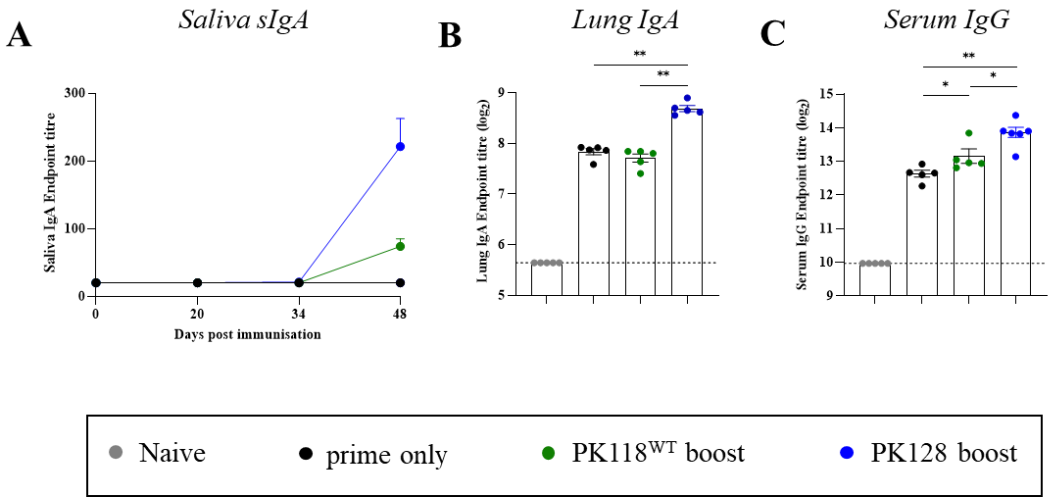

Figure 4.7: Intranasal boosting of a nucleocapsid subunit prime. Female BALB/C mice were primed (i.m.) with a recombinant spike (rN) followed by two intranasal boosts with 1 x 10^9 spores of either PK118 (naked spores, no antigen expression) or PK128 (CotB-N) three and five-weeks post-prime. Panels show rN-specific responses determined by ELISA (OD_{450nm}) 48-days post-immunisation. ***Panel A***) rN-specific sIgA in longitudinal saliva samples, ***Panel B***) rN-specific IgA in the lungs, ***Panel C***) rN-specific IgG in serum. Mann-Whitney, **$p < 0.01$, *** $p < 0.001$. This experiment was replicated three times.

4.2.7 Intranasal boosting with spores displaying the nucleocapsid domain results in mixed T-cell cytokine profile

To examine the subsets of T helper (Th) cells and cytotoxic T cells that were responding to the rN protein, splenocytes were harvested from animals (n = 3/group) that were primed with rN and then boosted with isogenic PK118 or PK128 (CotB-N) spores. Splenocytes were cultured with rN and assessed by flow cytometry for the production of

IL-2, IL-5, IL-6 and TNF-α, which are induced in Th1 (IL-2, IL-6 and TNF-α) and Th2 (IL-5) T-cell responses (Figure 4.8). As reported above for boosting with SporCoVax, boosting with PK128 (CotB-N) resulted in a similar cytokine profile. PK128-boosted animals exhibited a marked increase in the expression of all four cytokines compared to other groups. This data supports that intranasal spore boosting with spores expressing an antigen activates both Th1 and Th2 responses, as evidenced here.

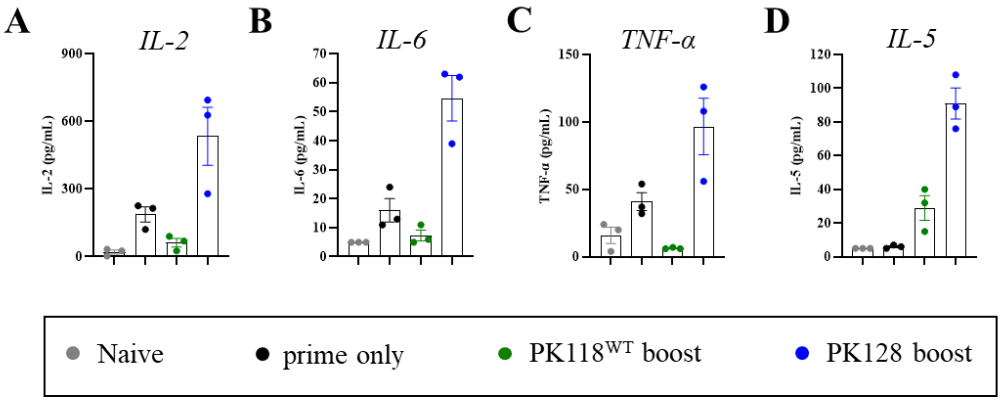

Figure 4.8: Cytokine profiles. BALB/C mice (female, aged 8 weeks; n = 3/gp) were primed (i.m.) with recombinant nucleocapsid (rN) followed by two intranasal boosts with 1 x 10^9 spores of either WT (naked spores, no antigen expression) or PK128 (CotB-N) three and five weeks post prime immunisation. Control groups included untreated animals (naïve) and animals primed only (rN). All mice were sacrificed 48 days post-prime, and their spleens were dissected and stimulated with 2.5 µg/mL rN protein for 72 h, and levels of the cytokines IL-2 (***Panel A***), IL-6 (***Panel B***) and TNF-α (***Panel C***) and IL-5 (***Panel D***) determined by flow cytometry.

4.2.8 Combination spore boost vaccination elicits robust N-specific and S-specific mucosal immune responses in mice

Section 4.2.6 demonstrated that PK128 (CotB-N) spores expressing N protein of SARS-CoV-2 following a systemic prime of rN elicited strong mucosal and cellular immunity. A further experiment was conducted evaluating whether a trivalent spore booster vaccine consisting of a combination of spores expressing N, RBD and HR1-HR2 fragments of SARS-CoV-2 (Wuhan-Hu-1) would induce a more robust systemic and mucosal immune responses. Thus, in addition to spores expressing N (PK128; CotB), PK120 (CotB-RBD^{Wuh}) and PK122 (CotC-HR1-HR2) spores from SporCoVax were combined. For simplicity, the trivalent spore booster vaccine is referred to henceforth as PK-S+N (consisting of a 1:1:1 mixture of CotB-RBD^{Wuh}, CotC-HR1-HR2 and CotB-N). Additionally, the systemic prime consisted of a 1:1 mixture of both rN and rS (rS+N). First, a mouse immunogenicity experiment was conducted, four groups of BALB/c mice (n = 5 per group) were vaccinated with an rN+S using intra-muscular injection (i.m. prime), followed by boost 21 and 35 days later through intranasal administration (i.n.) of spore vaccines. Briefly, two groups of mice (n = 5 per group) following a systemic prime received a boost of SporCoVax (a 1:1 mixture of CotB-RBD and CotC-HR1-HR2 spores) or PK-S+N (consisting of a 1:1:1 mixture of CotB-RBD, CotC-HR1-HR2 and CotB-N) spores at day 21 and 35. sIgA to rS or rN was measured by ELISA (Figure 4.9). Using ELISA plates coated with rS, it was evident that mice boosted with SporCoVax (alone) and mice boosted with the trivalent PK-S+N spores exhibited similar levels of rS^{Wuh}-specific sIgA in saliva samples, with both groups showing levels significantly higher than in animals primed only ($p < 0.001$; Figure 4.9A). Compared to SporCoVax-boosted mice

(alone), PK-S+N-boosted mice evoked antigen (rN)-specific sIgA in saliva samples which was at significantly higher levels than in the group dose with SporCoVax spore alone (p <0.01; Figure 4.9B). Due to resource limitations antigen specific IgG was not measured in serum samples obtained from these mice. On the other hand, section 4.2.2 and section 4.2.6 demonstrated that mice primed and boosted with spores expressing S or N specific antigens elicited rN or rS specific IgG, respectively, at levels significantly greater than mice primed only (Figure 4.2E and Figure 4.7C**).** To summarise, these results show that intranasal boosting with a combination of spores expressing S and N SARS-CoV-2 antigens following a systemic prime with a rS+N subunit protein evokes mucosal immunity that is specific to both rS and rN.

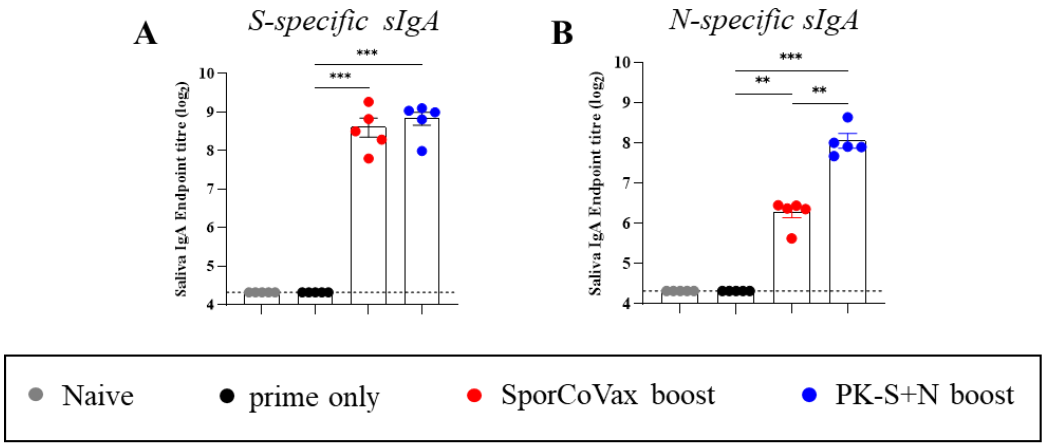

Figure 4.9: Intranasal boost of the S+N subunit vaccine with a combination of spores expressing S and N-specific antigens. Female BALB/C mice were primed (i.m.) with A combination of recombinant spike and nucleocapsid (rS+N) followed by two intranasal boosts with 1 x 10^9 spores of either SporCoVax alone (a 1:1 mixture of CotB-RBD and CotC-HR1-HR2) or PK-S+N trivalent spores (1:1:1 mixture of CotB-RBD, CotC-HR1-HR2 and CotB-N) three and five-weeks post-prime. Panels show rS^{Wuh} and rN-specific responses determined by ELISA (OD_{450nm}) 48-days post-immunisation. ***Panel A***) rS-specific sIgA in saliva samples, ***Panel B***) rN-specific IgA in the saliva sample. Mann-Whitney, ** p < 0.01, *** p < 0.001. This experiment was replicated twice.

4.2.9 Combination spore boost vaccination induces modest control of SARS-CoV-2 infection in hamsters

IRTA next investigated the protective efficacy of PK-S+N (1:1:1 mixture of CotB-RBD, CotC-HR1-HR2 and CotB-N) spore administered as an intranasal spore booster vaccine following a systemic prime with rS+N subunit protein against the SARS-CoV-2 D614G variant. First, Golden Syrian hamsters were primed via i.m. route (n = 12; Group 3) with rS+N (1:1 mixture, 10μg of S and 10μ of N) subunit proteins and then administered two intranasal boosts with 2.5×10^9 spores of PK-S+N (1:1:1 mixture of CotB-RBD, CotC-HR1-HR2 and CotB-N), Seven days after the last boost, animals were challenged intranasally with SARS-CoV-2 (D614G variant, 10^4 $TCID_{50}$/animal). Two additional groups, Group 1, being a naïve, unchallenged group (n = 6), and Group 2, a negative, unvaccinated control group (n = 12) which received PBS in place of immunogens (i.m. prime and two i.n. boosts). Seven days after the final boost, animals in groups 2 and group 3 were intranasally challenged with SARS-CoV-2 (D614G variant, 10^4 $TCID_{50}$/animal), and group 2 was used for comparison. Protection was analysed based on weight loss post-challenge (Figure 4.10A) and viral load (Figure 4.10C-D). Naïve (Group 1) animals showed no reduction in weight, whereas non-vaccinated animals (Group 2) challenged with SARS-CoV-2 showed progressive reductions in weight 2–7 dpi. By contrast, animals from Group 3 (PK-S+N boost) lost weight at 2 dpi and started to recover at 5 dpi, and finally regained ~80% of their initial weight at 7 dpi ($p < 0.01$). Analysis of viral RNA showed that after SARS-CoV-2 inoculation, viral RNA was detected at 2 dpi in OP swabs, nasal turbinates, and lungs in group 2 (non-vaccinated animals, and challenged) and group 3 (boosted with PK-S+N). The viral load in group 3 (boosted with PK-S+N)

decreased progressively at 7 dpi in NT ($p < 0.01$; Figure 4.10B), OP swabs ($p < 0.05$; Figure 4.10C**)** and lungs ($p < 0.05$; Figure 4.10D) compared to group 2 (non-vaccinated animals and challenged). This data indicates that PK-S+N administered intranasally following a systemic prime induces modest (partial) control of the SARS-CoV-2 D614G variant and significantly reduced viral RNA in the upper and lower respiratory tract. Therefore, the heterologous systemic-mucosal boost strategy targeting both S and N may also reduce the risk of transmission.

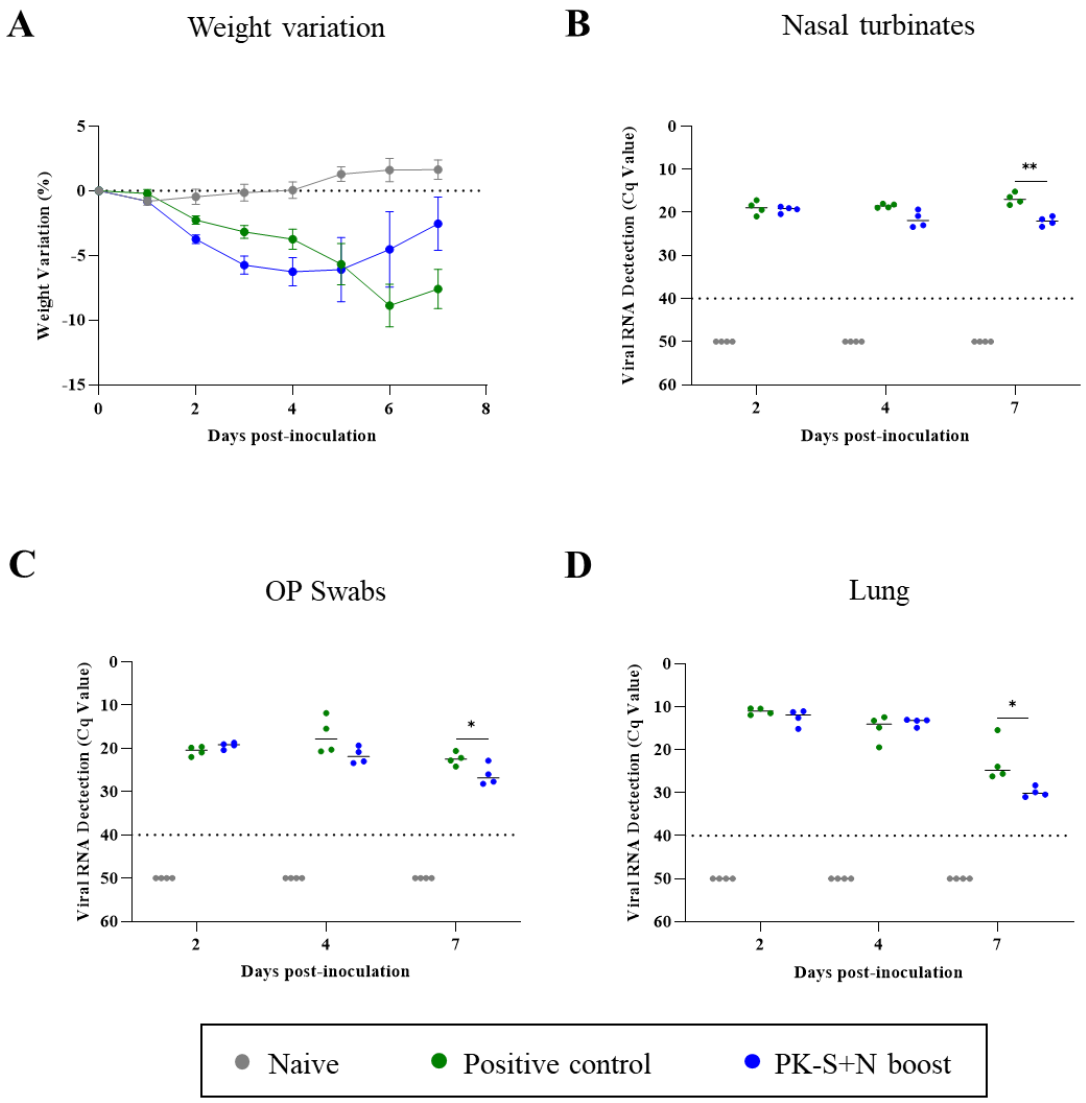

Figure 4.10. Protection in Golden Syrian hamsters following intranasal boosting with PK-S+N spores. Golden Syrian Hamsters were primed (i.m.) with recombinant spike (rS+rN) subunit protein followed by two intranasal boosts with 2.5 x 109 spores of PK-S+N (1:1:1 mixture of CotB-RBD, CotC-HR1-HR2 and CotB-N) (Group 3; blue). Negative, unvaccinated, (Group 2; green) and naive (Group 1; grey) control groups received PBS in place of immunogens. 7-days after the final boost animals in Groups 2 and 3 were intranasally challenged with SARS-CoV-2 (D614G variant, 104 TCID50/animal). Weight loss post-challenge is shown (***Panel A***). Animals were sequentially euthanised (2, 4, and 7 dpi), necropsies were performed, and viral load of SARS-CoV-2 were determined by qPCR in NT (***Panel B*****),** OP swabs (***Panel** C)* and lungs (***Panel** D*). Mann-Whitney, * $p < 0.05$, ** $p < 0.01$.

4.3 Discussion

4.3.1 SporCoVax spore booster vaccine confers protection against SARS-CoV-2

The continued dissemination of SARS-CoV-2 ensures that COVID-19 is likely to persist. To halt transmission, vaccine strategies must focus on blocking the interaction of the virus at the mucosa. Accomplishing this requires mucosal immunity and more specifically induction of sIgA that can block viral adhesion. At present, a few mucosal COVID-19 vaccines administered via the intranasal route have been investigated in preclinical studies, and have been shown to evoke both systemic and mucosal immunity (Jearanaiwitayakul *et al*., 2021; Lei *et al*., 2022). This chapter highlights that *B. subtilis* spores engineered to express SARS-CoV-2 S-specific antigens including RBD and HR1-HR2, when delivered as an intranasal boost following a systemic prime (either an S protein subunit or the AZD1222 COVID-19 vaccine) induced antigen-specific sIgA. This is further supported by a robust IL-5 response which is often associated with the innate and adaptive immunity. IL-5 is produced by Th2 cells (including, lymphocytes, eosinophils, and mast cells) and is often associated with the proliferation and differentiation of B cells and promotes sIgA production (Braciak *et al*., 2000; Mantis *et al*., 2011; Ramsay & Kohonen-Corish, 1993). In support of this, Braciak *et al.* (2000) demonstrated that adenovirus expressing murine IL-5 delivered intranasally resulted in an increase in viral-specific mucosal immune responses (sIgA) in lung lavages (Braciak *et al*., 2000). Surprisingly, the intranasal administration of SporCoVax (a 1:1 mixture of PK120 (CotB-RBD^{Wuh}) and PK122 (CotC-HR1-HR2)) spores as a booster vaccine could further boost the serum S-specific IgG responses. A possible explanation for this response is that activated Th1 cells produce IL-2 (Muraguchi *et al*., 1985) and TNF-α (Jelinek &

Lipsky, 1987) and it is well known that both cytokines can stimulate B cells to differentiate into antibody-producing plasma cells, which mainly produces IgG antibodies (Hubbell *et al*., 2009). Moreover, when this strategy was employed in hamsters, they showed signs of protection from viral challenge including recovery of body weight and a reduction in viral shedding. This is encouraging for a number of reasons but most importantly a reduction in viral shedding would lead to reduced transmission and dissemination ultimately providing a means to controlling this disease. The results presented here indicate that SporCoVax (a 1:1 mixture of CotB-RBD^{Wuh} (PK120) and CotC-HR1-HR2^{Wuh} (PK122)) as a booster following a systemic prime is safe and effective in mouse and hamster models. SporCoVax boost elicited robust cellular and humoral immune responses and protected vaccinated hamsters from SARS-CoV-2 infection. Although it is difficult to make a direct comparison to other preclinical studies that have used combination vaccines consisting of both the RBD and HR1-HR2 fragment, because of variations in experimental conditions and vaccination strategy designs, the immunogenicity, such as the nominal titers of vaccine-induced antibodies. Despite this the efficacy of SporCoVax are comparable to those reported in a small animal study using RBD-HR nanoparticle vaccine (X. Ma *et al*., 2020). In the study mentioned RBD-HR nanoparticle vaccine (consisting of RBD fused to HR1-HR2) delivered intramuscularly has been shown to induce potent neutralising antibodies and cellular immune response that protect against SARS-CoV-2 challenge in a transgenic mouse model (X. Ma *et al*., 2020). However, a limitation of the study mentioned above is the use of a transgenic mouse model which has been shown to have mild to moderate infections. In this chapter, a hamster model of infection was conducted. It is widely accepted that hamsters are a reliable model for SARS-CoV-2 challenge studies, they are highly susceptible to SARS-

CoV-2 infection and do not require prior adaptation, and develop severe pneumonia similar to COVID-19 patients, making this a strong model to study vaccine efficacy (Bi *et al*., 2021; Muñoz-Fontela *et al*., 2020).

4.3.2 Next generation SARS-CoV-2 spore booster vaccine induces mucosal immunity

The continuous emergence of variants of concern (VOCs) that have acquired a large number of mutations in various regions of the spike (S) protein, has posed challenges to the current COVID-19 (Dejnirattisai *et al*., 2021; Faulkner *et al*., 2021; Garcia-Beltran *et al*., 2021; Madhi *et al*., 2021; Supasa *et al*., 2021). This supports a rationale to develop next generation vaccine strategies that either replace the current COVID-19 vaccines or coexpress S and N antigens as a cocktail vaccine. The N protein is highly expressed during infection (after viral invasion) and plays an important role in genome packaging, transcription and replication (Bai *et al*., 2021; Dutta *et al*., 2020). Bioinformatic studies have demonstrated that the amino acid sequence of N protein is highly conserved and shares 90% homology with other coronaviruses (Oliveira *et al*., 2020; A. Wu *et al*., 2020). Studies have shown that individuals previously infected with SARS-COV-1 have long-lasting N-specific memory T-cells that remained detectable 17 years after the 2003 SARS outbreak; interestingly, these individuals displayed robust cross-reactivity to the N protein of SARS-CoV-2 (Bilich *et al*., 2021; Le Bert *et al*., 2020), suggesting that N-specific T cell immunity might provide long-lasting and broader protection (Lineburg *et al*., 2021). Although there is evidence that SARS-CoV-2 N-based vaccines can establish protective immunity in animals (Hajnik *et al*., 2022; Matchett *et al*., 2021), it should be noted that N-specific immune responses may not prevent breakthrough SARS-CoV-2

infection. However, many studies have suggested a potential role of N-specific $CD8^+$ T cells in viral control and providing protection against the ancestral SARS-CoV-2 and VOCs (Le Bert et al., 2020; P. Moss, 2022; Taus et al., 2022). This chapter demonstrated that the next generation spore booster vaccine expressing the N protein of SARS-CoV-2 (PK128; CotB-N), when administered as an intranasal boost following a systemic (i.m) prime with N protein subunit induced robust N-specific sIgA. This is further supported by a robust N-specific IL-5 responses which is associated with sIgA induction, as stated above. N-specific immune responses, specifically cellular immune responses (He *et al*., 2021; Taus *et al*., 2022) may provide antiviral activity by killing SARS-CoV-2 infected cells, which would lead to reduced transmission and dissemination. Moreover, the ability of N protein-based vaccines to elicit robust T cell responses provides an additional immunological benefit by potentially eliciting a more robust and protective immunity to current and future SARS-CoV-2 variants. Ni *et al* reported a strong correlation between the titers of neutralising antibodies and levels of virus-specific T cells, suggesting that a robust T cell response might be necessary for the induction of a humoral response (Ni *et al*., 2020).

Additionally, PK-S+N spore vaccine consisting of a combination of CotB-RBD, CotC-HR1-HR2 and CotB-N spores when used as an intranasal boost following a systemic prime with both S and N protein subunit also induced robust S and N-specific sIgA and IgG antibodies in immunised mice. Interestingly, when this strategy was employed in hamsters, PK-S+N spore booster vaccine following a systemic prime with S+N protein subunits induced a modest (partial) control of SARS-CoV-2 infection, evidenced by a steady increase in body weight, but did not result in complete recovery of body weight.

Whereas animals that received SporCoVax (a 1:1 mixture of CotB-RBD and CotC-HR1-HR2) booster alone following a systemic prime with S protein subunit induced a more robust control of SARS-CoV-2 infection evidenced by a full recovery of body weight. This was not expected because the dose of S-based spores (CotB-RBD and HR1-HR2) in PK-S+N and SporCoVax were identical. Explanations of these findings are not yet clear and warrant further investigation. A limitation in this challenge study was identified which could have helped to explain these findings. For operational reasons, animals were not assigned to a prime-only group and a group receiving a systemic prime of N-protein subunit alone followed by PK128 (CotB-N) intranasal spore boost. On the other hand, a recent data using N-based vaccines have reported that immunisation with N-expressing mRNA vaccine induced modest (partial) control of SARS-CoV-2 in Syrian hamsters, as evidenced by a reduction in viral load in the upper respiratory tract and increased body weight (Hajnik et al., 2022). Additionally, immunisations with mRNA expressing both S+N induced not only a more robust control of SARS-CoV-2 variants including Delta and Omicron variants but also reduced the viral load in the upper respiratory tract (Hajnik *et al*., 2022). Although this study indicates that PK128 (CotB-N) and PK-S+N spores administered intranasally after a systemic prime can augment mucosal immunity, it does not provide conclusive evidence of a correlation between vaccine-induced N specific-mucosal antibodies and protection against SARS-CoV-2 infection and this warrants further studies. Despite this, PK-S+N-boosted hamsters showed a significant reduction in SARS-CoV-2 RNA in the upper and lower respiratory tract, as well as a significant increase in body weight compared to the unvaccinated unimmunised-infected control group. This is encouraging because a reduction in viral shedding would lead to reduced transmission and dissemination ultimately providing a means to controlling this disease.

4.3.3 Naked spores induce SARS-CoV-2 antigen specific immunity

Intriguingly, nasal boosting with isogenic spores displaying no antigens also showed a clear augmentation of mucosal antigen-specific responses. The underlying mechanism is not fully understood but other studies have shown that *B. subtilis* spores carry natural adjuvant properties (Barnes *et al*., 2007; de Souza *et al*., 2014). Spores can be taken up by M-cells (Rhee *et al*., 2004), and persist within phagocytes (J.-M. Huang, La Ragione, Cooley, *et al*., 2008; J.-M. Huang, La Ragione, Nunez, *et al*., 2008) and intriguingly, inactivated spores are also able to stimulate the innate immune system when delivered nasally (Song *et al*., 2012). This is achieved by the interaction of spores with Toll-like receptors (TLR2 and TLR4), cytokine induction, recruitment of NK cells and maturation of Dendritic cells (J.-M. Huang, La Ragione, Nunez, *et al*., 2008; Song *et al*., 2012). What is interesting is that this data shows that 'naked' spores administered three weeks after the systemic prime are able to augment mucosal immunity suggesting that spore-induced innate immunity may be a factor. Although the mucosal responses are clearly greater when spores-displaying antigens are delivered, the long-term utility of non-recombinant spores may be more advantageous. It is worthwhile noting that previous work has also demonstrated that nasal administration of spores alone (naked spores) is sufficient to confer protection against influenza (H5N2) using both murine and ferret models of infection (James *et al*., 2022; Song *et al*., 2012). This observation is important since spores alone might provide a mechanism for evoking mucosal immunity and is currently being addressed in expanded animal and human studies.

4.4 Limitations of the study

This study has several limitations. First, vaccine efficacy was only examined in a hamster model of infection at 2 weeks after booster immunisation. Thus, the durability of protection against SARS-CoV-2 is uncertain. Second, this chapter demonstrated that intranasal boosting with SARS-CoV-2 spores provided protection against SARS-CoV-2 variant D614G, but our collaborators at IRTA were not able to test protection against more recent VOCs, such as Delta and Omicron due to time and resource limitations. Evaluation of SARS-CoV-2 spore booster vaccine against VOCs at longer intervals following booster immunisation would help to address this question. Third, vaccine efficacy was assessed in immunologically naïve animals (without pre-existing immunity). Given that a large number of the human population have been vaccinated with the first-generation vaccines comprised of S antigen from the Wuhan strain, it is important to assess the protective efficacy of SporCoVax booster in animal models or humans who have pre-existing immunity. Lastly, dose-dependent studies of SARS-CoV-2 spore vaccines was not conducted. The dose used in this study was based on a dose that has been widely used in previous studies that use an intranasal spore vaccine regimen (S. Lee *et al*., 2010; Y. Oh *et al*., 2020; Sibley *et al*., 2014; J. Wang *et al*., 2019). Nonetheless, it is still necessary to test the possible toxicity of SARS-CoV-2 spore vaccines at higher doses to ensure their safety. Therefore, an optimal dose for the SARS-CoV-2 spore vaccines providing sufficient protection against SARS-CoV-2 is yet to be examined.

4.5 Conclusion

A heterologous systemic prime-mucosal boost vaccination strategy is being considered as one of the most promising routes forward to enhance existing COVID-19 vaccines (Lapuente *et al*., 2021; R. Zhou *et al.*, 2022). This approach has attracted significant global interest in an attempt to address the concerns posed by the fluctuating vaccine supplies, serious adverse events (anaphylaxis and thromboembolic episodes following immunisations with the adenovirus vectored vaccine), the emergence of new VOCs and waning immunity. (Borobia *et al.*, 2021; X. Liu *et al*., 2021; Logunov *et al*., 2020). The underlying basis for how a mucosal boost induces a localised response is not yet fully understood but a "prime-pull" mechanism has been proposed where systemic memory cells (induced by the prime) are expanded during the recall response and then migrate to the mucosa followed by differentiation into tissue-resident memory cells (Çuburu *et al*., 2019). This chapter provide evidence that a heterologous systemic prime–mucosal boost strategy evoked SARS-CoV-2 antigen specific mucosal IgA and is potentially protective, as shown in the hamster model of SARS-CoV-2 infection. Intriguingly, antibodies resulting from a Wuhan-delivered antigen cross-react with the Omicron variant suggesting that the SporCoVax spore vaccine described here might be protective against emerging VOCs. Additionally, intranasal boosting with spores that display both S specific antigens and the highly conserved N protein evoked robust S and N-specific mucosal IgA and resulted in modest protection against the ancestral SARS-CoV-2 strain. This data suggests that the incorporation of a highly conserved immunogen such as N may promote protection against current and emerging VOCs and should be evaluated further. Spores are dormant entities that are found in soil and air and as such in humans

and animals also (Nicholson, 2002). As dominant members of the aerobiome (~10%) humans are continuously exposed to *Bacillus* spores with approximately 10–10^5 spores inhaled daily (Shaffer & Lighthart, 1997). In the gastrointestinal tract, they are present at constant levels of ~10^4 spores/g of faeces (Hong *et al.*, 2009). The spore vaccine platform has been well documented, and spores genetically engineered or adsorbed with heterologous antigens, evoke balanced Th1/Th2 responses as well as augmenting mucosal antibody responses (Barnes *et al*., 2007). This is supported by the studies here using a genetically engineered spore vaccine and resulting antibody and cytokine responses. Using the THY-X-CISE® cloning, spores, should they germinate, are unable to proliferate in the environment since live cells die immediately due to a 'thymine-less' death (Hosseini *et al.*, 2018). Biological containment is thus ensured. Spore vaccines can be constructed rapidly on a case-by-case basis although, as shown here. Coupled with their ease of production, heat stability, and extraordinary resistance properties, spore vaccines, used mucosally, lend themselves to pandemic situations, facilitating a method with which to both augment systemic immunity as well as induce mucosal immunity, a prerequisite for complete protection. As such, employed in a heterologous systemic prime–mucosal boost regimen, spore vaccines might have utility for current and future emerging diseases.

The work described in this chapter has been published in the Journal Vaccines (Katsande et al., 2022).

CHAPTER 5: Prophylactic immunisation to *Helicobacter pylori* infection using spore vectored vaccines

5.1 Introduction

Persistent gastric infection caused by *Helicobacter pylori* results in chronic inflammation of the stomach lining which in turn can progress to more serious gastrointestinal diseases such as gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa associated lymphoid tissue lymphoma (Nagy *et al*., 2016). Despite a decline in the prevalence of gastric cancer in certain regions, particularly in Western Countries, the disease remains a significant global health issue. With over 1 million new cases diagnosed each year, gastric cancer disease is recognised as the fifth most common cancer worldwide and is the third leading cause of cancer-related deaths. Approximately 770,000 deaths as a result of gastric cancer were estimated in 2020 (Bray *et al*., 2018; Morgan *et al*., 2022; Rawla & Barsouk, 2019). Global regions that have been reported to have the highest incidence rate of gastric cancer are in Eastern Asia, particularly, Mongolia (ASR 32.5 per 100,000, 24.6 per 100,000) which has the highest overall rate of gastric cancer, followed by Japan (ASR 31.6,) and South Korea (ASR 27.9) (Morgan *et al*., 2022). Unfortunately, the diagnosis of gastric cancer is often delayed because up to 80% of patients do not display specific symptoms during the early stages of cancer, moreover, most patients are diagnosed after gastric cancer has progressed and has invaded the muscularis and it is nearly impossible to eradicate the underlying infection (Layke & Lopez, 2004; Wroblewski *et al*., 2010).

While *H. pylori* infection can be treated in a majority of individuals using antibiotic therapy combined with a proton pump inhibitor, eradicating *H. pylori* using combinational antibiotic therapy is challenging particularly in regions with a high prevalence and/or antibiotic resistance (Savoldi *et al*., 2018). The prevalence of antimicrobial resistance (notably to clarithromycin and metronidazole) (Frenck & Clemens, 2003) is so high that many infected patients are now considered as having fully resistant infections (Graham & Shiotani, 2008). Importantly, antimicrobial therapy cannot protect against reinfection and the rate of reinfection is as high as 15-30% per year (Parsonnet, 2003).

It has been suggested that a 10-year vaccination program might significantly reduce the impact of *H. pylori* infection both with regard to symptoms, gastric cancer and the associated economic burden of disease management (Rupnow *et al*., 2001). Conceptually a vaccine would best be administered orally to enable the production of secretory IgA (sIgA) in the stomach mucosa preventing colonisation (Agarwal & Agarwal, 2008; Czinn & Blanchard, 2011; Svennerholm & Lundgren, 2007). However, other mucosal delivery routes (intranasal, rectal) have been successfully used. Based on the pathogenesis of *H. pylori* several putative protective antigens have been evaluated including urease (subunits UreA and UreB), flagellar antigens (FlaA and FlaB), cytotoxin-associated gene A (CagA), vacuolating toxin (VacA) and others. Vaccine formulations including subunit vaccines, live vector vaccines, DNA vaccines as well as other delivery systems have been evaluated (Agarwal & Agarwal, 2008; Czinn & Blanchard, 2011; Svennerholm & Lundgren, 2007). One of the major problems with oral immunisation is that the resulting immunity is weak. Accordingly, adjuvants such as cholera toxin (CT), the closely related

heat-liable (LT) of *E. coli* or the B subunit of CT (CTB) have been extensively evaluated (C. K. Lee, 2001; Raghavan *et al*., 2002). Although there has been considerable effort in vaccine development few human studies have demonstrated convincing levels of protective immunity (Czinn & Blanchard, 2011; Svennerholm & Lundgren, 2007). The one promising exception being a recently described oral vaccine consisting of an orally-administered protein formulation comprised of UreB fused to LT (Zeng *et al.*, 2015). Despite this, there is a case for vaccination where even reduced efficacy might shorten existing treatment regimens and help protect against reinfection (Svennerholm & Lundgren, 2007).

5.2 Results

5.2.1 Immune responses in mice dosed with spores expressing *H. pylori* antigens

Mice (C57 BL/6) were orally dosed (i.g.) four times (a single dose consisting of three consecutive daily oral administrations, totaling 12 oral administrations) with spores of PK82 (CotB-UreA), PK78 (CotB-UreB^{CT}) and the isogenic control PK118 strain that carried insertional disruption of both *thyA* and *thyB* but carried no chimeric genes. To enable a dose of 3×10^{10} spore (CFU) three daily administrations of 1×10^{10} spores were required since high concentrations of spores in suspension are typically overly viscous. A naïve group receiving only PBS provided a baseline. Measurement of antigen-specific sIgA in faecal samples showed seroconversion to UreA in mice dosed with PK82 (CotB-UreA) and UreB in mice dosed with PK78 (CotB-UreB^{CT}) (Figure 5.1). Maximal antibody responses were observed at day 61. Responses for both PK78 (Figure 5.1A) and PK82-dosed animals (Figure 5.1B), at day 61, were significantly ($p = 0.0001$) greater than

in mice dosed with PK118 spores or the naïve group. Very low levels of UreA-specific sIgA were observed in PK118-dosed mice but these were not statistically significant.

Interestingly, mice that received oral administrations of PK78 spores (CotB-UreB^{CT}) displayed high levels of UreB-specific sIgA in faecal samples at day 31 (after the second immunisation). From this data, it is possible that the number of doses that are required to elicit mucosal immune responses can be reduced from four immunisation to two immunisations (totaling 6 oral administrations) for the PK78 spore vaccine.

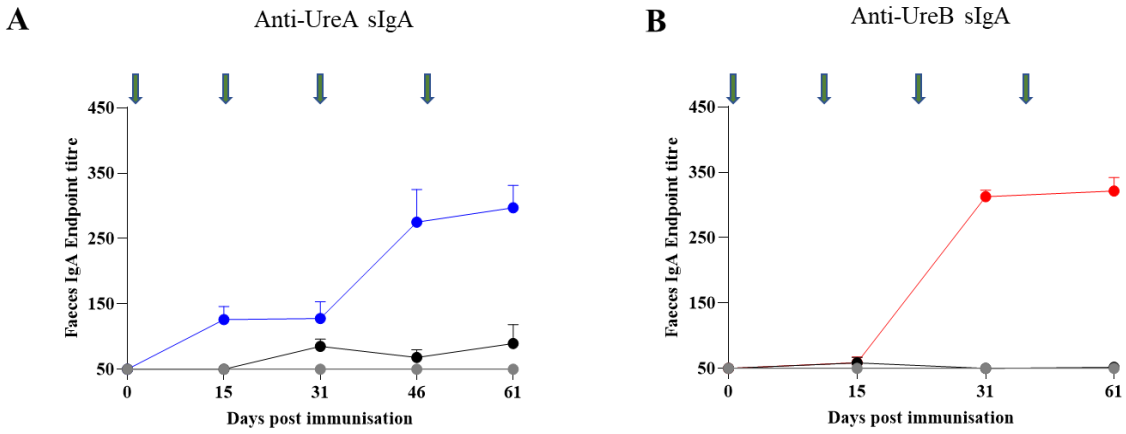

Figure 5.1: Mucosal responses following oral administration of spore vaccines expressing *H. pylori* antigens. Mice (C57 BL/6), were dosed (i.g.) with spores of PK118 (WT), PK82 (CotB-UreA) or PK78 (CotB-UreB^{CT}) four times (green arrows). Each dose comprised three separate administrations (1×10^{10} CFU/administration); dose 1 (days 1-3), dose 2 (days 16-18), dose 3 (days 32-34) and dose 4 (days 47-49). rUreA-specific sIgA in longitudinal faecal samples from PK82 (blue circles), PK118 (black circles) or naïve (grey circles) (***Panel A***) and rUreB-specific sIgA in longitudinal faecal samples from PK78 (red circles) or PK118 (black circles) dosed mice are shown (***Panel B***). This experiment was replicated twice.

To further assess the immunogenicity of PK78 (CotB-UreA) and PK82 (CotB-UreB^{CT}) spore vaccines. Serum samples were collected via terminal heart bleeds at day 61 and were used to measure rUreB and rUreA-specific IgG. Serum IgG responses measured on

day 61 showed that both PK78 (Figure 5.2B) and PK82 (Figure 5.2A) were able to induce systemic immunity. Mice that received oral administrations of PK78 spores (CotB-UreB^{CT}) displayed higher levels of UreB-specific IgG in faecal samples, compared to mice that received oral administrations of PK82 spores (CotB-UreA). This might suggest that PK78 spores expressing the C-terminal of UreB as the putative vaccine antigen might be better at inducing systemic immune responses than PK82 spores expressing the whole UreA vaccine antigen. Despite these differences, PK82 spores were able to elicit significantly higher levels of UreA-specific IgG in mice, compared to mice that received isogenic PK118 spores (WT control, no antigen expression) ($p < 0.001$). IgG levels were significantly higher ($p < 0.0001$) in mice that received oral administrations of PK78 than in mice receiving isogenic PK118 spores. Taken together, oral administration of spores expressing either UreA or UreB^{CT} on the spore surface can elicit robust mucosal and systemic responses.

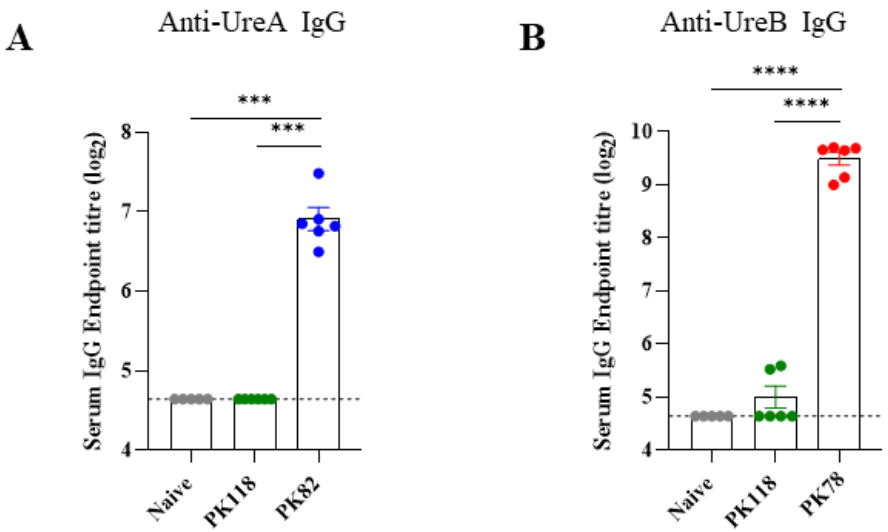

Figure 5.2: Systemic responses following oral administration of spore vaccines expressing *H. pylori* antigens. Mice (C57 BL/6) were dosed (i.g.) with spores of PK118 (WT), PK82 (CotB-UreA) or PK78 (CotB-UreB^{CT}) 4 times. Each dose comprised three separate administrations (1×10^{10} CFU/administration); dose 1 (days 1-3), dose 2 (days 16-18), dose 3 (days 32-34) and dose 4 (days 47-49). rUreA-specific (***Panel A***) and rUreB-specific (***Panel B***) IgG in serum samples taken on day-61 are shown. Mann-Whitney, ***, p = 0.001, ****, p = 0.0001. This experiment was replicated twice.

5.2.2 Protection in a murine colonisation model

The prophylactic effects of PK78 (CotB-UreB^{CT}) or PK82 (CotB-UreA) spore vaccines were assessed by counting the *H. pylori* bacterial load (CFU) in stomach samples. Mice were given four oral (a single dose consisting of three consecutive daily oral administrations) doses of spores (1×10^{10}/dose) of either PK118 (WT), PK78 (CotB-UreB^{CT}) or PK82 (CotB-UreA), as well as a naïve group, and then challenged with *H. pylori* (i.g.) using a challenge dose of ~ 10^8 CFU. This was performed by our collaborators at the Institute of Vaccines and Biological Medicals (IVAC). Stomach samples were taken 21 days post-challenge for enumeration of *H. pylori* CFU. The study was repeated and combined CFU data is shown in Figure 5.3. PK78 (CotB-UreB^{CT}) immunised animals showed the greatest reduction in *H. pylori* CFU of about 1-log. PK78-dosed animals showed a significant reduction in CFU (89%, median values, $p<0.0001$). PK82 (CotB-UreA) dosed animals also showed a significant reduction (~72%, median values; $p=0.0001$) in CFU. Although both PK78 and PK82 spores resulted in a significant reduction in *H. pylori* bacterial load (CFU), PK78 dosed animals displayed a higher level of protection than PK82-dosed animals. It has been reported that UreB-based vaccines seem to be more protective than UreA (Ferrero *et al*., 1994a) and this is evident in this work. Interestingly, animals dosed with ‘naked’ spores (PK118), i.e., spores displaying

no *H. pylori* antigens, also showed a reduction (~40%, median value, $p<0.01$) in *H. pylori* CFU compared to naïve animals (Figure 5.3). Overall, both spore vaccines (PK82; CotB-UreA and PK78; CotB-UreB^{CT}) expressing either UreA or UreB^{CT}, were able to reduce bacterial burden after challenge with *H. pylori*, thus both spores were able to confer protective immunity sufficient to reduce *H. pylori* colonisation in mice.

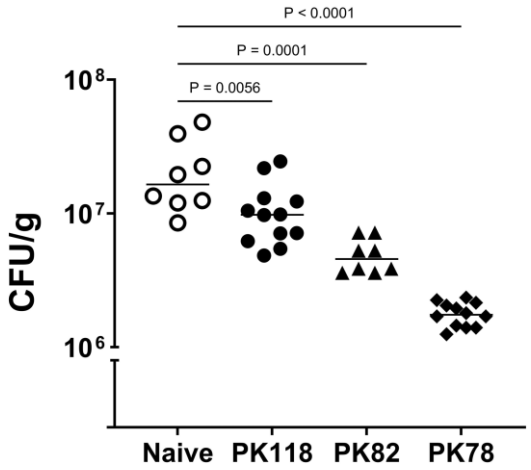

Figure 5.3. *H. pylori* colonisation in immunised mice. Bacterial loads of *H. pylori* HP34 in stomach samples 21 days post-challenge. Animals had been orally (i.g.) dosed 4-times with spores (1×10^{10} CFU/dose) of PK118 (WT), PK82 (CotB-UreA) or PK78 (CotB-UreB^{CT}) and challenged 7-8-9-days after the last immunisation. Naïve mice received PBS. The data combines samples from 2 independent repeat studies with P values as shown.

5.3 Discussion

For *H. pylori* vaccination two 'classical' antigens, UreA (urease A) and UreB (urease B) were evaluated since these have been used extensively in vaccine formulations and animal studies have shown evidence of protection (Bumann *et al*., 2001; Corthésy-Theulaz *et al*., 1998; Gómez-Duarte *et al*., 1998b; Hinc *et al*., 2010; Smythies *et al*., 2005; J. Yang *et al*., 2015; W.-Y. Zhou *et al*., 2009). The data show firstly that twelve oral doses of spores expressing either UreA or $UreB^{CT}$ evoked mucosal immunity evident from seroconversion of antigen-specific sIgA in faecal samples. It should be noted that IgG is also present in mucosal samples (Sano *et al*., 2022), however the levels of antigen specific IgG was not assessed in this study. UreB has previously been used for *H. pylori* vaccination utilising spores for oral delivery but this has incorporated the entire UreB polypeptide fused to the CotC spore coat anchor (Z. Zhou *et al*., 2015a, 2017b). Here, a truncated UreB domain, $UreB^{CT}$, was chosen to optimise spore expression since the use of the CotB anchor partner significantly increases the size of the resulting hybrid protein. Secondly, systemic antigen-specific IgG responses were also induced. Finally, when mice were dosed with UreA or $UreB^{CT}$ spores and then challenged with *H. pylori* the resulting counts of *H. pylori* CFU in the stomach were reduced by about 1-log (for CotB-$UreB^{CT}$). This data is broadly similar to those obtained by Zhou *et al* using spores expressing the full-length UreB protein (CotC-UreB) (Z. Zhou *et al*., 2017b) although there were some differences that need discussion. First, Zhou *et al* evaluated, in parallel, spores of an isogenic control strain that did not express any *H. pylori* antigens and in protection studies, mice showed no reduction in counts of *H. pylori* (Z. Zhou *et al*., 2017b). This is in marked contrast to the work presented here which showed that spores alone (i.e.,

PK118 spores) conferred a low level of protection (40% reduction in gastric CFU). These spores do not evoke antigen-specific sIgA so the most probable explanation is that of innate immunity. As previously mentioned, *Bacillus* spores have been well documented as being able to evoke innate immunity and for some pathogens such as influenza, this can be protective (de Souza *et al*., 2014; James *et al*., 2022; Song *et al*., 2012). This has included murine studies showing reduced colonisation by *C. difficile* following oral dosing with 'naked' spores (Permpoonpattana *et al*., 2011). It could be speculated that repeat dosing with *B. subtilis* spores may trigger an innate immune response sufficient to exert some level of protection. A second point is that Zhou *et al* also evaluated a trimeric fusion protein comprising a CotC anchor fused to CTB (cholera toxin subunit B) and UreB. This vaccine provided the highest reduction in gastric CFU of ~90% (Z. Zhou *et al*., 2017b) and was thus similar to our data found here for CotB-UreB^{CT}. CTB was employed as a mucosal adjuvant, but it is possible that the natural and well-documented microparticulate adjuvant properties of spores are sufficient to provide adjuvancy dispensing with the need for an auxiliary mucosal adjuvant component (Barnes *et al*., 2007; de Souza *et al*., 2014). It is challenging to directly compare the immunogenicity and efficacy of various vaccine formulations against *H. pylori* due to differences in experimental designs and animal models used for assessing vaccine efficacy. While many studies have investigated different vaccine candidates, including those utilising urease subunits (Bumann et al., 2001; DiPetrillo et al., 1999; Rizos et al., 2003), multi-epitope DNA vaccines with CpG oligonucleotides and heat-labile enterotoxin LTB (S. F. Moss et al., 2011), as well as constructs involving *H. pylori* opiA gene and IL-2 gene, along with adjuvants (J. Chen et al., 2012), and formulations containing CagA, VacA, and UreB proteins (K.-Y. Liu et al., 2011), none of the studies have comprehensively evaluated all

parameters, including humoral response, cellular response, and vaccine efficacy in murine models, in a single study. This lack of comprehensive evaluation hinders the ability to directly compare the efficacy of different vaccine formulations. Additionally, the use of diverse animal models further complicates comparisons. While some studies utilise mice, others may use different animal models, leading to variations in immune responses and vaccine efficacy. Therefore, future studies that employ standardised methodologies and assess multiple parameters are needed to facilitate meaningful comparisons and advancements in the development of effective *H. pylori* vaccines.

Neither of the two antigens reported here have previously been evaluated using spores (note that Zhou *et al* used complete UreB, (Z. Zhou *et al*., 2017b). Examination of other *in vivo* studies on *H. pylori* using live-vectored vaccines has shown that a 90% reduction in *H. pylori* CFU is close to the maximum that can be achieved. The fact that both UreA and UreB delivered on spores confer some level of protection reinforces the general finding that a variety of *H. pylori* antigens can be used for vaccination (Svennerholm & Lundgren, 2007). It also supports the notion that other elements of the immune system may be required to achieve full sterilising immunity (Agarwal & Agarwal, 2008; Chmiela & Michetti, 2006). It is well documented that cell-mediated immunity plays an important role in protection against *H. pylori* infection (Agarwal & Agarwal, 2008; Czinn & Blanchard, 2011). Gastric biopsy samples from infected patients display an increase in CD4+ T cells (Chmiela & Michetti, 2006) and a bias of Th1 cells has been considered necessary for protection (Eaton *et al.*, 2001). In addition, UreB has been shown to induce Th17 cells that, in turn, are responsible for the production of the proinflammatory cytokines, IL-17, IL-17F and IL-22 (J.-Y. Zhang *et al*., 2011). Oral administration of

Bacillus spores has been shown in mice to interact with components of the cellular immune system, notably Toll-like receptors (TLRs), with *in vivo* induction of proinflammatory cytokines (TNF-α and IL-6) (J.-M. Huang, La Ragione, Nunez, *et al*., 2008). Potentially, these phenotypes may be linked with the above-mentioned innate response and it is possible that the immunostimulatory properties of spores alone may also be contributing to the inhibition of *H. pylori* colonisation. Lastly, as humans are exposed to low levels of *Bacillus* on a daily basis future development of the spore platform must consider and address the issue of tolerance and suppression of the immune response (Homayun *et al*., 2019).

Our study has several limitations. First, the prophylactic effects of recombinant spore vaccine candidates (PK78; CotB-UreB^{CT}, PK82; CotB-UreA) was evaluated in Mlac: ICR male mice only. It has been reported that the prevalence of *H. pylori* infection is higher in male individuals than females, indicating that males are potentially more susceptible to *H. pylori* infection (Chitsazi *et al*., 2006). Aebischer *et al,* demonstrated that immunisations with a recombinant *Salmonella* vector expressing UreA and UreB subunit resulted in higher levels of protection in female mice than male mice (Aebischer *et al*., 2001). Although it was observed that both spore vaccine candidates were able to confer protective immunity sufficient to reduce *H. pylori* colonisation in male mice, further studies should be conducted to compare the potential differences in the efficiencies of PK78 (CotB-UreB^{CT}) and PK82 (CotB-UreA) vaccination in male and female mice. Additionally, immunogenicity of the recombinant spore vaccine candidates (PK78; CotB-UreB^{CT}, PK82; CotB-UreA) was evaluated in female C57BL/6 mice only, by measuring relative titres of ureA- or UreB-specific immunoglobulins in faecal and

serum samples. Further studies should be conducted that evaluate the immunogenicity of the recombinant spore vaccine candidates in both female and male C57BL/6 mice, to allow for comparisons in humoral immune responses in different genders. Second, oral-based vaccines require a higher dose of antigen to induce sufficient humoral and cellular immune responses (Permpoonpattana *et al*., 2011). In this work, mice received three daily oral administrations of 1×10^{10} spore CFU, with the total number of doses equating to 12 doses, which is very high. However, it was observed that mice that received oral administration of PK78 (CotB-UreB^{CT}) displayed high levels of antigen-specific sIgA after the second immunisation (6 doses), whereas for PK82 oral administrations, a total of three immunisations (9 doses) was required to elicit significantly high levels of antigen-specific sIgA in mice. From this data, it is possible that a lower dose of spores might be effective at stimulating robust mucosal and systemic immune responses, as well as providing protection against *H. pylori* infection. However, further studies evaluating the optimal dose of the *B. subtilis* spore vaccine candidates providing adequate protection against *H. pylori* are yet to be examined. Thirdly, histopathologic evaluations of gastritis and epithelial changes were not performed in the stomachs of mice that received oral administration of PK78 and PK82 and then challenged with *H. pylori*. Histopathological analysis of stomach samples is crucial to determine if the spore vaccines provide adequate protection against *H. pylori* and to understand the potential benefits or risks associated with the spore vaccines. This warrants further studies to evaluate the protective efficacy of the spore vaccine candidate. For instance, a decrease in tissue damage or inflammatory response following an immunisation and challenge with *H. pylori* might suggest that the vaccine is effective at preventing or mitigating *H. pylori* and its associated gastrointestinal damage.

For future work, it would be interesting to assess the immunogenicity and protection efficacy of a cocktail spore vaccine consisting of a combination of spores that express UreA and UreB, as well as spore expressing other protective immunogenic antigens including vacuolating cytotoxin (VacA) and/or the cytotoxin-associated antigen (CagA). VacA (T. Cover & Blaser, 1992) and CagA (Covacci *et al*., 1993) are important major virulence factors of *H. pylori* (CagA and VacA positive strains) and are associated with more severe disease and potentially increase the risk of developing ulcers and gastric cancer (El-Shenawy *et al*., 2017). Lui *et al*, showed that oral immunisation with an attenuated *Salmonella vector*, which expressed a fusion protein consisting of CagA, VacA and UreB significantly reduced *H. pylori* bacterial load in the stomach, and protection was related to specific $CD4^+$ T cell Th1 type responses, as well as mucosal (sIgA) and systemic (IgG) immune responses (K.-Y. Liu *et al.*, 2011). Thymine auxotrophic *Bacillus* strains carrying *cotB-cagA* (PK80; CotB-CagA) and *cotB-v*acA (PK84; CotB-VacA) insertion at the *thyA* loci have been constructed, however, the expression and display of these chimeric protein need to be confirmed by Western blotting and whole spore enzyme-linked immunosorbent assay (ELISA) before further evaluation and utilisation in immunisation studies.

5.4 Conclusion

The data demonstrated that oral administrations of spores carrying either the whole UreA antigen or C-terminal fragment of UreB lacking the active site showed antigen-specific mucosal responses (faecal sIgA) including seroconversion and hyper-immunity in mice. Moreover, both spore vaccines evoked systemic immune responses. When mice were orally immunised with PK78 and PK82 spores and then challenged with *H. pylori*,

colonisation by *H. pylori* was significantly reduced by up to 1-log. This study demonstrates the potential utility of bacterial spores for mucosal vaccination for *H. pylori* infection. The heat stability and robustness of *Bacillus* spores coupled with their existing use as probiotics make them an attractive solution for either protection against *H. pylori* infection or potentially for therapy and control of active infection. Additionally, the use of a THY-X-CISE system that ensures containment of GM probiotic spores is a further advantage primarily because the use of GMOs in humans remains contentious. Although not addressed here a therapeutic application of a *H. pylori* spore vaccine is worthwhile for consideration and is under current investigation. Finally, the spore platform enables other potential *H. pylori* antigens to be evaluated and potentially a multivalent vaccine to be formulated. Such an approach might further boost the levels of protection.

The work described in this chapter has been published in the Journal of Wiley Helicobacter (Katsande et al., 2023).

CHAPTER 6: General Discussion

6.1 Safety aspects of genetically modified organisms

In this work, a newly developed cloning system termed the 'THY-X-CISE' system was used to construct various recombinant strains that display heterologous antigens with immunological activities on the surface of the spore (Hosseini *et al*., 2018). However, strains constructed using this system are referred to as GMOs, which is a major drawback that stands in the way of its marketing and applications in vaccine and drug development. The subject of GMOs is a very controversial topic among scientists, the public and in the case of genetically modified food, food producers and consumers due to the potential biomedical risk and environmental side effects. Contrary, some still believe that genetic engineering might offer tremendous benefits in agriculture, as well as improving health benefits in humans and animals through the production of novel protein replacements, drugs and vaccines. For instance, the first produced GMO consumer product was human insulin (Humulin) in 1979, and was approved by the FDA in 1982 for use in humans for the treatment of diabetics and is widely accepted to this date (De Meyts *et al.*, 1981; Goeddel *et al*., 1979). Another example is the development of Golden Rice, which is genetically engineered rice that can be used as a cheap and effective way to supply dietary vitamin A to individuals in developing countries with vitamin A deficiency and thus at risk of developing blindness as a result of this deficiency (Potrykus, 2001; Sommer *et al*., 1981; X. Ye *et al*., 2000). The application of genetic engineering techniques in medicine is vast including the mass production of human insulin, Follistim which induces ovulation in women that cannot ovulate (infertile), monoclonal antibodies and many FDA approved

drugs and vaccines (Andrews *et al*., 2021; Dickey *et al*., 2003; Goeddel *et al*., 1979; Mullard, 2021; Shahryari *et al*., 2019; Tanne, 2022). However, social scientific evidence suggests that there is widespread public concern about the long-term safety and health effects of GMOs on the environment and humans and these misconceptions may have a negative impact on the public acceptance of GMOs (McFadden & Huffman, 2017). This is despite overwhelming scientific evidence that has shown that GMOs are safe for consumption, offer environmentally sustainable benefits by increasing yields, reduce cost and the environmental footprint of agriculture, as well as the potential to offer health benefits as exemplified above. In August 2013, the Philippine Government's International Rice Research Institute (IRRI) and other public sector partners that are in charge of managing a research field of Golden Rice (vitamin A enhance rice) were attacked by anti-GMO activists, and fields were vandalised. There was a strong scientific community condemnation following the 2013 incident, however, this reaction failed to achieve a consensus among the general public and some are in support of the actions of the protestors (Kupferschmidt, 2013). A survey study conducted by the Pew Research Center reported that 67% of respondents stated that they were convinced that scientists do not have a clear understanding of the risks and health benefits of GMOs. Furthermore, 48% of respondents stated that GM foods are harmful and 37% of respondents said they lack enough knowledge to make a decision (Blagoevska *et al*., 2021; Funk, 2015; Lucht, 2015). A possible explanation for this is the continuing lack of comprehensive understanding of the nature of GMOs, the health benefits and the difficulties that agriculture is currently facing with the growing population and climate change (Zilberman *et al.*, 2018).

Many studies have been conducted, comparing the effects and safety of GM foods in humans and animals to address some of the many questions that have been raised around the use of GM foods. The questions raised, mainly focus on human and environmental safety, food labelling, ethics, food security, environmental conversation, and poverty reduction (Bawa & Anilakumar, 2013). Finamore *et al*, reported that mice fed a diet containing GM maize (MON810) resulted in a significant increase in the expression of cytokines (including L-6, IL-13, IL-12p70, and MIP-1β) compared to mice fed GM-free maize (Finamore *et al*., 2008). These cytokines are associated with allergy and asthma (Alam *et al.*, 1992; Chung, 2001; Lambrecht & Hammad, 2013). A recent study identified 21 adverse events related to GM foods that have been approved in some countries and regions. These adverse events included mortality, reduced fertility, decreased cognitive function, tumour or cancer and some pathological abnormalities in the mammary glands, liver and kidney (Shen *et al*., 2022). Although GM foods are available and have been approved to be safe for animal and human consumption in many countries and regions, these studies highlight that there is still a potential health risk for both animals and humans and could also cause environmental damage.

The International Union for Conservation of Nature prepared a report in 2004 that addresses the potential risks associated with GMOs and the environment. They report that GMOs that are intended for use in an uncontrolled environment might increase the possibility of interbreeding with wild-type organisms which could result in a loss of novel traits that are essential to the wild-type organism unless it offers a selection benefit to the recipient organism. Additionally, GMOs that have been engineered to increase growth rates can reduce the genetic diversity of organisms in the environment by out-competing

them, thus increasing the likelihood of organism decline and possible extinction. The associated risk of interbreeding can also increase the risk of GMOs and interbred species spreading to new habitats and this can have a negative impact on the economy and might contribute to ecological damages (Young, 2004).

On the other hand, genetic modification techniques are widely used to develop drugs and vaccines for both animals and humans. For instance, a live attenuated influenza vaccine (LAIV) virus vector expressing various T-cell epitopes of SARS-CoV-2 antigen (LAIV/SARS-CoV-2), when administered intranasally protected hamsters against influenza virus and a high dose of SARS-CoV-2 variants (Isakova-Sivak *et al*., 2022). Another example, a live attenuated recombinant *Listeria monocytogenes* (rLm)-vectored TB vaccine expressing multiple immunogenic *Mycobacterium tuberculosis* antigens, when delivered intramuscularly in mice was shown to enhance antigen-specific CD4+ and CD8+ T-cell responses and was safe and protective against *M. tuberculosis* (Jia *et al*., 2022). However, a major concern regarding GM vaccines and crops is that the majority of genetic modification techniques used during the cloning stage require the insertion of antibiotic resistance gene markers for the selection of transgenic organisms or microorganisms. Another fundamental concern regarding GMOs relates to the possibility of horizontal gene transfer (HGT). Transfer of transgenic DNA and/or antibiotic resistance genes through HGT, in principle could potentially lead to novel pathogens with multi-drug resistance or with enhanced pathogenicity or could result in the development of new emerging diseases that could have a detrimental effect on human and animal health, as well as the economy if they result in an outbreak or a pandemic (Stirling & Silver, 2020; Vermeire, 2015). The spread of antibiotic resistance genes from

GMOs into pathogens poses a risk to human health and if multidrug-resistant pathogens or new pathogens emerge as a result of HGT they can potentially comprise the current animal and human therapies, increase treatment costs if new therapies are required (Bennett *et al*., 2004; WHO, 2017). In this work, GM *B. subtilis* spores have been developed as a potential prophylactic vaccine against *H. pylori* and a booster vaccine against SARS-CoV-2. An issue regarding the use of GM *B. subtilis* is that once they are administered *in vivo*, the spores can proliferate by germinating in the gut, followed by sporulation and can survive in the faeces thus increasing the risk of spreading GM spores in the environment. Once released in the environment spores can germinate under favourable conditions and can potentially spread the transgenic DNA and/or antibiotic-resistance genes into related species or other bacterial species. The THY-X-CISE biocontainment system that has been applied in this work, has the potential to address the concerns that have been highlighted above and might offer solutions that allow the use of GM *Bacillus* spores for both commercial and therapeutic purposes. This system might help to prevent the dissemination of transgenic DNA to other bacterial species and environmental dissemination of GM spores because the inactivation of both thymidylate synthase genes (*thyA* and *thyB*) generates a thymine auxotrophic strain, that is bactericidal in the absence of exogenous thymine supplementation. Therefore, if spores are released in the environment, they undergo thymineless death, thus reducing the risk of GM spores from spreading in the environment as well as, reducing the risk of interbreeding with wild-type species and HGT to other bacterial species. A recent study using a mouse colitis model showed that mice infected with streptomycin resistance-encoding mobilisable plasmid pRSF1010 (P3) of *Salmonella Typhimurium*, and once they colonised the gut lumen, were able to transfer the mobilisable plasmid P3 through

conjugation into other bacterial species including, *Escherichia coli* Z1331, *Yersinia enterocolitica* and *Citrobacter braakii* (Gaissmaier *et al*., 2022). This study shows that HGT of antibiotic resistance genes can occur. It is important to note that the risk of HGT and the spread of transgenic DNA and/or antibiotic resistance genes could be exacerbated in the context of *Bacillus* spores. Unlike, *Salmonella,* which is not a spore former, *Bacillus* has the ability to enter a dormant state and survive in the environment for prolonged periods. As the spores remain dormant, they may have a greater opportunity to come into contact with other bacteria in the gut. Moreover, the gut is not a typical environment for *Bacillus* spores, however, the introduction of GM spores containing antibiotic resistance gene markers into the gut via oral administrations could disrupt the microbial balance, potentially creating opportunities for interaction and HGT with other species and pathogenic bacteria. The THY-X-CISE system also addresses this issue, it does not require the use of antibiotic resistance gene as a selection marker, which implies that the risk of antibiotic resistance gene transfer is reduced because these genes are not present in the first place. This cloning system can also be incorporated in other bacterial species, virus and insects that are widely used as drug and vaccine delivery vehicles.

6.2 Therapeutic and commercial advantages of thymine auxotrophic strains

In this work, *B. subtilis* was selected as a live host for the delivery of immunogenic antigens on the spore surface. Numerous studies have shown that other species including *L. lactic, L. brevis, L. casei* and *L. salivarius* are available and can be genetically manipulated by inserting heterologous genes into *thy* genes, generating clones that are thymine auxotrophic and dependent on exogenous thymine supplementation (B. Ma *et al*., 2016; Steidler *et al*., 2003; H. Zhou *et al*., 2018). For instance, a thymine auxotrophic

strain of *L. lactis* that expresses lactoferricin showed excellent antimicrobial and antiviral activity with good genetic stability, with the potential use as a feed additive in live stock (H. Zhou *et al.*, 2018). Another study constructed a thymine auxotrophic strain of *L. salivarius* as a delivery vehicle that secretes multi-epitope antigens of infectious bronchitis virus (IBV) and could potentially be used as an oral vaccine that protects chicken against IBV (B. Ma *et al.*, 2016). However, *B. subtilis* might be a better host candidate compared to these species, for numerous reasons. First, is that these species are non-spore formers and are likely to be more sensitive to harsh environmental conditions compared to spores of *B. subtilis* which are highly resistant to various environmental factors, as previously mentioned. Second, these species carry a single *thy* gene, therefore, only one copy of the heterologous gene can be inserted in the *thyA* loci. Contrary, *B. subtilis* carries two *thy genes* which allow the insertion of two copies of the same chimeric gene to be inserted into the *thyA* and *thyB* genes. In this work, a single copy of a chimeric gene was inserted into the *thyA* gene, but Hosseini *et al* demonstrated that two copies of a chimeric gene can be inserted into the *thyA* and *thyB* gene increasing the number of chimeric proteins that are being displayed on the spore surface. For instance, a strain (SH14) carrying *cotB-tcdA*$^{26–39}$ insertions at the *thyA* and *thyB* loci expressed the TcdA protein at significantly higher levels compared to a strain carrying a single copy of *cotB-tcdA*$^{26–39}$ chimeric gene at the *thyA* locus (Hosseini *et al*., 2018). Although, the thymine auxotrophic strains constructed in this work carried a single copy of a chimeric gene and when the spore candidate vaccine was administered as a mucosal vaccine or as a mucosal booster vaccine resulted in protective immunity in animal models of *H. pylori* and SARS-CoV-2 infection. *H. pylori* immunogenic antigens such as UreA and UreB have been previously displayed on the *B. subtilis* spore surface using conventional cloning methods

(Bumann *et al*., 2001; Corthésy-Theulaz *et al*., 1998; Gómez-Duarte *et al*., 1998b; Hinc *et al*., 2010; Smythies *et al*., 2005; J. Yang *et al*., 2015; W.-Y. Zhou *et al*., 2009). Although these spore vaccines have shown potential as a protective mucosal vaccine against *H. pylori*, they have not made it into the market, nor have they been approved for human use. A possible explanation is that these spore vaccines do not offer biocontainment and require antibiotic gene markers for the selection of positive transformation, which is a major concern considering that the current treatment for *H. pylori* infection consists of antibiotics of which some are no longer effective at eradicating the pathogen due to AMR. The use of antibiotic resistance genes as a selection marker, and if these GM spores are shed in faeces they increase the risk of spreading antibiotic resistance genes which could further contribute to the development of multidrug-resistant *H. pylori* strains and other bacterial species in the environment. In chapter 5, thymine auxotrophic strains (PK78; *thyA*::*cotB-ureB*^{CT}) and (PK82; *thyA*::*cotB-ureA*) showed protective immunity against *H. pylori* in a mouse colonisation model, and if these spores are released into the environment, they under thymineless death because thymine is not readily available in the environment. In chapters 4, thymine auxotrophic strains expressing SARS-CoV-2 immunogenic antigens (PK120, *thyA*::*cotB*-RBD^{Wuh}; PK122, *thyA*::*cotC*-HR1-HR2^{Wuh}; PK128, *thyA*::*cotB*-N and PK230, *thyA*::*cotB*-RBD^{Omi}) when administered as an intranasal booster following an intramuscular prime COVID-19 vaccine or protein subunit conferred protective humoral and cellular immunity in a hamster model of SARS-CoV-2 challenge. Although these thymine auxotrophic spore vaccines seem promising as vaccine delivery vehicles in small animals, the stability, safety and efficacy of these thymine auxotrophic spore-based vaccines need to be explored further in larger animals and eventually in humans before they can be considered

for application as vaccines in humans. Hosseini *et al*, demonstrated that a thymine auxotrophic strain of *B. subtilis* expressing subtilisin E, an alkaline protease that is commonly incorporated into animal feeds retained its enzymatic activity. In the same study, they also showed that thymine auxotrophic strains expressing streptavidin could bind to biotinylated TcdA26–39 antibodies, and reduced toxicity by 90% when incorporated into crude cell-free lysate containing *C. difficile* toxins. Both, this work and Hosseini *et al*, exemplify the possibilities of using thymine auxotrophic GM spores as vaccine delivery vehicles against various diseases by inserting immunogenic antigens into *thy* genes or if the spore is expressing streptavidin, could act as a drug carrier to target biomarkers on cancer cells by conjugating with specific biotinylated antibodies. Furthermore, thymine auxotrophic spores can also be used as feed additives in the farming industries to improve the quality of feed and the health and performance of animals. Their bactericidal properties in the absence of thymine might significantly reduce or prevent the unintended proliferation and survival of GM *Bacillus* spores in the natural ecosystem. Additionally, the THY-X-CISE biocontainment strategy does not require antibiotic resistance gene markers for selection which reduces the risk of transferring antibiotic resistance genes to wild-type strains, other bacterial species and organisms in the environment.

6.3 The role of thymine auxotrophic *B. subtilis* spores in inducing mucosal immunity

Two different immunisation strategies were employed in this work. Chapter 5 demonstrated that thymine auxotrophic strains expressing *H. pylori* urease antigens could be use used as a primary, oral-based vaccine against *H. pylori* infection. *In vivo*, data showed that the oral delivery of thymine auxotrophic spores expressing UreA and $UreB^{CT}$ (lacking the active site) confer protection in a mouse model of *H. pylori* infection. Although, other studies have highlighted the importance of UreA and UreB as a target for vaccine development and has previously been shown to be protective. Studies have also reported that the active site of UreB is the most desirable and immunogenic region for vaccine development. However, these studies have not developed a vaccine that consists of the C-terminal region (lacking the active site) and does not compare the full-length UreB vaccine to a UreB vaccine lacking the active site (Corthésy-Theulaz *et al*., 1998; Ferrero *et al*., 1994b; Mobley, 2001). This is the first study to demonstrate that the C-terminal region of UreB lacking the active site induced robust antigen specific mucosal responses in faecal samples and also conferred protection in a mouse model of *H. pylori* infection.

Chapter 4, demonstrated that thymine auxotrophic strains expressing SARS-CoV-2 immunogenic antigens (RBD, HR1-HR2 subunit of spike protein and NC) could be used as an intranasal spore booster, following an intramuscular prime immunisation with protein subunit COVID-19 vaccine. Spores expressing SARS-CoV-2 antigens when administered as an intranasal booster conferred protection in a hamster model of infection and induced robust mucosal and cellular immune responses in mice compared to animals vaccinated using the parenteral route only. Three interesting findings were reported in

this work. First, the demonstration that the delivery of spores expressing the RBD from the Wuhan-Hu-1 strain resulted in mucosal antibodies that are cross-reactive to the RBD from the Omicron variant. Intriguingly, wild-type thymine auxotrophic spores (no antigen) also elicited modest mucosal immune responses, although, at levels significantly lower than groups vaccinated with spores expressing SARS-CoV-2 antigens. This data shows that even wild-type spores can be used to boost pre-existing humoral antibodies, specifically, mucosal antibodies when administered via the mucosal route. Moreover, wild-type *B. subtilis* spores can enhance or augment humoral immune response, most importantly mucosal immune responses, and these responses are consistent with data observed in other studies showing that *Bacillus* spore carries adjuvant properties (Barnes *et al*., 2007; J.-M. Huang *et al*., 2010; Song *et al*., 2012). A recent study showed that oral co-administration of H9N2 influenza whole inactivated virus and *B. subtilis* spores enhanced local mucosal and systemic immune responses. Additionally, they reported that *B. subtilis* were acting as an adjuvant by inducing memory T-cell formation through the activation of intercellular adhesion molecule-1 (ICAM1) expressed by DCs (Lin *et al*., 2021). Dendritic cells (DCs) are important submucosal APCs which have been shown to provide a link between the innate immune response and the adaptive immune response. Other studies have also shown that spores can prime the innate immune system by interacting with TLRs (TLR2 and TLR4) which initiates the recruitment of NK cells and triggers the activation of the NK-κB pathway (de Souza *et al*., 2014; J.-M. Huang, La Ragione, Nunez, *et al.*, 2008; Song *et al*., 2012).

In conclusion, this work provides evidence that thymine auxotrophic *Bacillus* spores that express *H. pylori* immunogenic antigens, when used as an oral prophylactic vaccine could

elicit robust mucosal and systemic immune responses and conferred protection in a mouse model of *H. pylori* infection. Additionally, a heterologous systemic prime–mucosal boost strategy, consisting of thymine auxotrophic spores expressing SARS-CoV-2 antigens, when delivered as an intranasal booster evoked mucosal IgA, enhanced systemic IgG responses and is potentially protective, as shown in the hamster model of SARS-CoV-2 infection. Despite being GMOs, the spore vaccine platform is attractive and promising since it offers biological containment and since antibiotic resistance genes are not required in the cloning stage reduces the risk of spreading antibiotic resistance genes into wild-type species and other bacterial species. The THY-X-CISE system offers a means of engineering *B. subtilis* whiles ensuring biocontainment and can potentially result in improved strains that could enhance the use of GM spores as engineered probiotics, therapeutics for humans and animals, in animal feeds and as cell factories for the microbial production of chemicals and enzymes. Moreover, while the THY-X-CISE system has been used to develop various spore vaccine candidates, the assessment of their biocontainment was not within the scope of this study. As the study primarily focused on proving the concept of vaccine efficacy, the spores utilised were reliant on external thymine supplementation. Thus, the lack of experimental evidence regarding the biocontainment efficacy of these spores has been recognised. Future research is necessary to evaluate their biocontainment capabilities under different environmental conditions, given the potential differences in behavior between laboratory and natural environments.

References

Abayasingam, A., Balachandran, H., Agapiou, D., Hammoud, M., Rodrigo, C., Keoshkerian, E., Li, H., Brasher, N. A., Christ, D., Rouet, R., Burnet, D., Grubor-Bauk, B., Rawlinson, W., Turville, S., Aggarwal, A., Stella, A. O., Fichter, C., Brilot, F., Mina, M., … COSIN Study Group. (2021). Long-term persistence of RBD+ memory B cells encoding neutralizing antibodies in SARS-CoV-2 infection. *Cell Reports. Medicine*, *2*(4), 100228. https://doi.org/10.1016/j.xcrm.2021.100228

Aebischer, T., Laforsch, S., Hurwitz, R., Brombacher, F., & Meyer, T. F. (2001). Immunity against *Helicobacter pylor*i: Significance of Interleukin-4 Receptor α Chain Status and Gender of Infected Mice. *Infection and Immunity*, *69*(1), 556–558. https://doi.org/10.1128/IAI.69.1.556-558.2001

Agarwal, K., & Agarwal, S. (2008). *Helicobacter pylori* vaccine: From past to future. *Mayo Clinic Proceedings*, *83*(2), 169–175. https://doi.org/10.4065/83.2.169

Ahmad, F. B. (2022). Provisional Mortality Data—United States, 2021. *MMWR. Morbidity and Mortality Weekly Report*, *71*. https://doi.org/10.15585/mmwr.mm7117e1

Ahn, J. H., Kim, J., Hong, S. P., Choi, S. Y., Yang, M. J., Ju, Y. S., Kim, Y. T., Kim, H. M., Rahman, M. T., Chung, M. K., Hong, S. D., Bae, H., Lee, C.-S., & Koh, G. Y. (2021). Nasal ciliated cells are primary targets for SARS-CoV-2 replication in the early stage of COVID-19. *The Journal of Clinical Investigation*, *131*(13), e148517. https://doi.org/10.1172/JCI148517

Akada, J. K., Shirai, M., Takeuchi, H., Tsuda, M., & Nakazawa, T. (2000). Identification of the urease operon in *Helicobacter pylori* and its control by mRNA decay in response to pH. *Molecular Microbiology*, *36*(5), 1071–1084. https://doi.org/10.1046/j.1365-2958.2000.01918.x

Alam, R., Forsythe, P. A., Stafford, S., Lett-Brown, M. A., & Grant, J. A. (1992). Macrophage inflammatory protein-1 alpha activates basophils and mast cells. *The*

Journal of Experimental Medicine, *176*(3), 781–786. https://doi.org/10.1084/jem.176.3.781

Alaofi, A. L., & Shahid, M. (2021). Mutations of SARS-CoV-2 RBD May Alter Its Molecular Structure to Improve Its Infection Efficiency. *Biomolecules*, *11*(9), Article 9. https://doi.org/10.3390/biom11091273

Alba, C., Blanco, A., & Blanco, T. (2017). Antibiotic resistance in *Helicobacter pylori*. *Current Opinion in Infectious Diseases*, *30*(5). https://doi.org/10.1097/QCO.0000000000000396

Alexander, S. M., Retnakumar, R. J., Chouhan, D., Devi, T. N. B., Dharmaseelan, S., Devadas, K., Thapa, N., Tamang, J. P., Lamtha, S. C., & Chattopadhyay, S. (2021). Helicobacter pylori in Human Stomach: The Inconsistencies in Clinical Outcomes and the Probable Causes. *Frontiers in Microbiology*, *12*. https://www.frontiersin.org/articles/10.3389/fmicb.2021.713955

Alhazzani, W., Møller, M. H., Arabi, Y. M., Loeb, M., Gong, M. N., Fan, E., Oczkowski, S., Levy, M. M., Derde, L., Dzierba, A., Du, B., Aboodi, M., Wunsch, H., Cecconi, M., Koh, Y., Chertow, D. S., Maitland, K., Alshamsi, F., Belley-Cote, E., … Rhodes, A. (2020). Surviving Sepsis Campaign: Guidelines on the management of critically ill adults with Coronavirus Disease 2019 (COVID-19). *Intensive Care Medicine*, *46*(5), 854–887. https://doi.org/10.1007/s00134-020-06022-5

Alu, A., Chen, L., Lei, H., Wei, Y., Tian, X., & Wei, X. (2022). Intranasal COVID-19 vaccines: From bench to bed. *EBioMedicine*, *76*, 103841. https://doi.org/10.1016/j.ebiom.2022.103841

Amuguni, H., Lee, S., Kerstein, K., Brown, D., Belitsky, B., Herrmann, J., Keusch, G., Sonenshein, A., & Tzipori, S. (2012). Sublingual immunization with an engineered *Bacillus subtilis* strain expressing tetanus toxin fragment C induces systemic and mucosal immune responses in piglets. *Microbes and Infection*, *14*(5), 447–456. https://doi.org/10.1016/j.micinf.2011.12.001

Andrews, N., Stowe, J., Kirsebom, F., Toffa, S., Rickeard, T., Gallagher, E., Gower, C., Kall, M., Groves, N., O'Connell, A.-M., Simons, D., Blomquist, P. B., Zaidi, A., Nash, S., Iwani Binti Abdul Aziz, N., Thelwall, S., Dabrera, G., Myers, R.,

Amirthalingam, G., … Lopez Bernal, J. (2022). Covid-19 Vaccine Effectiveness against the Omicron (B.1.1.529) Variant. *The New England Journal of Medicine*, *386*(16), 1532–1546. https://doi.org/10.1056/NEJMoa2119451

Andrews, N., Stowe, J., Kirsebom, F., Toffa, S., Rickeard, T., Gallagher, E., Gower, C., Kall, M., Groves, N., O'Connell, A.-M., Simons, D., Blomquist, P., Zaidi, A., Nash, S., Aziz, N. I. B. A., Thelwall, S., Dabrera, G., Myers, R., Amirthalingam, G., … Bernal, J. L. (2021). *Effectiveness of COVID-19 vaccines against the Omicron (B.1.1.529) variant of concer*. https://doi.org/10.1101/2021.12.14.21267615

Angert, E. R. (2005). Alternatives to binary fission in bacteria. *Nature Reviews. Microbiology*, *3*(3), 214–224. https://doi.org/10.1038/nrmicro1096

Antos, D., Crone, J., Konstantopoulos, N., & Koletzko, S. (2005). Evaluation of a novel rapid one-step immunochromatographic assay for detection of monoclonal *Helicobacter pylori* antigen in stool samples from children. *Journal of Clinical Microbiology*, *43*(6), 2598–2601. https://doi.org/10.1128/JCM.43.6.2598-2601.2005

Aranda, J., Poza, M., Pardo, B. G., Rumbo, S., Rumbo, C., Parreira, J. R., Rodríguez-Velo, P., & Bou, G. (2010). A rapid and simple method for constructing stable mutants of *Acinetobacter baumannii*. *BMC Microbiology*, *10*(1), 279. https://doi.org/10.1186/1471-2180-10-279

Asahi, M., Azuma, T., Ito, S., Ito, Y., Suto, S., Nagai, Y., Tsubokawa, M., Tohyama, Y., Maeda, S., Omata, M., Suzuki, T., & Sasakawa, C. (2000). *Helicobacter pylori* CagA protein can be tyrosine phosphorylated in gastric epithelial cells. *The Journal of Experimental Medicine*, *191*(4). https://doi.org/10.1084/jem.191.4.593

Atherton, J. C., Cao, P., Peek, R. M., Tummuru, M. K., Blaser, M. J., & Cover, T. L. (1995). Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific vacA types with cytotoxin production and peptic ulceration. *The Journal of Biological Chemistry*, *270*(30), 17771–17777. https://doi.org/10.1074/jbc.270.30.17771

Awuku, Y. A., Simpong, D. L., Alhassan, I. K., Tuoyire, D. A., Afaa, T., & Adu, P. (2017). Prevalence of *helicobacter pylori* infection among children living in a rural

setting in Sub-Saharan Africa. *BMC Public Health*, *17*(1), 360. https://doi.org/10.1186/s12889-017-4274-z

Aziz, M. A., & Islam, M. S. (2022). Association of ACE1 I/D rs1799752 and ACE2 rs2285666 polymorphisms with the infection and severity of COVID-19: A meta-analysis. *Molecular Genetics & Genomic Medicine*, *10*(11), e2063. https://doi.org/10.1002/mgg3.2063

Baden, L. R., El Sahly, H. M., Essink, B., Kotloff, K., Frey, S., Novak, R., Diemert, D., Spector, S. A., Rouphael, N., Creech, C. B., McGettigan, J., Khetan, S., Segall, N., Solis, J., Brosz, A., Fierro, C., Schwartz, H., Neuzil, K., Corey, L., … Zaks, T. (2021). Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *New England Journal of Medicine*, *384*(5), 403–416. https://doi.org/10.1056/NEJMoa2035389

Bai, Z., Cao, Y., Liu, W., & Li, J. (2021). The SARS-CoV-2 Nucleocapsid Protein and Its Role in Viral Structure, Biological Functions, and a Potential Target for Drug or Vaccine Mitigation. *Viruses*, *13*(6), 1115. https://doi.org/10.3390/v13061115

Barnes, A. G. C., Cerovic, V., Hobson, P. S., & Klavinskis, L. S. (2007). *Bacillus subtilis* spores: A novel microparticle adjuvant which can instruct a balanced Th1 and Th2 immune response to specific antigen. *European Journal of Immunology*, *37*(6), 1538–1547. https://doi.org/10.1002/eji.200636875

Bawa, A. S., & Anilakumar, K. R. (2013). Genetically modified foods: Safety, risks and public concerns—a review. *Journal of Food Science and Technology*, *50*(6), 1035–1046. https://doi.org/10.1007/s13197-012-0899-1

Benini, S., Rypniewski, W. R., Wilson, K. S., Miletti, S., Ciurli, S., & Mangani, S. (1999). A new proposal for urease mechanism based on the crystal structures of the native and inhibited enzyme from *Bacillus pasteurii*: Why urea hydrolysis costs two nickels. *Structure*, *7*(2), 205–216. https://doi.org/10.1016/S0969-2126(99)80026-4

Bennett, P. M., Livesey, C. T., Nathwani, D., Reeves, D. S., Saunders, J. R., Wise, R., & Working Party of the British Society for Antimicrobial Chemotherapy. (2004). An assessment of the risks associated with the use of antibiotic resistance genes in genetically modified plants: Report of the Working Party of the British Society for

Antimicrobial Chemotherapy. *The Journal of Antimicrobial Chemotherapy*, *53*(3), 418–431. https://doi.org/10.1093/jac/dkh087

Ben-Yehuda, S., & Losick, R. (2002). Asymmetric Cell Division in *B. subtilis* Involves a Spiral-like Intermediate of the Cytokinetic Protein FtsZ. *Cell*, *109*(2), 257–266. https://doi.org/10.1016/S0092-8674(02)00698-0

Berlin, D. A., Gulick, R. M., & Martinez, F. J. (2020). Severe Covid-19. *New England Journal of Medicine*, *383*(25), 2451–2460. https://doi.org/10.1056/NEJMcp2009575

Beumer, J., Geurts, M. H., Lamers, M. M., Puschhof, J., Zhang, J., van der Vaart, J., Mykytyn, A. Z., Breugem, T. I., Riesebosch, S., Schipper, D., van den Doel, P. B., de Lau, W., Pleguezuelos-Manzano, C., Busslinger, G., Haagmans, B. L., & Clevers, H. (2021). A CRISPR/Cas9 genetically engineered organoid biobank reveals essential host factors for coronaviruses. *Nature Communications*, *12*(1), 5498. https://doi.org/10.1038/s41467-021-25729-7

Bhatnagar, T., Chaudhuri, S., Ponnaiah, M., Yadav, P. D., Sabarinathan, R., Sahay, R. R., Ahmed, F., Aswathy, S., Bhardwaj, P., Bilimale, A., Kumar, M. S., Logaraj, M., Narlawar, U., Palanivel, C., Patel, P., Rai, S. K., Saxena, V., Singh, A., Thangaraj, J. W., … Murhekar, M. V. (2022). Effectiveness of BBV152/Covaxin and AZD1222/Covishield vaccines against severe COVID-19 and B.1.617.2/Delta variant in India, 2021: A multi-centric hospital-based case-control study. *International Journal of Infectious Diseases*, *122*, 693–702. https://doi.org/10.1016/j.ijid.2022.07.033

Bi, Z., Hong, W., Yang, J., Lu, S., & Peng, X. (2021). Animal models for SARS-CoV-2 infection and pathology. *MedComm*, *2*(4), 548–568. https://doi.org/10.1002/mco2.98

Bilich, T., Nelde, A., Heitmann, J. S., Maringer, Y., Roerden, M., Bauer, J., Rieth, J., Wacker, M., Peter, A., Hörber, S., Rachfalski, D., Märklin, M., Stevanović, S., Rammensee, H.-G., Salih, H. R., & Walz, J. S. (2021). T cell and antibody kinetics delineate SARS-CoV-2 peptides mediating long-term immune responses in

COVID-19 convalescent individuals. *Science Translational Medicine*, *13*(590), eabf7517. https://doi.org/10.1126/scitranslmed.abf7517

Binda, F., Galazzi, A., Marelli, F., Gambazza, S., Villa, L., Vinci, E., Adamini, I., & Laquintana, D. (2021). Complications of prone positioning in patients with COVID-19: A cross-sectional study. *Intensive & Critical Care Nursing*, *67*, 103088. https://doi.org/10.1016/j.iccn.2021.103088

Binh, T. T., Shiota, S., Nguyen, L. T., Ho, D. D. Q., Hoang, H. H., Ta, L., Trinh, D. T., Fujioka, T., & Yamaoka, Y. (2013). The incidence of primary antibiotic resistance of Helicobacter pylori in Vietnam. *Journal of Clinical Gastroenterology*, *47*(3), 233–238. https://doi.org/10.1097/MCG.0b013e3182676e2b

Bj, B., Be, M., R, V. D. Z., J, L., Bj, H., C, V., Aj, H., R, D. G., Ad, O., & Pj, R. (2004). Severe acute respiratory syndrome coronavirus (SARS-CoV) infection inhibition using spike protein heptad repeat-derived peptides. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(22). https://doi.org/10.1073/pnas.0400576101

Blagoevska, K., Ilievska, G., Jankuloski, D., Dimzoska, B. S., Crceva, R., Nikolovska, & Angeleska, A. (2021). The controversies of genetically modified food. *IOP Conference Series: Earth and Environmental Science*, *854*(1), 012009. https://doi.org/10.1088/1755-1315/854/1/012009

Bleier, B. S., Ramanathan, M., & Lane, A. P. (2021). COVID-19 Vaccines May Not Prevent Nasal SARS-CoV-2 Infection and Asymptomatic Transmission. *Otolaryngology–Head and Neck Surgery*, *164*(2), 305–307. https://doi.org/10.1177/0194599820982633

Bloor, A. E., & Cranenburgh, R. M. (2006). An Efficient Method of Selectable Marker Gene Excision by Xer Recombination for Gene Replacement in Bacterial Chromosomes. *Applied and Environmental Microbiology*, *72*(4), 2520–2525. https://doi.org/10.1128/AEM.72.4.2520-2525.2006

Boder, E. T., & Wittrup, K. D. (1997). Yeast surface display for screening combinatorial polypeptide libraries. *Nature Biotechnology*, *15*(6), Article 6. https://doi.org/10.1038/nbt0697-553

Bond, K., Nicholson, S., Lim, S. M., Karapanagiotidis, T., Williams, E., Johnson, D., Hoang, T., Sia, C., Purcell, D., Mordant, F., Lewin, S. R., Catton, M., Subbarao, K., Howden, B. P., & Williamson, D. A. (2020). Evaluation of Serological Tests for SARS-CoV-2: Implications for Serology Testing in a Low-Prevalence Setting. *The Journal of Infectious Diseases*, jiaa467. https://doi.org/10.1093/infdis/jiaa467

Borobia, A. M., Carcas, A. J., Pérez-Olmeda, M., Castaño, L., Bertran, M. J., García-Pérez, J., Campins, M., Portolés, A., González-Pérez, M., García Morales, M. T., Arana-Arri, E., Aldea, M., Díez-Fuertes, F., Fuentes, I., Ascaso, A., Lora, D., Imaz-Ayo, N., Barón-Mira, L. E., Agustí, A., … Frías, J. (2021). Immunogenicity and reactogenicity of BNT162b2 booster in ChAdOx1-S-primed participants (CombiVacS): A multicentre, open-label, randomised, controlled, phase 2 trial. *Lancet (London, England)*, *398*(10295), 121–130. https://doi.org/10.1016/S0140-6736(21)01420-3

Borody, T. J., Carrick, J., & Hazell, S. L. (1987). Symptoms improve after the eradication of gastric *Campylobacter pyloridis*. *The Medical Journal of Australia*, *146*(8), 450–451. https://doi.org/10.5694/j.1326-5377.1987.tb120344.x

Borody, T. J., Cole, P., Noonan, S., Morgan, A., Lenne, J., Hyland, L., Brandl, S., Borody, E. G., & George, L. L. (1989). Recurrence of duodenal ulcer and *Campylobacter pylori* infection after eradication. *The Medical Journal of Australia*, *151*(8), 431–435. https://doi.org/10.5694/j.1326-5377.1989.tb101251.x

Boyanova, L., Hadzhiyski, P., Kandilarov, N., Markovska, R., & Mitov, I. (2019). Multidrug resistance in *Helicobacter pylori*: Current state and future directions. *Expert Review of Clinical Pharmacology*, *12*(9), 909–915. https://doi.org/10.1080/17512433.2019.1654858

Braciak, T. A., Gallichan, W. S., Graham, F. L., Richards, C. D., Ramsay, A. J., Rosenthal, K. L., & Gauldie, J. (2000). Recombinant adenovirus vectors expressing interleukin-5 and -6 specifically enhance mucosal immunoglobulin A responses in the lung. *Immunology*, *101*(3), 388–396. https://doi.org/10.1046/j.1365-2567.2000.00116.x

Braun, A., Farber, M. J., Klase, Z. A., Berget, P. B., & Myers, K. A. (2018). A cell surface display fluorescent biosensor for measuring MMP14 activity in real-time. *Scientific Reports*, *8*(1), Article 1. https://doi.org/10.1038/s41598-018-24080-0

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, *68*(6), 394–424. https://doi.org/10.3322/caac.21492

Brosh-Nissimov, T., Orenbuch-Harroch, E., Chowers, M., Elbaz, M., Nesher, L., Stein, M., Maor, Y., Cohen, R., Hussein, K., Weinberger, M., Zimhony, O., Chazan, B., Najjar, R., Zayyad, H., Rahav, G., & Wiener-Well, Y. (2021). BNT162b2 vaccine breakthrough: Clinical characteristics of 152 fully vaccinated hospitalized COVID-19 patients in Israel. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *27*(11), 1652–1657. https://doi.org/10.1016/j.cmi.2021.06.036

Bruxvoort, K. J., Sy, L. S., Qian, L., Ackerson, B. K., Luo, Y., Lee, G. S., Tian, Y., Florea, A., Aragones, M., Tubert, J. E., Takhar, H. S., Ku, J. H., Paila, Y. D., Talarico, C. A., & Tseng, H. F. (2021). Effectiveness of mRNA-1273 against delta, mu, and other emerging variants of SARS-CoV-2: Test negative case-control study. *BMJ*, *375*, e068848. https://doi.org/10.1136/bmj-2021-068848

Bücker, R., Azevedo-Vethacke, M., Groll, C., Garten, D., Josenhans, C., Suerbaum, S., & Schreiber, S. (2012). *Helicobacter pylori* colonization critically depends on postprandial gastric conditions. *Scientific Reports*, *2*(1), Article 1. https://doi.org/10.1038/srep00994

Bumann, D., Metzger, W. G., Mansouri, E., Palme, O., Wendland, M., Hurwitz, R., Haas, G., Aebischer, T., von Specht, B. U., & Meyer, T. F. (2001). Safety and immunogenicity of live recombinant *Salmonella enterica* serovar Typhi Ty21a expressing urease A and B from Helicobacter pylori in human volunteers. *Vaccine*, *20*(5–6), 845–852. https://doi.org/10.1016/s0264-410x(01)00391-7

Cai, Y., Zhang, J., Xiao, T., Lavine, C. L., Rawson, S., Peng, H., Zhu, H., Anand, K., Tong, P., Gautam, A., Lu, S., Sterling, S. M., Walsh, R. M., Rits-Volloch, S., Lu,

J., Wesemann, D. R., Yang, W., Seaman, M. S., & Chen, B. (2021). Structural basis for enhanced infectivity and immune evasion of SARS-CoV-2 variants. *Science (New York, N.Y.)*, *373*(6555), 642–648. https://doi.org/10.1126/science.abi9745

Callaway, E. (2021). Heavily mutated Omicron variant puts scientists on alert. *Nature*, *600*(7887), 21. https://doi.org/10.1038/d41586-021-03552-w

Cano, R. J., & Borucki, M. K. (1995). Revival and identification of bacterial spores in 25- to 40-million-year-old Dominican amber. *Science (New York, N.Y.)*, *268*(5213), 1060–1064. https://doi.org/10.1126/science.7538699

Cascella, M., Rajnik, M., Aleem, A., Dulebohn, S. C., & Napoli, R. D. (2023). Features, Evaluation, and Treatment of Coronavirus (COVID-19). In *StatPearls [Internet]*. StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK554776/

Castillo, F., Benmohamed, A., & Szatmari, G. (2017). Xer Site Specific Recombination: Double and Single Recombinase Systems. *Frontiers in Microbiology*, *8*. https://www.frontiersin.org/articles/10.3389/fmicb.2017.00453

Castillo, J., Goodson, B., & Winter, J. (2001). T7 displayed peptides as targets for selecting peptide specific scFvs from M13 scFv display libraries. *Journal of Immunological Methods*, *257*(1), 117–122. https://doi.org/10.1016/S0022-1759(01)00454-9

CDC. (2020, February 11). *Coronavirus Disease 2019 (COVID-19)*. Centers for Disease Control and Prevention. https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-classifications.html

Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Rappuoli, R., & Covacci, A. (1996). Cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(25), 14648. https://doi.org/10.1073/pnas.93.25.14648

Cerda, O. A., Núñez-Villena, F., Soto, S. E., Ugalde, J. M., López-Solís, R., & Toledo, H. (2011). TlpA gene expression is required for arginine and bicarbonate

chemotaxis in Helicobacter pylori. *Biological Research*, *44*(3), 277–282. https://doi.org/10.4067/S0716-97602011000300009

Chan, C.-P., & Jin, D.-Y. (2022). Cytoplasmic RNA sensors and their interplay with RNA-binding partners in innate antiviral response: Theme and variations. *RNA*, *28*(4), 449–477. https://doi.org/10.1261/rna.079016.121

Chan, J. F. W., Lau, S. K. P., To, K. K. W., Cheng, V. C. C., Woo, P. C. Y., & Yuen, K.-Y. (2015). Middle East Respiratory Syndrome Coronavirus: Another Zoonotic Betacoronavirus Causing SARS-Like Disease. *Clinical Microbiology Reviews*, *28*(2), 465–522. https://doi.org/10.1128/CMR.00102-14

Chan, J. F.-W., Yuan, S., Kok, K.-H., To, K. K.-W., Chu, H., Yang, J., Xing, F., Liu, J., Yip, C. C.-Y., Poon, R. W.-S., Tsoi, H.-W., Lo, S. K.-F., Chan, K.-H., Poon, V. K.-M., Chan, W.-M., Ip, J. D., Cai, J.-P., Cheng, V. C.-C., Chen, H., … Yuen, K.-Y. (2020). A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: A study of a family cluster. *Lancet (London, England)*, *395*(10223), 514–523. https://doi.org/10.1016/S0140-6736(20)30154-9

Chang, J. Y., Shim, K.-N., Tae, C. H., Lee, K. E., Lee, J., Lee, K. H., Moon, C. M., Kim, S.-E., Jung, H.-K., & Jung, S.-A. (2017). Triple therapy versus sequential therapy for the first-line Helicobacter pylori eradication. *BMC Gastroenterology*, *17*(1), 16. https://doi.org/10.1186/s12876-017-0579-8

Chatterjee, I., Kriegeskorte, A., Fischer, A., Deiwick, S., Theimann, N., Proctor, R. A., Peters, G., Herrmann, M., & Kahl, B. C. (2008). *In Vivo* Mutations of Thymidylate Synthase (Encoded by thyA) Are Responsible for Thymidine Dependency in Clinical Small-Colony Variants of *Staphylococcus aureus*. *Journal of Bacteriology*, *190*(3), 834–842. https://doi.org/10.1128/JB.00912-07

Chavda, V. P., Patel, A. B., & Vaghasiya, D. D. (2022). SARS-CoV-2 variants and vulnerability at the global level. *Journal of Medical Virology*, *94*(7), 2986–3005. https://doi.org/10.1002/jmv.27717

Chemaitelly, H., Yassine, H. M., Benslimane, F. M., Al Khatib, H. A., Tang, P., Hasan, M. R., Malek, J. A., Coyle, P., Ayoub, H. H., Al Kanaani, Z., Al Kuwari, E.,

Jeremijenko, A., Kaleeckal, A. H., Latif, A. N., Shaik, R. M., Abdul Rahim, H. F., Nasrallah, G. K., Al Kuwari, M. G., Al Romaihi, H. E., … Abu-Raddad, L. J. (2021). MRNA-1273 COVID-19 vaccine effectiveness against the B.1.1.7 and B.1.351 variants and severe COVID-19 disease in Qatar. *Nature Medicine*, *27*(9), Article 9. https://doi.org/10.1038/s41591-021-01446-y

Chen, H., Ullah, J., & Jia, J. (2017). Progress in *Bacillus subtilis* Spore Surface Display Technology towards Environment, Vaccine Development, and Biocatalysis. *Microbial Physiology*, *27*(3), 159–167. https://doi.org/10.1159/000475177

Chen, J., Lin, L., Li, N., & She, F. (2012). Enhancement of *Helicobacter pylori* outer inflammatory protein DNA vaccine efficacy by co-delivery of interleukin-2 and B subunit heat-labile toxin gene encoded plasmids. *Microbiology and Immunology*, *56*(2), 85–92. https://doi.org/10.1111/j.1348-0421.2011.00409.x

Chen, J., Wang, R., Gilby, N. B., & Wei, G.-W. (2021). Omicron (B.1.1.529): Infectivity, vaccine breakthrough, and antibody resistance. *ArXiv*, arXiv:2112.01318v1.

Chen, N., Zhou, M., Dong, X., Qu, J., Gong, F., Han, Y., Qiu, Y., Wang, J., Liu, Y., Wei, Y., Xia, J., Yu, T., Zhang, X., & Zhang, L. (2020). Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: A descriptive study. *Lancet (London, England)*, *395*(10223), 507–513. https://doi.org/10.1016/S0140-6736(20)30211-7

Chen, R. E., Zhang, X., Case, J. B., Winkler, E. S., Liu, Y., VanBlargan, L. A., Liu, J., Errico, J. M., Xie, X., Suryadevara, N., Gilchuk, P., Zost, S. J., Tahan, S., Droit, L., Turner, J. S., Kim, W., Schmitz, A. J., Thapa, M., Wang, D., … Diamond, M. S. (2021). Resistance of SARS-CoV-2 variants to neutralization by monoclonal and serum-derived polyclonal antibodies. *Nature Medicine*, *27*(4), Article 4. https://doi.org/10.1038/s41591-021-01294-w

Cheng, V. C. C., Lau, S. K. P., Woo, P. C. Y., & Yuen, K. Y. (2007). Severe Acute Respiratory Syndrome Coronavirus as an Agent of Emerging and Reemerging Infection. *Clinical Microbiology Reviews*, *20*(4), 660–694. https://doi.org/10.1128/CMR.00023-07

Chey, W. D., Wong, B. C. Y., & Practice Parameters Committee of the American College of Gastroenterology. (2007). American College of Gastroenterology guideline on the management of Helicobacter pylori infection. *The American Journal of Gastroenterology*, *102*(8), 1808–1825. https://doi.org/10.1111/j.1572-0241.2007.01393.x

Chikanza, I. C. (2002). Mechanisms of corticosteroid resistance in rheumatoid arthritis: A putative role for the corticosteroid receptor beta isoform. *Annals of the New York Academy of Sciences*, *966*, 39–48. https://doi.org/10.1111/j.1749-6632.2002.tb04200.x

Chirico, F., da Silva, J. A. T., Tsigaris, P., & Sharun, K. (2022). Safety & effectiveness of COVID-19 vaccines: A narrative review. *The Indian Journal of Medical Research*, *155*(1), 91–104. https://doi.org/10.4103/ijmr.IJMR_474_21

Chitsazi, M. T., Fattahi, E., Farahani, R. M. Z., & Fattahi, S. (2006). *Helicobacter pylori* in the dental plaque: Is it of diagnostic value for gastric infection? *Helicobacter Pylori*.

Chiuppesi, F., Salazar, M. d'Alincourt, Contreras, H., Nguyen, V. H., Martinez, J., Park, Y., Nguyen, J., Kha, M., Iniguez, A., Zhou, Q., Kaltcheva, T., Levytskyy, R., Ebelt, N. D., Kang, T. H., Wu, X., Rogers, T. F., Manuel, E. R., Shostak, Y., Diamond, D. J., & Wussow, F. (2020). Development of a multi-antigenic SARS-CoV-2 vaccine candidate using a synthetic poxvirus platform. *Nature Communications*, *11*(1), 6121. https://doi.org/10.1038/s41467-020-19819-1

Chmiela, M., & Michetti, P. (2006). Inflammation, Immunity, Vaccines for *Helicobacter Infection*. *Helicobacter*, *11*(s1), 21–26. https://doi.org/10.1111/j.1478-405X.2006.00422.x

Choi, A., Koch, M., Wu, K., Chu, L., Ma, L., Hill, A., Nunna, N., Huang, W., Oestreicher, J., Colpitts, T., Bennett, H., Legault, H., Paila, Y., Nestorova, B., Ding, B., Montefiori, D., Pajon, R., Miller, J. M., Leav, B., … Edwards, D. K. (2021). Safety and immunogenicity of SARS-CoV-2 variant mRNA vaccine boosters in healthy adults: An interim analysis. *Nature Medicine*, *27*(11), 2025–2031. https://doi.org/10.1038/s41591-021-01527-y

Choi, S.-W., & Mason, J. B. (2000). Folate and Carcinogenesis: An Integrated Scheme. *The Journal of Nutrition*, *130*(2), 129–132. https://doi.org/10.1093/jn/130.2.129

Choi, Y., Kim, N., Kim, K. W., Jo, H. H., Park, J., Yoon, H., Shin, C. M., Park, Y. S., & Lee, D. H. (2022). Gastric Cancer in Older Patients: A Retrospective Study and Literature Review. *Annals of Geriatric Medicine and Research*, *26*(1), 33. https://doi.org/10.4235/agmr.21.0144

Chon, H. J., Hyung, W. J., Kim, C., Park, S., Kim, J.-H., Park, C. H., Ahn, J. B., Kim, H., Chung, H. C., Rha, S. Y., Noh, S. H., & Jeung, H.-C. (2017). Differential Prognostic Implications of Gastric Signet Ring Cell Carcinoma: Stage Adjusted Analysis From a Single High-volume Center in Asia. *Annals of Surgery*, *265*(5), 946. https://doi.org/10.1097/SLA.0000000000001793

Chow, W. H., Blaser, M. J., Blot, W. J., Gammon, M. D., Vaughan, T. L., Risch, H. A., Perez-Perez, G. I., Schoenberg, J. B., Stanford, J. L., Rotterdam, H., West, A. B., & Fraumeni, J. F. (1998). An inverse relation between cagA+ strains of *Helicobacter pylori* infection and risk of esophageal and gastric cardia adenocarcinoma. *Cancer Research*, *58*(4), 588–590.

Christensen, W. B. (1946). Urea Decomposition as a Means of Differentiating Proteus and Paracolon Cultures from Each Other and from *Salmonella* and *Shigella* Types 1. *Journal of Bacteriology*, *52*(4), 461–466.

Chu, K. M., Kwok, K. F., Law, S. Y., & Wong, J. (2000). One-week once-daily triple therapy for *Helicobacter pylori*—A pilot study. *Hepato-Gastroenterology*, *47*(36), 1624–1626.

Chung, F. (2001). Anti-inflammatory cytokines in asthma and allergy: Interleukin-10, interleukin-12, interferon-gamma. *Mediators of Inflammation*, *10*(2), 51–59. https://doi.org/10.1080/09629350120054518

Ciabattini, A., Parigi, R., Isticato, R., Oggioni, M. R., & Pozzi, G. (2004). Oral priming of mice by recombinant spores of *Bacillus subtilis*. *Vaccine*, *22*(31–32), 4139–4143. https://doi.org/10.1016/j.vaccine.2004.05.001

Cohn, F. (1872). Untersuchungen über Bakterien. *Beitr Biol Pflanzen*, *1*, 127–224.

Collins, K. D., Andermann, T. M., Draper, J., Sanders, L., Williams, S. M., Araghi, C., & Ottemann, K. M. (2016). The *Helicobacter pylori* CZB Cytoplasmic Chemoreceptor TlpD Forms an Autonomous Polar Chemotaxis Signaling Complex That Mediates a Tactic Response to Oxidative Stress. *Journal of Bacteriology*, *198*(11), 1563–1575. https://doi.org/10.1128/JB.00071-16

Coopersmith, C. M., Antonelli, M., Bauer, S. R., Deutschman, C. S., Evans, L. E., Ferrer, R., Hellman, J., Jog, S., Kesecioglu, J., Kissoon, N., Martin-Loeches, I., Nunnally, M. E., Prescott, H. C., Rhodes, A., Talmor, D., Tissieres, P., & De Backer, D. (2021). The Surviving Sepsis Campaign: Research Priorities for Coronavirus Disease 2019 in Critical Illness. *Critical Care Medicine*, *49*(4), 598–622. https://doi.org/10.1097/CCM.0000000000004895

Copland, A., Diogo, G. R., Hart, P., Harris, S., Tran, A. C., Paul, M. J., Singh, M., Cutting, S. M., & Reljic, R. (2018). Mucosal Delivery of Fusion Proteins with *Bacillus subtilis* Spores Enhances Protection against Tuberculosis by Bacillus Calmette-Guérin. *Frontiers in Immunology*, *9*, 346. https://doi.org/10.3389/fimmu.2018.00346

Corman, V. M., Muth, D., Niemeyer, D., & Drosten, C. (2018). Hosts and Sources of Endemic Human Coronaviruses. *Advances in Virus Research*, *100*, 163–188. https://doi.org/10.1016/bs.aivir.2018.01.001

Correa, P. (1992). Human gastric carcinogenesis: A multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Research*, *52*(24), 6735–6740.

Correa, P., & Piazuelo, M. B. (2008). Natural history of *Helicobacter pylori* infection. *Digestive and Liver Disease: Official Journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver*, *40*(7), 490–496. https://doi.org/10.1016/j.dld.2008.02.035

Correa, P., & Piazuelo, M. B. (2012). The gastric precancerous cascade. *Journal of Digestive Diseases*, *13*(1), 2–9. https://doi.org/10.1111/j.1751-2980.2011.00550.x

Correa, P., Piazuelo, M. B., & Wilson, K. T. (2010). Pathology of Gastric Intestinal Metaplasia: Clinical Implications. *The American Journal of Gastroenterology*, *105*(3), 493. https://doi.org/10.1038/ajg.2009.728

Corthésy-Theulaz, I. E., Hopkins, S., Bachmann, D., Saldinger, P. F., Porta, N., Haas, R., Zheng-Xin, Y., Meyer, T., Bouzourène, H., Blum, A. L., & Kraehenbuhl, J. P. (1998). Mice are protected from *Helicobacter pylori* infection by nasal immunization with attenuated *Salmonella typhimurium* phoPc expressing urease A and B subunits. *Infection and Immunity*, *66*(2), 581–586. https://doi.org/10.1128/IAI.66.2.581-586.1998

Covacci, A., Censini, S., Bugnoli, M., Petracca, R., Burroni, D., Macchia, G., Massone, A., Papini, E., Xiang, Z., & Figura, N. (1993). Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. *Proceedings of the National Academy of Sciences*, *90*(12), 5791–5795. https://doi.org/10.1073/pnas.90.12.5791

Cover, T., & Blaser, M. J. (1992). Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. *The Journal of Biological Chemistry, 267*, 10570–10575. https://doi.org/10.1016/S0021-9258(19)50054-0

Cover, T. L., & Blanke, S. R. (2005). *Helicobacter pylori* VacA, a paradigm for toxin multifunctionality. *Nature Reviews. Microbiology*, *3*(4), 320–332. https://doi.org/10.1038/nrmicro1095

Cover, T. L., Tummuru, M. K., Cao, P., Thompson, S. A., & Blaser, M. J. (1994). Divergence of genetic sequences for the vacuolating cytotoxin among *Helicobacter pylori* strains. *The Journal of Biological Chemistry*, *269*(14), 10566–10573.

Crackower, M. A., Sarao, R., Oudit, G. Y., Yagil, C., Kozieradzki, I., Scanga, S. E., Oliveira-dos-Santos, A. J., da Costa, J., Zhang, L., Pei, Y., Scholey, J., Ferrario, C. M., Manoukian, A. S., Chappell, M. C., Backx, P. H., Yagil, Y., & Penninger, J. M. (2002). Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature, 417*(6891), 822–828. https://doi.org/10.1038/nature00786

Croxtall, J. D., Choudhury, Q., & Flower, R. J. (2000). Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a

receptor-dependent, transcription-independent mechanism. *British Journal of Pharmacology*, *130*(2), 289–298. https://doi.org/10.1038/sj.bjp.0703272

Cruz-Ramos, H., Glaser, P., Wray, L. V., & Fisher, S. H. (1997). The *Bacillus subtilis* ureABC operon. *Journal of Bacteriology*, *179*(10), 3371–3373. https://doi.org/10.1128/jb.179.10.3371-3373.1997

Çuburu, N., Kim, R., Guittard, G. C., Thompson, C. D., Day, P. M., Hamm, D. E., Pang, Y.-Y. S., Graham, B. S., Lowy, D. R., & Schiller, J. T. (2019). A Prime-Pull-Amplify Vaccination Strategy to Maximize Induction of Circulating and Genital-Resident Intraepithelial CD8+ Memory T Cells. *Journal of Immunology (Baltimore, Md. : 1950)*, *202*(4), 1250–1264. https://doi.org/10.4049/jimmunol.1800219

Cussac, V., Ferrero, R. L., & Labigne, A. (1992). Expression of *Helicobacter pylori* urease genes in *Escherichia coli* grown under nitrogen-limiting conditions. *Journal of Bacteriology*, *174*(8), 2466–2473. https://doi.org/10.1128/jb.174.8.2466-2473.1992

Cutting, S. M. (1990). *Genetic analysis.* John Wiley & Sons, Ltd.

Cutting, S. M. (2011). *Bacillus* probiotics. *Food Microbiology*, *28*(2), 214–220. https://doi.org/10.1016/j.fm.2010.03.007

Czinn, S. J., & Blanchard, T. (2011). Vaccinating against *Helicobacter pylori* infection. *Nature Reviews. Gastroenterology & Hepatology*, *8*(3), 133–140. https://doi.org/10.1038/nrgastro.2011.1

D, P., Mm, S., N, C., Js, L., Ma, T., Mp, H., J, N., Ac, W., Je, B., B, G., Le, R., J, di I., J, J., H, K., S, I., S, J., N, S., K, C., R, A., … D, V. (2021). Broad betacoronavirus neutralization by a stem helix-specific human antibody. *Science (New York, N.Y.)*, *373*(6559). https://doi.org/10.1126/science.abj3321

Dangi, T., Class, J., Palacio, N., Richner, J. M., & Penaloza MacMaster, P. (2021). Combining spike- and nucleocapsid-based vaccines improves distal control of SARS-CoV-2. *Cell Reports*, *36*(10), 109664. https://doi.org/10.1016/j.celrep.2021.109664

Dangi, T., Sanchez, S., Class, J., Richner, M., Visvabharathy, L., Chung, Y. R., Bentley, K., Stanton, R. J., Koralnik, I. J., Richner, J. M., & Penaloza-MacMaster, P. (2022). Improved control of SARS-CoV-2 by treatment with a nucleocapsid-specific monoclonal antibody. *The Journal of Clinical Investigation*, *132*(23). https://doi.org/10.1172/JCI162282

Danilczyk, U., Sarao, R., Remy, C., Benabbas, C., Stange, G., Richter, A., Arya, S., Pospisilik, J. A., Singer, D., Camargo, S. M. R., Makrides, V., Ramadan, T., Verrey, F., Wagner, C. A., & Penninger, J. M. (2006). Essential role for collectrin in renal amino acid transport. *Nature*, *444*(7122), 1088–1091. https://doi.org/10.1038/nature05475

De Francesco, V., Giorgio, F., Hassan, C., Manes, G., Vannella, L., Panella, C., Ierardi, E., & Zullo, A. (2010). Worldwide *H. pylori* antibiotic resistance: A systematic review. *Journal of Gastrointestinal and Liver Diseases: JGLD*, *19*(4), 409–414.

De Francesco, V., Zullo, A., Ierardi, E., & Vaira, D. (2009). Minimal inhibitory concentration (MIC) values and different point mutations in the 23S rRNA gene for clarithromycin resistance in *Helicobacter pylori. Digestive and Liver Disease: Official Journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver*, *41*(8), 610–611. https://doi.org/10.1016/j.dld.2009.01.001

De Meyts, P., Halban, P., & Hepp, K. D. (1981). *In vitro* studies on biosynthetic human insulin: An overview. *Diabetes Care*, *4*(2), 144–146. https://doi.org/10.2337/diacare.4.2.144

de Souza, R. D., Batista, M. T., Luiz, W. B., Cavalcante, R. C. M., Amorim, J. H., Bizerra, R. S. P., Martins, E. G., & Ferreira, L. C. de S. (2014). *Bacillus subtilis* spores as vaccine adjuvants: Further insights into the mechanisms of action. *PloS One*, *9*(1), e87454. https://doi.org/10.1371/journal.pone.0087454

Dejnirattisai, W., Zhou, D., Supasa, P., Liu, C., Mentzer, A. J., Ginn, H. M., Zhao, Y., Duyvesteyn, H. M. E., Tuekprakhon, A., Nutalai, R., Wang, B., López-Camacho, C., Slon-Campos, J., Walter, T. S., Skelly, D., Costa Clemens, S. A., Naveca, F. G., Nascimento, V., Nascimento, F., … Screaton, G. R. (2021). Antibody evasion by

the P.1 strain of SARS-CoV-2. *Cell*, *184*(11), 2939-2954.e9. https://doi.org/10.1016/j.cell.2021.03.055

Del Valle, D. M., Kim-Schulze, S., Huang, H.-H., Beckmann, N. D., Nirenberg, S., Wang, B., Lavin, Y., Swartz, T. H., Madduri, D., Stock, A., Marron, T. U., Xie, H., Patel, M., Tuballes, K., Van Oekelen, O., Rahman, A., Kovatch, P., Aberg, J. A., Schadt, E., … Gnjatic, S. (2020). An inflammatory cytokine signature predicts COVID-19 severity and survival. *Nature Medicine*, *26*(10), 1636–1643. https://doi.org/10.1038/s41591-020-1051-9

Diamond, M. S., & Kanneganti, T.-D. (2022). Innate immunity: The first line of defense against SARS-CoV-2. *Nature Immunology*, *23*(2), Article 2. https://doi.org/10.1038/s41590-021-01091-0

Dickey, R. P., Nichols, J. E., Steinkampf, M. P., Gocial, B., Thornton, M., Webster, B. W., Bello, S. M., Crain, J., & Marshall, D. C. (2003). Highly purified human-derived follicle-stimulating hormone (Bravelle®) has equivalent efficacy to follitropin-beta (Follistim ®) in infertile women undergoing *in vitro* fertilization. *Reproductive Biology and Endocrinology : RB&E*, *1*, 63. https://doi.org/10.1186/1477-7827-1-63

Ding, Y., He, L., Zhang, Q., Huang, Z., Che, X., Hou, J., Wang, H., Shen, H., Qiu, L., Li, Z., Geng, J., Cai, J., Han, H., Li, X., Kang, W., Weng, D., Liang, P., & Jiang, S. (2004). Organ distribution of severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV) in SARS patients: Implications for pathogenesis and virus transmission pathways. *The Journal of Pathology*, *203*(2), 622–630. https://doi.org/10.1002/path.1560

DiPetrillo, M. D., Tibbetts, T., Kleanthous, H., Killeen, K. P., & Hohmann, E. L. (1999). Safety and immunogenicity of phoP/phoQ-deleted *Salmonella typhi* expressing *Helicobacter pylori* urease in adult volunteers. *Vaccine*, *18*(5–6), 449–459. https://doi.org/10.1016/s0264-410x(99)00246-7

Dong, E., Du, H., & Gardner, L. (2020). An interactive web-based dashboard to track COVID-19 in real time. *The Lancet Infectious Diseases*, *20*(5), 533–534. https://doi.org/10.1016/S1473-3099(20)30120-1

Dorer, M., Talarico, S., & Salama, N. (2009). Helicobacter pylori's unconventional role in health and disease. *PLoS Pathogens*, *5*(10). https://doi.org/10.1371/journal.ppat.1000544

Dorji, D., Dendup, T., Malaty, H. M., Wangchuk, K., Yangzom, D., & Richter, J. M. (2014). Epidemiology of *Helicobacter pylori* in Bhutan: The Role of Environment and Geographic Location. *Helicobacter*, *19*(1), 69–73. https://doi.org/10.1111/hel.12088

Dougan, M., Azizad, M., Chen, P., Feldman, B., Frieman, M., Igbinadolor, A., Kumar, P., Morris, J., Potts, J., Baracco, L., Macpherson, L., Kallewaard, N. L., Patel, D. R., Hufford, M. M., Wietecha, L., Chigutsa, E., Demmon, S. L., Jones, B. E., Nirula, A., … Gottlieb, R. L. (2022). *Bebtelovimab, alone or together with bamlanivimab and etesevimab, as a broadly neutralizing monoclonal antibody treatment for mild to moderate, ambulatory COVID-19* (p. 2022.03.10.22272100). medRxiv. https://doi.org/10.1101/2022.03.10.22272100

Dougan, M., Nirula, A., Azizad, M., Mocherla, B., Gottlieb, R. L., Chen, P., Hebert, C., Perry, R., Boscia, J., Heller, B., Morris, J., Crystal, C., Igbinadolor, A., Huhn, G., Cardona, J., Shawa, I., Kumar, P., Adams, A. C., Van Naarden, J., … Skovronsky, D. M. (2021). Bamlanivimab plus Etesevimab in Mild or Moderate Covid-19. *New England Journal of Medicine*, *385*(15), 1382–1392. https://doi.org/10.1056/NEJMoa2102685

Driks, A. (1999). *Bacillus subtilis* Spore Coat. *Microbiology and Molecular Biology Reviews*, *63*(1), 1. https://doi.org/10.1128/mmbr.63.1.1-20.1999

Du, L., He, Y., Zhou, Y., Liu, S., Zheng, B.-J., & Jiang, S. (2009). The spike protein of SARS-CoV — a target for vaccine and therapeutic development. *Nature Reviews. Microbiology*, *7*(3), 226–236. https://doi.org/10.1038/nrmicro2090

Du, Y., Xu, Y., Feng, J., Hu, L., Zhang, Y., Zhang, B., Guo, W., Mai, R., Chen, L., Fang, J., Zhang, H., & Peng, T. (2021). Intranasal administration of a recombinant RBD vaccine induced protective immunity against SARS-CoV-2 in mouse. *Vaccine*, *39*(16), 2280–2287. https://doi.org/10.1016/j.vaccine.2021.03.006

Dubnau, D., & Davidoff-Abelson, R. (1971). Fate of transforming DNA following uptake by competent *Bacillus subtilis*: I. Formation and properties of the donor-recipient complex. *Journal of Molecular Biology*, *56*(2), 209–221. https://doi.org/10.1016/0022-2836(71)90460-8

Duc, L. H., Hong, H. A., Atkins, H. S., Flick-Smith, H. C., Durrani, Z., Rijpkema, S., Titball, R. W., & Cutting, S. M. (2007). Immunization against anthrax using *Bacillus subtilis* spores expressing the anthrax protective antigen. *Vaccine*, *25*(2), 346–355. https://doi.org/10.1016/j.vaccine.2006.07.037

Duc, L. H., Hong, H. A., Fairweather, N., Ricca, E., & Cutting, S. M. (2003). Bacterial Spores as Vaccine Vehicles. *Infection and Immunity*, *71*(5), 2810–2818. https://doi.org/10.1128/IAI.71.5.2810-2818.2003

Duc, L. H., Hong, H. A., Uyen, N. Q., & Cutting, S. M. (2004). Intracellular fate and immunogenicity of *B. subtilis* spores. *Vaccine*, *22*(15–16), 1873–1885. https://doi.org/10.1016/j.vaccine.2003.11.021

Dutta, N. K., Mazumdar, K., & Gordy, J. T. (2020). The Nucleocapsid Protein of SARS-CoV-2: A Target for Vaccine Development. *Journal of Virology*, *94*(13), e00647-20. https://doi.org/10.1128/JVI.00647-20

Eaton, K. A., Mefford, M., & Thevenot, T. (2001). The role of T cell subsets and cytokines in the pathogenesis of *Helicobacter pylori* gastritis in mice. *Journal of Immunology (Baltimore, Md.: 1950)*, *166*(12), 7456–7461. https://doi.org/10.4049/jimmunol.166.12.7456

ECDC. (2020). *Risk assessment: Outbreak of acute respiratory syndrome associated with a novel coronavirus, Wuhan, China; first update*. https://www.ecdc.europa.eu/en/publications-data/risk-assessment-outbreak-acute-respiratory-syndrome-associated-novel-coronavirus

Edara, V. V., Norwood, C., Floyd, K., Lai, L., Davis-Gardner, M. E., Hudson, W. H., Mantus, G., Nyhoff, L. E., Adelman, M. W., Fineman, R., Patel, S., Byram, R., Gomes, D. N., Michael, G., Abdullahi, H., Beydoun, N., Panganiban, B., McNair, N., Hellmeister, K., … Suthar, M. S. (2021). Infection- and vaccine-induced

antibody binding and neutralization of the B.1.351 SARS-CoV-2 variant. *Cell Host & Microbe, 29*(4), 516-521.e3. https://doi.org/10.1016/j.chom.2021.03.009

Ehrenberg, C. G. (1835). *Abhandlungen der Königlichen Akademie der Wissenschaften zu Berlin* (pp. 1–1402). Realschul-Buchhandlung. https://www.biodiversitylibrary.org/item/94127

Ella, R., Vadrevu, K. M., Jogdand, H., Prasad, S., Reddy, S., Sarangi, V., Ganneru, B., Sapkal, G., Yadav, P., Abraham, P., Panda, S., Gupta, N., Reddy, P., Verma, S., Kumar Rai, S., Singh, C., Redkar, S. V., Gillurkar, C. S., Kushwaha, J. S., … Bhargava, B. (2021). Safety and immunogenicity of an inactivated SARS-CoV-2 vaccine, BBV152: A double-blind, randomised, phase 1 trial. *The Lancet. Infectious Diseases*, *21*(5), 637–646. https://doi.org/10.1016/S1473-3099(20)30942-7

El-Omar, E., Oien, K., El-Nujumi, A., Gillen, D., Wirz, A., Dahill, S., Williams, C., Ardill, J., & McColl, K. (1997). *Helicobacter pylori* infection and chronic gastric acid hyposecretion. *Gastroenterology*, *113*(1), 15–24. https://doi.org/10.1016/S0016-5085(97)70075-1

Elshabrawy, H. A., Coughlin, M. M., Baker, S. C., & Prabhakar, B. S. (2012). Human monoclonal antibodies against highly conserved HR1 and HR2 domains of the SARS-CoV spike protein are more broadly neutralizing. *PloS One, 7*(11), e50366. https://doi.org/10.1371/journal.pone.0050366

El-Shenawy, A., Diab, M., Shemis, M., El-Ghannam, M., Salem, D., Abdelnasser, M., Shahin, M., Abdel-Hady, M., El-Sherbini, E., & Saber, M. (2017). Detection of *Helicobacter pylori* vacA, cagA and iceA1 virulence genes associated with gastric diseases in Egyptian patients. *Egyptian Journal of Medical Human Genetics*, *18*(4), 365–371. https://doi.org/10.1016/j.ejmhg.2017.04.003

Emary, K. R. W., Golubchik, T., Aley, P. K., Ariani, C. V., Angus, B., Bibi, S., Blane, B., Bonsall, D., Cicconi, P., Charlton, S., Clutterbuck, E. A., Collins, A. M., Cox, T., Darton, T. C., Dold, C., Douglas, A. D., Duncan, C. J. A., Ewer, K. J., Flaxman, A. L., … Oxford COVID-19 Vaccine Trial Group. (2021). Efficacy of ChAdOx1 nCoV-19 (AZD1222) vaccine against SARS-CoV-2 variant of concern 202012/01 (B.1.1.7): An exploratory analysis of a randomised controlled trial. *Lancet (London,*

England), *397*(10282), 1351–1362. https://doi.org/10.1016/S0140-6736(21)00628-0

Errington, J. (2003). Regulation of endospore formation in *Bacillus subtilis*. *Nature Reviews Microbiology*, *1*(2), Article 2. https://doi.org/10.1038/nrmicro750

Eusebi, L. H., Zagari, R. M., & Bazzoli, F. (2014). Epidemiology of *Helicobacter pylori* infection. *Helicobacter*, *19 Suppl 1*, 1–5. https://doi.org/10.1111/hel.12165

Falsafi, T., Mobasheri, F., Nariman, F., & Najafi, M. (2004). Susceptibilities to different antibiotics of *Helicobacter pylori* strains isolated from patients at the pediatric medical center of Tehran, Iran. *Journal of Clinical Microbiology*, *42*(1). https://doi.org/10.1128/JCM.42.1.387-389.2004

Falsey, A. R., Sobieszczyk, M. E., Hirsch, I., Sproule, S., Robb, M. L., Corey, L., Neuzil, K. M., Hahn, W., Hunt, J., Mulligan, M. J., McEvoy, C., DeJesus, E., Hassman, M., Little, S. J., Pahud, B. A., Durbin, A., Pickrell, P., Daar, E. S., Bush, L., … Gonzalez-Lopez, A. (2021). Phase 3 Safety and Efficacy of AZD1222 (ChAdOx1 nCoV-19) Covid-19 Vaccine. *New England Journal of Medicine*, *385*(25), 2348–2360. https://doi.org/10.1056/NEJMoa2105290

Fang, Y.-J., Chen, M.-J., Chen, C.-C., Lee, J.-Y., Yang, T.-H., Yu, C.-C., Chiu, M.-C., Kuo, C.-C., Weng, Y.-J., Bair, M.-J., Wu, M.-S., Luo, J.-C., & Liou, J.-M. (2020). Accuracy of rapid *Helicobacter pylori* antigen tests for the surveillance of the updated prevalence of H. pylori in Taiwan. *Journal of the Formosan Medical Association = Taiwan Yi Zhi*, *119*(11), 1626–1633. https://doi.org/10.1016/j.jfma.2019.12.003

Farinholt, T., Doddapaneni, H., Qin, X., Menon, V., Meng, Q., Metcalf, G., Chao, H., Gingras, M.-C., Avadhanula, V., Farinholt, P., Agrawal, C., Muzny, D. M., Piedra, P. A., Gibbs, R. A., & Petrosino, J. (2021). Transmission event of SARS-CoV-2 delta variant reveals multiple vaccine breakthrough infections. *BMC Medicine*, *19*(1), 255. https://doi.org/10.1186/s12916-021-02103-4

Faulkner, N., Ng, K. W., Wu, M. Y., Harvey, R., Margaritis, M., Paraskevopoulou, S., Houlihan, C., Hussain, S., Greco, M., Bolland, W., Warchal, S., Heaney, J., Rickman, H., Spyer, M., Frampton, D., Byott, M., de Oliveira, T., Sigal, A., Kjaer,

S., … Kassiotis, G. (2021). Reduced antibody cross-reactivity following infection with B.1.1.7 than with parental SARS-CoV-2 strains. *ELife*, *10*, e69317. https://doi.org/10.7554/eLife.69317

Fazeli, Z., Alebouyeh, M., Tavirani, M. R., Yadegar, A., Farzi, N., & Zali, M. R. (2017). Functional Cytotoxin Associated Gene A in *Helicobacter pylori* Strains and Its Association with Integrity of Cag-pathogenicity Island and Histopathological Changes of Gastric Tissue. *Archives of Clinical Infectious Diseases*, *12*(3), Article 3. https://doi.org/10.5812/archcid.62955

Fels, U., Gevaert, K., & Van Damme, P. (2020). Bacterial Genetic Engineering by Means of Recombineering for Reverse Genetics. *Frontiers in Microbiology*, *11*, 548410. https://doi.org/10.3389/fmicb.2020.548410

Feng, Y., Ling, Y., Bai, T., Xie, Y., Huang, J., Li, J., Xiong, W., Yang, D., Chen, R., Lu, F., Lu, Y., Liu, X., Chen, Y., Li, X., Li, Y., Summah, H. D., Lin, H., Yan, J., Zhou, M., … Qu, J. (2020). COVID-19 with Different Severities: A Multicenter Study of Clinical Features. *American Journal of Respiratory and Critical Care Medicine*, *201*(11), 1380–1388. https://doi.org/10.1164/rccm.202002-0445OC

Ferdinands, J. M., Rao, S., Dixon, B. E., Mitchell, P. K., DeSilva, M. B., Irving, S. A., Lewis, N., Natarajan, K., Stenehjem, E., Grannis, S. J., Han, J., McEvoy, C., Ong, T. C., Naleway, A. L., Reese, S. E., Embi, P. J., Dascomb, K., Klein, N. P., Griggs, E. P., … Fireman, B. (2022). Waning 2-Dose and 3-Dose Effectiveness of mRNA Vaccines Against COVID-19–Associated Emergency Department and Urgent Care Encounters and Hospitalizations Among Adults During Periods of Delta and Omicron Variant Predominance—VISION Network, 10 States, August 2021–January 2022. *Morbidity and Mortality Weekly Report*, *71*(7), 255–263. https://doi.org/10.15585/mmwr.mm7107e2

Ferrero, R. L., Thiberge, J. M., Huerre, M., & Labigne, A. (1994a). Recombinant antigens prepared from the urease subunits of *Helicobacter* spp.: Evidence of protection in a mouse model of gastric infection. *Infection and Immunity*, *62*(11), 4981–4989.

Ferrero, R. L., Thiberge, J. M., Huerre, M., & Labigne, A. (1994b). Recombinant antigens prepared from the urease subunits of Helicobacter spp.: Evidence of protection in a

mouse model of gastric infection. *Infection and Immunity*, *62*(11), 4981–4989. https://doi.org/10.1128/iai.62.11.4981-4989.1994

Finamore, A., Roselli, M., Britti, S., Monastra, G., Ambra, R., Turrini, A., & Mengheri, E. (2008). Intestinal and Peripheral Immune Response to MON810 Maize Ingestion in Weaning and Old Mice. *Journal of Agricultural and Food Chemistry*, *56*(23), 11533–11539. https://doi.org/10.1021/jf802059w

Fischer, W., Buhrdorf, R., Gerland, G., & Haas, R. (2001). Outer membrane targeting of passenger proteins by the vacuolating cytotoxin autotransporter of *Helicobacter pylori*. *Infection and Immunity*, *69*(11). https://doi.org/10.1128/IAI.69.11.6769-6775.2001

Fontana, L., Bermudez-Brito, M., Plaza-Diaz, J., Muñoz-Quezada, S., & Gil, A. (2013). Sources, isolation, characterisation and evaluation of probiotics. *The British Journal of Nutrition*, *109 Suppl 2*. https://doi.org/10.1017/S0007114512004011

Foynes, S., Dorrell, N., Ward, S. J., Stabler, R. A., McColm, A. A., Rycroft, A. N., & Wren, B. W. (2000). *Helicobacter pylori* possesses two CheY response regulators and a histidine kinase sensor, CheA, which are essential for chemotaxis and colonization of the gastric mucosa. *Infection and Immunity*, *68*(4), 2016–2023. https://doi.org/10.1128/IAI.68.4.2016-2023.2000

Frenck, R. W., & Clemens, J. (2003). *Helicobacter* in the developing world. *Microbes and Infection*, *5*(8), 705–713. https://doi.org/10.1016/s1286-4579(03)00112-6

Freudl, R., MacIntyre, S., Degen, M., & Henning, U. (1986). Cell surface exposure of the outer membrane protein OmpA of *Escherichia col*i K-12. *Journal of Molecular Biology*, *188*(3), 491–494. https://doi.org/10.1016/0022-2836(86)90171-3

Fu, X., & Xu, J. (2000). Development of a Chromosome-Plasmid Balanced Lethal System for *Lactobacillus acidophilus* with thyA Gene as Selective Marker. *Microbiology and Immunology*, *44*(7), 551–556. https://doi.org/10.1111/j.1348-0421.2000.tb02533.x

Funk, C. (2015, January 29). Public and Scientists' Views on Science and Society. *Pew Research Center Science & Society*.

https://www.pewresearch.org/science/2015/01/29/public-and-scientists-views-on-science-and-society/

Gaissmaier, M. S., Laganenka, L., Herzog, M. K.-M., Bakkeren, E., & Hardt, W.-D. (2022). The Mobilizable Plasmid P3 of *Salmonella enterica* Serovar Typhimurium SL1344 Depends on the P2 Plasmid for Conjugative Transfer into a Broad Range of Bacteria *In Vitro* and *In Vivo*. *Journal of Bacteriology*, *204*(12), e00347-22. https://doi.org/10.1128/jb.00347-22

Gan, J., Bornhorst, G. M., Henrick, B. M., & German, J. B. (2018). Protein digestion of baby foods: Study approaches and implications for infant health. *Molecular Nutrition & Food Research*, *62*(1). https://doi.org/10.1002/mnfr.201700231

Garcia-Beltran, W. F., Lam, E. C., Denis, K. S., Nitido, A. D., Garcia, Z. H., Hauser, B. M., Feldman, J., Pavlovic, M. N., Gregory, D. J., Poznansky, M. C., Sigal, A., Schmidt, A. G., Iafrate, A. J., Naranbhai, V., & Balazs, A. B. (2021). Multiple SARS-CoV-2 variants escape neutralization by vaccine-induced humoral immunity. *Cell*, *184*(9), 2372-2383.e9. https://doi.org/10.1016/j.cell.2021.03.013

Gatta, L., Vakil, N., Leandro, G., Di Mario, F., & Vaira, D. (2009). Sequential therapy or triple therapy for *Helicobacter pylori* infection: Systematic review and meta-analysis of randomized controlled trials in adults and children. *The American Journal of Gastroenterology*, *104*(12), 3069–3079; quiz 1080. https://doi.org/10.1038/ajg.2009.555

Georgiou, G., Stathopoulos, C., Daugherty, P. S., Nayak, A. R., Iverson, B. L., & Iii, R. C. (1997). Display of heterologous proteins on the surface of microorganisms: From the screening of combinatorial libraries to live recombinant vaccines. *Nature Biotechnology*, *15*(1), Article 1. https://doi.org/10.1038/nbt0197-29

Gerges Harb, J., Noureldine, H. A., Chedid, G., Eldine, M. N., Abdallah, D. A., Chedid, N. F., & Nour-Eldine, W. (2020). SARS, MERS and COVID-19: Clinical manifestations and organ-system complications: a mini review. *Pathogens and Disease*, *78*(4), ftaa033. https://doi.org/10.1093/femspd/ftaa033

Ghorbani, N., Assmar, M., Amirmozafari, N., & Issazadeh, K. (2021). Investigating the Efficiency of Recombinant FliC-Loaded *Bacillus subtilis* Spores in Mice

Immunization against *Salmonella enterica* Serovar Typhi. *Iranian Journal of Public Health*, *50*(7), 1474–1482. https://doi.org/10.18502/ijph.v50i7.6638

Gisbert, J. P., Perez-Aisa, A., Rodrigo, L., Molina-Infante, J., Modolell, I., Bermejo, F., Castro-Fernández, M., Antón, R., Sacristán, B., Cosme, A., Barrio, J., Harb, Y., Gonzalez-Barcenas, M., Fernandez-Bermejo, M., Algaba, A., Marín, A. C., McNicholl, A. G., & *H. pylori* Study Group of the Spanish Gastroenterology Association. (2014). Third-line rescue therapy with bismuth-containing quadruple regimen after failure of two treatments (with clarithromycin and levofloxacin) for *H. pylori* infection. *Digestive Diseases and Sciences*, *59*(2), 383–389. https://doi.org/10.1007/s10620-013-2900-x

Goderska, K., Agudo Pena, S., & Alarcon, T. (2018). *Helicobacter pylori* treatment: Antibiotics or probiotics. *Applied Microbiology and Biotechnology*, *102*(1), 1–7. https://doi.org/10.1007/s00253-017-8535-7

Goeddel, D. V., Kleid, D. G., Bolivar, F., Heyneker, H. L., Yansura, D. G., Crea, R., Hirose, T., Kraszewski, A., Itakura, K., & Riggs, A. D. (1979). Expression in *Escherichia coli* of chemically synthesized genes for human insulin. *Proceedings of the National Academy of Sciences of the United States of America*, *76*(1), 106–110. https://doi.org/10.1073/pnas.76.1.106

Gómez-Duarte, O. G., Lucas, B., Yan, Z. X., Panthel, K., Haas, R., & Meyer, T. F. (1998a). Protection of mice against gastric colonization by *Helicobacter pylori* by single oral dose immunization with attenuated *Salmonella typhimurium* producing urease subunits A and B. *Vaccine*, *16*(5), 460–471. https://doi.org/10.1016/s0264-410x(97)00247-8

Goo, T., Akiba, Y., & Kaunitz, J. D. (2010). Mechanisms of intragastric pH sensing. *Current Gastroenterology Reports*, *12*(6), 465–470. https://doi.org/10.1007/s11894-010-0147-7

Goodwin, C. S., Armstrong, J. A., Chilvers, T., Peters, M., Collins, M. D., Sly, L., McConnell, W., & Harper, W. E. S. (1989). Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. Nov. As *Helicobacter pylori* comb. Nov. And *Helicobacter mustelae* comb, nov., Respectively. *International Journal*

of Systematic and Evolutionary Microbiology, *39*(4), 397–405. https://doi.org/10.1099/00207713-39-4-397

Goodwin, C. S., Armstrong, J. A., & Marshall, B. J. (1986). *Campylobacter pyloridis*, gastritis, and peptic ulceration. *Journal of Clinical Pathology*, *39*(4), 353–365.

Gorbalenya, A. E., Baker, S. C., Baric, R. S., Groot, R. J. de, Drosten, C., Gulyaeva, A. A., Haagmans, B. L., Lauber, C., Leontovich, A. M., Neuman, B. W., Penzar, D., Perlman, S., Poon, L. L. M., Samborskiy, D. V., Sidorov, I. A., Sola, I., Ziebuhr, J., & Grp, C. S. (2020). The species Severe acute respiratory syndrome-related coronavirus: Classifying 2019-nCoV and naming it SARS-CoV-2. *Nature Microbiology*, *5*(4), 536–544. https://doi.org/10.1038/s41564-020-0695-z

Gottlieb, R. L., Nirula, A., Chen, P., Boscia, J., Heller, B., Morris, J., Huhn, G., Cardona, J., Mocherla, B., Stosor, V., Shawa, I., Kumar, P., Adams, A. C., Van Naarden, J., Custer, K. L., Durante, M., Oakley, G., Schade, A. E., Holzer, T. R., … Skovronsky, D. M. (2021). Effect of Bamlanivimab as Monotherapy or in Combination With Etesevimab on Viral Load in Patients With Mild to Moderate COVID-19: A Randomized Clinical Trial. *JAMA*, *325*(7), 632–644. https://doi.org/10.1001/jama.2021.0202

Gottlieb, R. L., Vaca, C. E., Paredes, R., Mera, J., Webb, B. J., Perez, G., Oguchi, G., Ryan, P., Nielsen, B. U., Brown, M., Hidalgo, A., Sachdeva, Y., Mittal, S., Osiyemi, O., Skarbinski, J., Juneja, K., Hyland, R. H., Osinusi, A., Chen, S., … GS-US-540-9012 (PINETREE) Investigators. (2022). Early Remdesivir to Prevent Progression to Severe Covid-19 in Outpatients. *The New England Journal of Medicine*, *386*(4), 305–315. https://doi.org/10.1056/NEJMoa2116846

Graham, D. Y., Alpert, L. C., Smith, J. L., & Yoshimura, H. H. (1988). Iatrogenic *Campylobacter pylori* infection is a cause of epidemic achlorhydria. *The American Journal of Gastroenterology*, *83*(9), 974–980.

Graham, D. Y., Opekun, A. R., Osato, M. S., El-Zimaity, H. M. T., Lee, C. K., Yamaoka, Y., Qureshi, W. A., Cadoz, M., & Monath, T. P. (2004). Challenge model for *Helicobacter pylori* infection in human volunteers. *Gut*, *53*(9), 1235–1243. https://doi.org/10.1136/gut.2003.037499

Graham, D. Y., & Shiotani, A. (2008). New concepts of resistance in the treatment of *Helicobacter pylori* infections. *Nature Clinical Practice. Gastroenterology & Hepatology*, *5*(6), 321–331. https://doi.org/10.1038/ncpgasthep1138

Grifoni, A., Sidney, J., Zhang, Y., Scheuermann, R. H., Peters, B., & Sette, A. (2020). A Sequence Homology and Bioinformatic Approach Can Predict Candidate Targets for Immune Responses to SARS-CoV-2. *Cell Host & Microbe*, *27*(4), 671-680.e2. https://doi.org/10.1016/j.chom.2020.03.002

Grifoni, A., Weiskopf, D., Ramirez, S. I., Mateus, J., Dan, J. M., Moderbacher, C. R., Rawlings, S. A., Sutherland, A., Premkumar, L., Jadi, R. S., Marrama, D., de Silva, A. M., Frazier, A., Carlin, A. F., Greenbaum, J. A., Peters, B., Krammer, F., Smith, D. M., Crotty, S., & Sette, A. (2020). Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans with COVID-19 Disease and Unexposed Individuals. *Cell*, *181*(7), 1489-1501.e15. https://doi.org/10.1016/j.cell.2020.05.015

Grubaugh, N. D., Hodcroft, E. B., Fauver, J. R., Phelan, A. L., & Cevik, M. (2021). Public health actions to control new SARS-CoV-2 variants. *Cell*, *184*(5), 1127–1132. https://doi.org/10.1016/j.cell.2021.01.044

Gu, H., Krishnan, P., Ng, D. Y. M., Chang, L. D. J., Liu, G. Y. Z., Cheng, S. S. M., Hui, M. M. Y., Fan, M. C. Y., Wan, J. H. L., Lau, L. H. K., Cowling, B. J., Peiris, M., & Poon, L. L. M. (2022). Probable Transmission of SARS-CoV-2 Omicron Variant in Quarantine Hotel, Hong Kong, China, November 2021. *Emerging Infectious Diseases*, *28*(2), 460–462. https://doi.org/10.3201/eid2802.212422

Gu, J., Gong, E., Zhang, B., Zheng, J., Gao, Z., Zhong, Y., Zou, W., Zhan, J., Wang, S., Xie, Z., Zhuang, H., Wu, B., Zhong, H., Shao, H., Fang, W., Gao, D., Pei, F., Li, X., He, Z., … Leong, A. S.-Y. (2005). Multiple organ infection and the pathogenesis of SARS. *The Journal of Experimental Medicine*, *202*(3), 415–424. https://doi.org/10.1084/jem.20050828

Guan, W.-J., Ni, Z.-Y., Hu, Y., Liang, W.-H., Ou, C.-Q., He, J.-X., Liu, L., Shan, H., Lei, C.-L., Hui, D. S. C., Du, B., Li, L.-J., Zeng, G., Yuen, K.-Y., Chen, R.-C., Tang, C.-L., Wang, T., Chen, P.-Y., Xiang, J., … China Medical Treatment Expert Group for Covid-19. (2020). Clinical Characteristics of Coronavirus Disease 2019 in

China. *The New England Journal of Medicine*, *382*(18), 1708–1720. https://doi.org/10.1056/NEJMoa2002032

Guérout-Fleury, A. M., Frandsen, N., & Stragier, P. (1996). Plasmids for ectopic integration in *Bacillus subtilis*. *Gene*, *180*(1–2), 57–61. https://doi.org/10.1016/s0378-1119(96)00404-0

Guo, L., Ren, L., Yang, S., Xiao, M., Chang, D., Yang, F., Dela Cruz, C. S., Wang, Y., Wu, C., Xiao, Y., Zhang, L., Han, L., Dang, S., Xu, Y., Yang, Q.-W., Xu, S.-Y., Zhu, H.-D., Xu, Y.-C., Jin, Q., … Wang, J. (2020). Profiling Early Humoral Response to Diagnose Novel Coronavirus Disease (COVID-19). *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, ciaa310. https://doi.org/10.1093/cid/ciaa310

Guo, Z.-D., Wang, Z.-Y., Zhang, S.-F., Li, X., Li, L., Li, C., Cui, Y., Fu, R.-B., Dong, Y.-Z., Chi, X.-Y., Zhang, M.-Y., Liu, K., Cao, C., Liu, B., Zhang, K., Gao, Y.-W., Lu, B., & Chen, W. (2020). Aerosol and Surface Distribution of Severe Acute Respiratory Syndrome Coronavirus 2 in Hospital Wards, Wuhan, China, 2020. *Emerging Infectious Diseases*, *26*(7), 1583–1591. https://doi.org/10.3201/eid2607.200885

Gupta, A., Gonzalez-Rojas, Y., Juarez, E., Crespo Casal, M., Moya, J., Falci, D. R., Sarkis, E., Solis, J., Zheng, H., Scott, N., Cathcart, A. L., Hebner, C. M., Sager, J., Mogalian, E., Tipple, C., Peppercorn, A., Alexander, E., Pang, P. S., Free, A., … Shapiro, A. E. (2021). Early Treatment for Covid-19 with SARS-CoV-2 Neutralizing Antibody Sotrovimab. *New England Journal of Medicine*, *385*(21), 1941–1950. https://doi.org/10.1056/NEJMoa2107934

Gupta, A., Madhavan, M. V., Sehgal, K., Nair, N., Mahajan, S., Sehrawat, T. S., Bikdeli, B., Ahluwalia, N., Ausiello, J. C., Wan, E. Y., Freedberg, D. E., Kirtane, A. J., Parikh, S. A., Maurer, M. S., Nordvig, A. S., Accili, D., Bathon, J. M., Mohan, S., Bauer, K. A., … Landry, D. W. (2020). Extrapulmonary manifestations of COVID-19. *Nature Medicine*, *26*(7), Article 7. https://doi.org/10.1038/s41591-020-0968-3

Hajnik, R. L., Plante, J. A., Liang, Y., Alameh, M.-G., Tang, J., Bonam, S. R., Zhong, C., Adam, A., Scharton, D., Rafael, G. H., Liu, Y., Hazell, N. C., Sun, J., Soong, L.,

Shi, P.-Y., Wang, T., Walker, D. H., Sun, J., Weissman, D., … Hu, H. (2022). Dual spike and nucleocapsid mRNA vaccination confer protection against SARS-CoV-2 Omicron and Delta variants in preclinical models. *Science Translational Medicine*, *14*(662), eabq1945. https://doi.org/10.1126/scitranslmed.abq1945

Halland, M., Haque, R., Langhorst, J., Boone, J. H., & Petri, W. A. (2021). Clinical performance of the H. PYLORI QUIK CHEK™ and H. PYLORI CHEK™ assays, novel stool antigen tests for diagnosis of *Helicobacter pylori*. *European Journal of Clinical Microbiology & Infectious Diseases*, *40*(5), 1023. https://doi.org/10.1007/s10096-020-04137-7

Han, L., Zhao, Y., Cui, S., & Liang, B. (2018). Redesigning of Microbial Cell Surface and Its Application to Whole-Cell Biocatalysis and Biosensors. *Applied Biochemistry and Biotechnology*, *185*(2), 396–418. https://doi.org/10.1007/s12010-017-2662-6

Han, L., Zhuang, M.-W., Deng, J., Zheng, Y., Zhang, J., Nan, M.-L., Zhang, X.-J., Gao, C., & Wang, P.-H. (2021). SARS-CoV-2 ORF9b antagonizes type I and III interferons by targeting multiple components of the RIG-I/MDA-5-MAVS, TLR3-TRIF, and cGAS-STING signaling pathways. *Journal of Medical Virology*, *93*(9), 5376–5389. https://doi.org/10.1002/jmv.27050

Hanafi, M. I., & Mohamed, A. M. (2013). *Helicobacter pylori* infection: Seroprevalence and predictors among healthy individuals in Al Madinah, Saudi Arabia. *The Journal of the Egyptian Public Health Association*, *88*(1), 40–45. https://doi.org/10.1097/01.EPX.0000427043.99834.a4

Hansen, S., Melby, K. K., Aase, S., Jellum, E., & Vollset, S. E. (1999). *Helicobacter pylori* infection and risk of cardia cancer and non-cardia gastric cancer. A nested case-control study. *Scandinavian Journal of Gastroenterology*, *34*(4), 353–360. https://doi.org/10.1080/003655299750026353

Hansson, L. E., Nyrén, O., Hsing, A. W., Bergström, R., Josefsson, S., Chow, W. H., Fraumeni, J. F., & Adami, H. O. (1996). The risk of stomach cancer in patients with gastric or duodenal ulcer disease. *The New England Journal of Medicine*, *335*(4), 242–249. https://doi.org/10.1056/NEJM199607253350404

Harwood, C. R. (1992). *Bacillus subtilis* and its relatives: Molecular biological and industrial workhorses. *Trends in Biotechnology*, *10*(7), 247–256. https://doi.org/10.1016/0167-7799(92)90233-l

Harwood, C. R., & Cutting, S. M. (1990). *Molecular biological methods for Bacillus* ((Eds)). John Wiley & Sons Ltd.

He, J., Huang, J.-R., Zhang, Y.-L., & Zhang, J. (2021). SARS-CoV-2 nucleocapsid protein intranasal inoculation induces local and systemic T cell responses in mice. *Journal of Medical Virology*, *93*(4), 1923–1925. https://doi.org/10.1002/jmv.26769

Heap, J. T., Ehsaan, M., Cooksley, C. M., Ng, Y.-K., Cartman, S. T., Winzer, K., & Minton, N. P. (2012). Integration of DNA into bacterial chromosomes from plasmids without a counter-selection marker. *Nucleic Acids Research*, *40*(8), e59. https://doi.org/10.1093/nar/gkr1321

Heinz, F. X., & Stiasny, K. (2021). Distinguishing features of current COVID-19 vaccines: Knowns and unknowns of antigen presentation and modes of action. *Npj Vaccines*, *6*(1), Article 1. https://doi.org/10.1038/s41541-021-00369-6

Henriques, A. O., & Moran, C. P. (2007). Structure, assembly, and function of the spore surface layers. *Annual Review of Microbiology*, *61*, 555–588. https://doi.org/10.1146/annurev.micro.61.080706.093224

Hentzien, M., Autran, B., Piroth, L., Yazdanpanah, Y., & Calmy, A. (2022). A monoclonal antibody stands out against omicron subvariants: A call to action for a wider access to bebtelovimab. *The Lancet Infectious Diseases*, *22*(9), 1278. https://doi.org/10.1016/S1473-3099(22)00495-9

Hermosilla, E., Coma, E., Xie, J., Feng, S., Cabezas, C., Méndez-Boo, L., Fina, F., Ballo, E., Martínez, M., Medina-Peralta, M., Argimon, J. M., & Prieto-Alhambra, D. (2022). Comparative effectiveness and safety of homologous two-dose ChAdOx1 versus heterologous vaccination with ChAdOx1 and BNT162b2. *Nature Communications*, *13*(1), Article 1. https://doi.org/10.1038/s41467-022-29301-9

Higashi, H., Tsutsumi, R., Muto, S., Sugiyama, T., Azuma, T., Asaka, M., & Hatakeyama, M. (2002). SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter*

pylori CagA protein. *Science (New York, N.Y.)*, *295*(5555), 683–686. https://doi.org/10.1126/science.1067147

Hinc, K., Isticato, R., Dembek, M., Karczewska, J., Iwanicki, A., Peszyńska-Sularz, G., De Felice, M., Obuchowski, M., & Ricca, E. (2010). Expression and display of UreA of *Helicobacter acinonychis* on the surface of *Bacillus subtilis* spores. *Microbial Cell Factories*, *9*(1), 2. https://doi.org/10.1186/1475-2859-9-2

Hino, B., Eliakim, R., Levine, A., Sprecher, H., Berkowitz, B., Hartman, C., Eshach-Adiv, O., & Shamir, R. (2004). Comparison of invasive and non-invasive tests diagnosis and monitoring of *Helicobacter pylori* infection in children. *Journal of Pediatric Gastroenterology and Nutrition*, *39*(5). https://doi.org/10.1097/00005176-200411000-00013

Hirayama, F., Takagi, S., Kusuhara, H., Iwao, E., Yokoyama, Y., & Ikeda, Y. (1996). Induction of gastric ulcer and intestinal metaplasia in mongolian gerbils infected with *Helicobacter pylori*. *Journal of Gastroenterology*, *31*(5), 755–757. https://doi.org/10.1007/BF02347631

Hoang, T. H., Hong, H. A., Clark, G. C., Titball, R. W., & Cutting, S. M. (2008). Recombinant *Bacillus subtilis* expressing the *Clostridium perfringens* alpha toxoid is a candidate orally delivered vaccine against necrotic enteritis. *Infection and Immunity*, *76*(11), 5257–5265. https://doi.org/10.1128/IAI.00686-08

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., Schiergens, T. S., Herrler, G., Wu, N.-H., Nitsche, A., Müller, M. A., Drosten, C., & Pöhlmann, S. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell*, *181*(2), 271-280.e8. https://doi.org/10.1016/j.cell.2020.02.052

Homayun, B., Lin, X., & Choi, H.-J. (2019). Challenges and Recent Progress in Oral Drug Delivery Systems for Biopharmaceuticals. *Pharmaceutics*, *11*(3), 129. https://doi.org/10.3390/pharmaceutics11030129

Honda, S., Fujioka, T., Tokieda, M., Gotoh, T., Nishizono, A., & Nasu, M. (1998). Gastric ulcer, atrophic gastritis, and intestinal metaplasia caused by *Helicobacter pylori*

infection in Mongolian gerbils. *Scandinavian Journal of Gastroenterology*, *33*(5). https://doi.org/10.1080/00365529850171990

Hong, H. A., Duc, L. H., & Cutting, S. M. (2005). The use of bacterial spore formers as probiotics. *FEMS Microbiology Reviews*, *29*(4), 813–835. https://doi.org/10.1016/j.femsre.2004.12.001

Hong, H. A., Hitri, K., Hosseini, S., Kotowicz, N., Bryan, D., Mawas, F., Wilkinson, A. J., Broekhoven, A. van, Kearsey, J., & Cutting, S. M. (2017). Mucosal Antibodies to the C Terminus of Toxin A Prevent Colonization of *Clostridium difficile*. *Infection and Immunity*, *85*(4). https://doi.org/10.1128/IAI.01060-16

Hong, H. A., To, E., Fakhry, S., Baccigalupi, L., Ricca, E., & Cutting, S. M. (2009). Defining the natural habitat of *Bacillus* spore-formers. *Research in Microbiology*, *160*(6), 375–379. https://doi.org/10.1016/j.resmic.2009.06.006

Hooi, J. K. Y., Lai, W. Y., Ng, W. K., Suen, M. M. Y., Underwood, F. E., Tanyingoh, D., Malfertheiner, P., Graham, D. Y., Wong, V. W. S., Wu, J. C. Y., Chan, F. K. L., Sung, J. J. Y., Kaplan, G. G., & Ng, S. C. (2017). Global Prevalence of *Helicobacter pylori* Infection: Systematic Review and Meta-Analysis. *Gastroenterology*, *153*(2), 420–429. https://doi.org/10.1053/j.gastro.2017.04.022

Horby, P., Lim, W. S., Emberson, J., Mafham, M., Bell, J., Linsell, L., Staplin, N., Brightling, C., Ustianowski, A., Elmahi, E., Prudon, B., Green, C., Felton, T., Chadwick, D., Rege, K., Fegan, C., Chappell, L. C., Faust, S. N., Jaki, T., … Group, R. C. (2020). *Effect of Dexamethasone in Hospitalized Patients with COVID-19 – Preliminary Report* (p. 2020.06.22.20137273). medRxiv. https://doi.org/10.1101/2020.06.22.20137273

Hosoya, S., Lu, Z., Ozaki, Y., Takeuchi, M., & Sato, T. (2007). Cytological Analysis of the Mother Cell Death Process during Sporulation in *Bacillus subtilis*. *Journal of Bacteriology*, *189*(6), 2561–2565. https://doi.org/10.1128/JB.01738-06

Hosseini, S., Curilovs, A., & Cutting, S. M. (2018). Biological Containment of Genetically Modified *Bacillus subtilis*. *Applied and Environmental Microbiology*, *84*(3), e02334-17. https://doi.org/10.1128/AEM.02334-17

Hou, Y. J., Okuda, K., Edwards, C. E., Martinez, D. R., Asakura, T., Dinnon, K. H., Kato, T., Lee, R. E., Yount, B. L., Mascenik, T. M., Chen, G., Olivier, K. N., Ghio, A., Tse, L. V., Leist, S. R., Gralinski, L. E., Schäfer, A., Dang, H., Gilmore, R., … Baric, R. S. (2020). SARS-CoV-2 Reverse Genetics Reveals a Variable Infection Gradient in the Respiratory Tract. *Cell*, *182*(2), 429-446.e14. https://doi.org/10.1016/j.cell.2020.05.042

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., Zhang, L., Fan, G., Xu, J., Gu, X., Cheng, Z., Yu, T., Xia, J., Wei, Y., Wu, W., Xie, X., Yin, W., Li, H., Liu, M., … Cao, B. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *The Lancet*, *395*(10223), 497–506. https://doi.org/10.1016/S0140-6736(20)30183-5

Huang, J. Y., Sweeney, E. G., Sigal, M., Zhang, H. C., Remington, S. J., Cantrell, M. A., Kuo, C. J., Guillemin, K., & Amieva, M. R. (2015). Chemodetection and Destruction of Host Urea Allows *Helicobacter pylori* to Locate the Epithelium. *Cell Host & Microbe*, *18*(2), 147–156. https://doi.org/10.1016/j.chom.2015.07.002

Huang, J.-M., Hong, H. A., Van Tong, H., Hoang, T. H., Brisson, A., & Cutting, S. M. (2010). Mucosal delivery of antigens using adsorption to bacterial spores. *Vaccine*, *28*(4), 1021–1030. https://doi.org/10.1016/j.vaccine.2009.10.127

Huang, J.-M., La Ragione, R. M., Cooley, W. A., Todryk, S., & Cutting, S. M. (2008). Cytoplasmic delivery of antigens, by *Bacillus subtilis* enhances Th1 responses. *Vaccine*, *26*(48), 6043–6052. https://doi.org/10.1016/j.vaccine.2008.09.024

Huang, J.-M., La Ragione, R. M., Nunez, A., & Cutting, S. M. (2008). Immunostimulatory activity of *Bacillus* spores. *FEMS Immunology and Medical Microbiology*, *53*(2), 195–203. https://doi.org/10.1111/j.1574-695X.2008.00415.x

Huang, Y., Yang, C., Xu, X., Xu, W., & Liu, S. (2020). Structural and functional properties of SARS-CoV-2 spike protein: Potential antivirus drug development for COVID-19. *Acta Pharmacologica Sinica*, *41*(9), Article 9. https://doi.org/10.1038/s41401-020-0485-4

Hubbell, J. A., Thomas, S. N., & Swartz, M. A. (2009). Materials engineering for immunomodulation. *Nature*, *462*(7272), 449–460. https://doi.org/10.1038/nature08604

Huovinen, P., Sundström, L., Swedberg, G., & Sköld, O. (1995). Trimethoprim and sulfonamide resistance. *Antimicrobial Agents and Chemotherapy*, *39*(2), 279–289.

IARC. (1994). Schistosomes, liver flukes and *Helicobacter pylori*. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994. *Iarc Monographs on the Evaluation of Carcinogenic Risks to Humans*, *61*, 1.

Imae, Y., Strominger, M., & Strominger, jl. (1976). Electron microscope studies of conditional spore cortexless mutants of *Bacillus sphaericus*. *Journal of Bacteriology*, *127*(3). https://doi.org/10.1128/jb.127.3.1568-1570.1976

Imrie, C., Rowland, M., Bourke, B., & Drumm, B. (2001). Limitations to carbon 13-labeled urea breath testing for *Helicobacter pylori* in infants. *The Journal of Pediatrics*, *139*(5). https://doi.org/10.1067/mpd.2001.118398

Isakova-Sivak, I., Stepanova, E., Matyushenko, V., Niskanen, S., Mezhenskaya, D., Bazhenova, E., Krutikova, E., Kotomina, T., Prokopenko, P., Neterebskii, B., Doronin, A., Vinogradova, E., Yakovlev, K., Sivak, K., & Rudenko, L. (2022). Development of a T Cell-Based COVID-19 Vaccine Using a Live Attenuated Influenza Vaccine Viral Vector. *Vaccines*, *10*(7), Article 7. https://doi.org/10.3390/vaccines10071142

Isticato, R., Cangiano, G., Tran, H. T., Ciabattini, A., Medaglini, D., Oggioni, M. R., De Felice, M., Pozzi, G., & Ricca, E. (2001). Surface display of recombinant proteins on *Bacillus subtilis* spores. *Journal of Bacteriology*, *183*(21), 6294–6301. https://doi.org/10.1128/JB.183.21.6294-6301.2001

Isticato, R., Scotto Di Mase, D., Mauriello, E. M. F., De Felice, M., & Ricca, E. (2007). Amino terminal fusion of heterologous proteins to CotC increases display efficiencies in the *Bacillus subtilis* spore system. *BioTechniques*, *42*(2), 151–156. https://doi.org/10.2144/000112329

Iwanicki, A., Piątek, I., Stasiłojć, M., Grela, A., Łęga, T., Obuchowski, M., & Hinc, K. (2014). A system of vectors for *Bacillus subtilis* spore surface display. *Microbial Cell Factories, 13*(1), 30. https://doi.org/10.1186/1475-2859-13-30

Jabri, E., Carr, M. B., Hausinger, R. P., & Karplus, P. A. (1995). The Crystal Structure of Urease from *Klebsiella aerogenes*. *Science, 268*(5213), 998–1004. https://doi.org/10.1126/science.7754395

James, J., Meyer, S. M., Hong, H. A., Dang, C., Linh, H. T. Y., Ferreira, W., Katsande, P. M., Vo, L., Hynes, D., Love, W., Banyard, A. C., & Cutting, S. M. (2022). Intranasal Treatment of Ferrets with Inert Bacterial Spores Reduces Disease Caused by a Challenging H7N9 Avian Influenza Virus. *Vaccines, 10*(9), 1559. https://doi.org/10.3390/vaccines10091559

Jearanaiwitayakul, T., Seesen, M., Chawengkirttikul, R., Limthongkul, J., Apichirapokey, S., Sapsutthipas, S., Phumiamorn, S., Sunintaboon, P., & Ubol, S. (2021). Intranasal Administration of RBD Nanoparticles Confers Induction of Mucosal and Systemic Immunity against SARS-CoV-2. *Vaccines, 9*(7), 768. https://doi.org/10.3390/vaccines9070768

Jelinek, D. F., & Lipsky, P. E. (1987). Enhancement of human B cell proliferation and differentiation by tumor necrosis factor-alpha and interleukin 1. *Journal of Immunology (Baltimore, Md.: 1950), 139*(9), 2970–2976.

Jencks, D. S., Adam, J. D., Borum, M. L., Koh, J. M., Stephen, S., & Doman, D. B. (2018). Overview of Current Concepts in Gastric Intestinal Metaplasia and Gastric Cancer. *Gastroenterology & Hepatology, 14*(2), 92–101.

Jia, Q., Masleša-Galić, S., Nava, S., & Horwitz, M. A. (2022). Listeria-vectored multi-antigenic tuberculosis vaccine protects C57BL/6 and BALB/c mice and guinea pigs against Mycobacterium tuberculosis challenge. *Communications Biology, 5*(1), Article 1. https://doi.org/10.1038/s42003-022-04345-1

John Albert, M., Al-Mekhaizeem, K., Neil, L., Dhar, R., Dhar, P. M., Al-Ali, M., Al-Abkal, H. M., & Haridas, S. (2006). High prevalence and level of resistance to metronidazole, but lack of resistance to other antimicrobials in *Helicobacter pylori*, isolated from a multiracial population in Kuwait. *Alimentary Pharmacology &*

Therapeutics, *24*(9), 1359–1366. https://doi.org/10.1111/j.1365-2036.2006.03144.x

Johnson, S., Martinez, C. I., Tedjakusuma, S. N., Peinovich, N., Dora, E. G., Birch, S. M., Kajon, A. E., Werts, A. D., & Tucker, S. N. (2022). Oral Vaccination Protects Against Severe Acute Respiratory Syndrome Coronavirus 2 in a Syrian Hamster Challenge Model. *The Journal of Infectious Diseases*, *225*(1), 34–41. https://doi.org/10.1093/infdis/jiab561

Kalinke, U., Barouch, D. H., Rizzi, R., Lagkadinou, E., Türeci, Ö., Pather, S., & Neels, P. (2022). Clinical development and approval of COVID-19 vaccines. *Expert Review of Vaccines*, *21*(5), 609–619. https://doi.org/10.1080/14760584.2022.2042257

Kampf, G., Brüggemann, Y., Kaba, H. E. J., Steinmann, J., Pfaender, S., Scheithauer, S., & Steinmann, E. (2020). Potential sources, modes of transmission and effectiveness of prevention measures against SARS-CoV-2. *The Journal of Hospital Infection*, *106*(4), 678–697. https://doi.org/10.1016/j.jhin.2020.09.022

Karimi, S., Rashidian, E., Birjandi, M., & Mahmoodnia, L. (2018). Antagonistic effect of isolated probiotic bacteria from natural sources against intestinal *Escherichia coli* pathotypes. *Electronic Physician*, *10*(3), 6534. https://doi.org/10.19082/6534

Kasuga, Y., Zhu, B., Jang, K.-J., & Yoo, J.-S. (2021). Innate immune sensing of coronavirus and viral evasion strategies. *Experimental & Molecular Medicine*, *53*(5), 723–736. https://doi.org/10.1038/s12276-021-00602-1

Katsande, P. M., Fernández-Bastit, L., Ferreira, W. T., Vergara-Alert, J., Hess, M., Lloyd-Jones, K., Hong, H. A., Segales, J., & Cutting, S. M. (2022). Heterologous Systemic Prime–Intranasal Boosting Using a Spore SARS-CoV-2 Vaccine Confers Mucosal Immunity and Cross-Reactive Antibodies in Mice as well as Protection in Hamsters. *Vaccines*, *10*(11), 1900. https://doi.org/10.3390/vaccines10111900

Katsande, P. M., Nguyen, V. D., Nguyen, T. L. P., Nguyen, T. K. C., Mills, G., Bailey, D. M. D., Christie, G., Hong, H. A., & Cutting, S. M. (2023). Prophylactic immunization to *Helicobacter pylori* infection using spore vectored vaccines. *Helicobacter*, e12997. https://doi.org/10.1111/hel.12997

Kayali, S., Aloe, R., Bonaguri, C., Gaiani, F., Manfredi, M., Leandro, G., Fornaroli, F., Di Mario, F., & De' Angelis, G. L. (2018). Non-invasive tests for the diagnosis of helicobacter pylori: State of the art. *Acta Bio-Medica: Atenei Parmensis*, *89*(8-S), 58–64. https://doi.org/10.23750/abm.v89i8-S.7910

Kayesh, M. E. H., Kohara, M., & Tsukiyama-Kohara, K. (2021). An Overview of Recent Insights into the Response of TLR to SARS-CoV-2 Infection and the Potential of TLR Agonists as SARS-CoV-2 Vaccine Adjuvants. *Viruses*, *13*(11), 2302. https://doi.org/10.3390/v13112302

Keates, S., Keates, A., Warny, M., Peek, R., Murray, P., & Kelly, C. (1999). Differential activation of mitogen-activated protein kinases in AGS gastric epithelial cells by cag+ and cag- *Helicobacter pylori. Journal of Immunology (Baltimore, Md. : 1950)*, *163*(10). https://pubmed.ncbi.nlm.nih.gov/10553083/

Keilberg, D., & Ottemann, K. M. (2016). How *Helicobacter pylori* senses, targets and interacts with the gastric epithelium. *Environmental Microbiology*, *18*(3), 791–806. https://doi.org/10.1111/1462-2920.13222

Khan, M., Yoo, S.-J., Clijsters, M., Backaert, W., Vanstapel, A., Speleman, K., Lietaer, C., Choi, S., Hether, T. D., Marcelis, L., Nam, A., Pan, L., Reeves, J. W., Van Bulck, P., Zhou, H., Bourgeois, M., Debaveye, Y., De Munter, P., Gunst, J., … Van Gerven, L. (2021). Visualizing in deceased COVID-19 patients how SARS-CoV-2 attacks the respiratory and olfactory mucosae but spares the olfactory bulb. *Cell*, *184*(24), 5932-5949.e15. https://doi.org/10.1016/j.cell.2021.10.027

Khanna, K., Lopez-Garrido, J., & Pogliano, K. (2020). Shaping an Endospore: Architectural Transformations During *Bacillus subtilis* Sporulation. *Annual Review of Microbiology*, *74*, 361–386. https://doi.org/10.1146/annurev-micro-022520-074650

Kikkert, M. (2020). Innate Immune Evasion by Human Respiratory RNA Viruses. *Journal of Innate Immunity*, *12*(1), 4–20. https://doi.org/10.1159/000503030

Kim, J., & Schumann, W. (2009). Display of proteins on *Bacillus subtilis* endospores. *Cellular and Molecular Life Sciences: CMLS*, *66*(19), 3127–3136. https://doi.org/10.1007/s00018-009-0067-6

Kim, J.-H., Lee, C.-S., & Kim, B.-G. (2005). Spore-displayed streptavidin: A live diagnostic tool in biotechnology. *Biochemical and Biophysical Research Communications*, *331*(1), 210–214. https://doi.org/10.1016/j.bbrc.2005.03.144

Kirsebom, F. C. M., Andrews, N., Sachdeva, R., Stowe, J., Ramsay, M., & Lopez Bernal, J. (2022). Effectiveness of ChAdOx1-S COVID-19 booster vaccination against the Omicron and Delta variants in England. *Nature Communications*, *13*(1), Article 1. https://doi.org/10.1038/s41467-022-35168-7

Knoops, K., Kikkert, M., Worm, S. H. E. van den, Zevenhoven-Dobbe, J. C., van der Meer, Y., Koster, A. J., Mommaas, A. M., & Snijder, E. J. (2008). SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum. *PLoS Biology*, *6*(9), e226. https://doi.org/10.1371/journal.pbio.0060226

Kociolek, L. K., & Gerding, D. N. (2016). Breakthroughs in the treatment and prevention of *Clostridium difficile* infection. *Nature Reviews. Gastroenterology & Hepatology*, *13*(3), 150–160. https://doi.org/10.1038/nrgastro.2015.220

Kotloff, K. L., Wasserman, S. S., Losonsky, G. A., Thomas, W., Nichols, R., Edelman, R., Bridwell, M., & Monath, T. P. (2001). Safety and Immunogenicity of Increasing Doses of a *Clostridium difficile* Toxoid Vaccine Administered to Healthy Adults. *Infection and Immunity*, *69*(2), 988–995. https://doi.org/10.1128/IAI.69.2.988-995.2001

Kreimendahl, S., & Rassow, J. (2020). The Mitochondrial Outer Membrane Protein Tom70-Mediator in Protein Traffic, Membrane Contact Sites and Innate Immunity. *International Journal of Molecular Sciences*, *21*(19), 7262. https://doi.org/10.3390/ijms21197262

Ksiazek, T. G., Erdman, D., Goldsmith, C. S., Zaki, S. R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J. A., Lim, W., Rollin, P. E., Dowell, S. F., Ling, A.-E., Humphrey, C. D., Shieh, W.-J., Guarner, J., Paddock, C. D., Rota, P., Fields, B., … SARS Working Group. (2003). A novel coronavirus associated with severe acute respiratory syndrome. *The New England Journal of Medicine*, *348*(20), 1953–1966. https://doi.org/10.1056/NEJMoa030781

Kucirka, L. M., Lauer, S. A., Laeyendecker, O., Boon, D., & Lessler, J. (2020). Variation in False-Negative Rate of Reverse Transcriptase Polymerase Chain Reaction–Based SARS-CoV-2 Tests by Time Since Exposure. *Annals of Internal Medicine*, M20-1495. https://doi.org/10.7326/M20-1495

Kuipers, E. J., Lundell, L., Klinkenberg-Knol, E. C., Havu, N., Festen, H. P., Liedman, B., Lamers, C. B., Jansen, J. B., Dalenback, J., Snel, P., Nelis, G. F., & Meuwissen, S. G. (1996). Atrophic gastritis and *Helicobacter pylori* infection in patients with reflux esophagitis treated with omeprazole or fundoplication. *The New England Journal of Medicine*, *334*(16), 1018–1022. https://doi.org/10.1056/NEJM199604183341603

Kuipers, E. J., Uyterlinde, A. M., Peña, A. S., Roosendaal, R., Pals, G., Nelis, G. F., Festen, H. P., & Meuwissen, S. G. (1995). Long-term sequelae of *Helicobacter pylori* gastritis. *Lancet (London, England)*, *345*(8964), 1525–1528. https://doi.org/10.1016/s0140-6736(95)91084-0

Kuloğlu, Z., Kansu, A., Kırsaçlıoğlu, C. T., Üstündağ, G., Aysev, D., Ensari, A., Küçük, N. Ö., & Girgin, N. (2008). A rapid lateral flow stool antigen immunoassay and 14C-urea breath test for the diagnosis and eradication of *Helicobacter pylori* infection in children. *Diagnostic Microbiology and Infectious Disease*, *62*(4), 351–356. https://doi.org/10.1016/j.diagmicrobio.2008.07.006

Kupferschmidt, K. (2013). *Activists Destroy 'Golden Rice' Field Trial*. https://www.science.org/content/article/activists-destroy-golden-rice-field-trial

Kuzminov, A. (2011). Homologous Recombination—Experimental Systems, Analysis, and Significance. *EcoSal Plus*, *4*(2), 10.1128/ecosalplus.7.2.6. https://doi.org/10.1128/ecosalplus.7.2.6

Lam, R., Romanov, V., Johns, K., Battaile, K. P., Wu-Brown, J., Guthrie, J. L., Hausinger, R. P., Pai, E. F., & Chirgadze, N. Y. (2010). Crystal Structure of a Truncated Urease Accessory Protein UreF From *Helicobacter pylori*. *Proteins*, *78*(13), 2839–2848. https://doi.org/10.1002/prot.22802

Lamb, A., & Chen, L.-F. (2013). Role of the *Helicobacter pylori*-induced inflammatory response in the development of gastric cancer. *Journal of Cellular Biochemistry*, *114*(3), 491–497. https://doi.org/10.1002/jcb.24389

Lambrecht, B. N., & Hammad, H. (2013). Asthma: The importance of dysregulated barrier immunity. *European Journal of Immunology*, *43*(12), 3125–3137. https://doi.org/10.1002/eji.201343730

Lamers, M. M., & Haagmans, B. L. (2022). SARS-CoV-2 pathogenesis. *Nature Reviews Microbiology*, *20*(5), Article 5. https://doi.org/10.1038/s41579-022-00713-0

Lamers, M. M., Mykytyn, A. Z., Breugem, T. I., Wang, Y., Wu, D. C., Riesebosch, S., van den Doel, P. B., Schipper, D., Bestebroer, T., Wu, N. C., & Haagmans, B. L. (2021). Human airway cells prevent SARS-CoV-2 multibasic cleavage site cell culture adaptation. *ELife*, *10*, e66815. https://doi.org/10.7554/eLife.66815

Lapuente, D., Fuchs, J., Willar, J., Vieira Antão, A., Eberlein, V., Uhlig, N., Issmail, L., Schmidt, A., Oltmanns, F., Peter, A. S., Mueller-Schmucker, S., Irrgang, P., Fraedrich, K., Cara, A., Hoffmann, M., Pöhlmann, S., Ensser, A., Pertl, C., Willert, T., … Tenbusch, M. (2021). Protective mucosal immunity against SARS-CoV-2 after heterologous systemic prime-mucosal boost immunization. *Nature Communications*, *12*(1), 6871. https://doi.org/10.1038/s41467-021-27063-4

Lauer, S. A., Grantz, K. H., Bi, Q., Jones, F. K., Zheng, Q., Meredith, H. R., Azman, A. S., Reich, N. G., & Lessler, J. (2020). The Incubation Period of Coronavirus Disease 2019 (COVID-19) From Publicly Reported Confirmed Cases: Estimation and Application. *Annals of Internal Medicine*, *172*(9), 577–582. https://doi.org/10.7326/M20-0504

Lavin, M., Elder, P. T., O'Keeffe, D., Enright, H., Ryan, E., Kelly, A., El Hassadi, E., McNicholl, F. P., Benson, G., Le, G. N., Byrne, M., Ryan, K., O'Connell, N. M., & O'Donnell, J. S. (2021). Vaccine-induced immune thrombotic thrombocytopenia (VITT) – a novel clinico-pathological entity with heterogeneous clinical presentations. *British Journal of Haematology*, *195*(1), 76–84. https://doi.org/10.1111/bjh.17613

Layke, J. C., & Lopez, P. P. (2004). Gastric Cancer: Diagnosis and Treatment Options. *American Family Physician*, *69*(5), 1133–1141.

Le Bert, N., Tan, A. T., Kunasegaran, K., Tham, C. Y. L., Hafezi, M., Chia, A., Chng, M. H. Y., Lin, M., Tan, N., Linster, M., Chia, W. N., Chen, M. I.-C., Wang, L.-F., Ooi, E. E., Kalimuddin, S., Tambyah, P. A., Low, J. G.-H., Tan, Y.-J., & Bertoletti, A. (2020). SARS-CoV-2-specific T cell immunity in cases of COVID-19 and SARS, and uninfected controls. *Nature*, *584*(7821), Article 7821. https://doi.org/10.1038/s41586-020-2550-z

Lechien, J. R., Chiesa-Estomba, C. M., Place, S., Van Laethem, Y., Cabaraux, P., Mat, Q., Huet, K., Plzak, J., Horoi, M., Hans, S., Rosaria Barillari, M., Cammaroto, G., Fakhry, N., Martiny, D., Ayad, T., Jouffe, L., Hopkins, C., Saussez, S., & Yo-Ifos, C.-19 T. F. of. (2020). Clinical and epidemiological characteristics of 1420 European patients with mild-to-moderate coronavirus disease 2019. *Journal of Internal Medicine*, *288*(3), 335–344. https://doi.org/10.1111/joim.13089

Lee, C. K. (2001). Vaccination against Helicobacter pylori in non-human primate models and humans. *Scandinavian Journal of Immunology*, *53*(5), 437–442. https://doi.org/10.1046/j.1365-3083.2001.00911.x

Lee, J. Y., Wing, P. A., Gala, D. S., Noerenberg, M., Järvelin, A. I., Titlow, J., Zhuang, X., Palmalux, N., Iselin, L., Thompson, M. K., Parton, R. M., Prange-Barczynska, M., Wainman, A., Salguero, F. J., Bishop, T., Agranoff, D., James, W., Castello, A., McKeating, J. A., & Davis, I. (2022). Absolute quantitation of individual SARS-CoV-2 RNA molecules provides a new paradigm for infection dynamics and variant differences. *ELife*, *11*, e74153. https://doi.org/10.7554/eLife.74153

Lee, S., Belitsky, B. R., Brinker, J. P., Kerstein, K. O., Brown, D. W., Clements, J. D., Keusch, G. T., Tzipori, S., Sonenshein, A. L., & Herrmann, J. E. (2010). Development of a *Bacillus subtilis*-Based Rotavirus Vaccine. *Clinical and Vaccine Immunology : CVI*, *17*(11), 1647–1655. https://doi.org/10.1128/CVI.00135-10

Lei, H., Alu, A., Yang, J., Ren, W., He, C., Lan, T., He, X., Yang, L., Li, J., Wang, Z., Song, X., Wang, W., Lu, G., & Wei, X. (2022). Intranasal administration of a recombinant RBD vaccine induces long-term immunity against Omicron-included

SARS-CoV-2 variants. *Signal Transduction and Targeted Therapy*, *7*, 159. https://doi.org/10.1038/s41392-022-01002-1

Letley, D. P., Rhead, J. L., Twells, R. J., Dove, B., & Atherton, J. C. (2003). Determinants of non-toxicity in the gastric pathogen *Helicobacter pylori*. *The Journal of Biological Chemistry*, *278*(29), 26734–26741. https://doi.org/10.1074/jbc.M304071200

Levin, P. A., Shim, J. J., & Grossman, A. D. (1998). Effect of minCD on FtsZ Ring Position and Polar Septation in *Bacillus subtilis*. *Journal of Bacteriology*, *180*(22), 6048–6051.

Levine-Tiefenbrun, M., Yelin, I., Katz, R., Herzel, E., Golan, Z., Schreiber, L., Wolf, T., Nadler, V., Ben-Tov, A., Kuint, J., Gazit, S., Patalon, T., Chodick, G., & Kishony, R. (2021). Initial report of decreased SARS-CoV-2 viral load after inoculation with the BNT162b2 vaccine. *Nature Medicine*, *27*(5), Article 5. https://doi.org/10.1038/s41591-021-01316-7

Levy, I., & Gralnek, I. M. (2016). Complications of diagnostic colonoscopy, upper endoscopy, and enteroscopy. *Best Practice & Research. Clinical Gastroenterology*, *30*(5), 705–718. https://doi.org/10.1016/j.bpg.2016.09.005

Leyton, D. L., Rossiter, A. E., & Henderson, I. R. (2012). From self sufficiency to dependence: Mechanisms and factors important for autotransporter biogenesis. *Nature Reviews Microbiology*, *10*(3), Article 3. https://doi.org/10.1038/nrmicro2733

Li, F. (2016). Structure, Function, and Evolution of Coronavirus Spike Proteins. *Annual Review of Virology*, *3*(1), 237–261. https://doi.org/10.1146/annurev-virology-110615-042301

Li, G., Cappuccini, F., Marchevsky, N. G., Aley, P. K., Aley, R., Anslow, R., Bibi, S., Cathie, K., Clutterbuck, E., Faust, S. N., Feng, S., Heath, P. T., Kerridge, S., Lelliott, A., Mujadidi, Y., Ng, K. F., Rhead, S., Roberts, H., Robinson, H., … Team, B. C. R. N. (2022). Safety and immunogenicity of the ChAdOx1 nCoV-19 (AZD1222) vaccine in children aged 6–17 years: A preliminary report of COV006,

a phase 2 single-blind, randomised, controlled trial. *The Lancet*, *399*(10342), 2212–2225. https://doi.org/10.1016/S0140-6736(22)00770-X

Li, J.-Y., Liao, C.-H., Wang, Q., Tan, Y.-J., Luo, R., Qiu, Y., & Ge, X.-Y. (2020). The ORF6, ORF8 and nucleocapsid proteins of SARS-CoV-2 inhibit type I interferon signaling pathway. *Virus Research*, *286*, 198074. https://doi.org/10.1016/j.virusres.2020.198074

Li, L., Hu, X., Wu, Z., Xiong, S., Zhou, Z., Wang, X., Xu, J., Lu, F., & Yu, X. (2009). Immunogenicity of self-adjuvanticity oral vaccine candidate based on use of *Bacillus subtilis* spore displaying Schistosoma japonicum 26 KDa GST protein. *Parasitology Research*, *105*(6), 1643–1651. https://doi.org/10.1007/s00436-009-1606-7

Li, Q., Guan, X., Wu, P., Wang, X., Zhou, L., Tong, Y., Ren, R., Leung, K. S. M., Lau, E. H. Y., Wong, J. Y., Xing, X., Xiang, N., Wu, Y., Li, C., Chen, Q., Li, D., Liu, T., Zhao, J., Liu, M., … Feng, Z. (2020). Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus-Infected Pneumonia. *The New England Journal of Medicine*, *382*(13), 1199–1207. https://doi.org/10.1056/NEJMoa2001316

Li, W., Moore, M. J., Vasilieva, N., Sui, J., Wong, S. K., Berne, M. A., Somasundaran, M., Sullivan, J. L., Luzuriaga, K., Greenough, T. C., Choe, H., & Farzan, M. (2003). Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature*, *426*(6965), 450–454. https://doi.org/10.1038/nature02145

Li, Y., Ma, M.-L., Lei, Q., Wang, F., Hong, W., Lai, D.-Y., Hou, H., Xu, Z.-W., Zhang, B., Chen, H., Yu, C., Xue, J.-B., Zheng, Y.-X., Wang, X.-N., Jiang, H.-W., Zhang, H.-N., Qi, H., Guo, S.-J., Zhang, Y., … Tao, S.-C. (2021). Linear epitope landscape of the SARS-CoV-2 Spike protein constructed from 1,051 COVID-19 patients. *Cell Reports*, *34*(13), 108915. https://doi.org/10.1016/j.celrep.2021.108915

Liang, C.-M., Cheng, J.-W., Kuo, C.-M., Chang, K.-C., Wu, K.-L., Tai, W.-C., Chiu, K.-W., Chiou, S.-S., Lin, M.-T., Hu, T.-H., & Chuah, S.-K. (2014). Levofloxacin-containing second-line anti-*Helicobacter pylori* eradication in Taiwanese real-world practice. *Biomedical Journal*, *37*(5), 326–330. https://doi.org/10.4103/2319-4170.125650

Lin, J., Huang, L., Li, Y., Zhang, P., Yu, Q., & Yang, Q. (2021). *Bacillus subtilis* Spore-Trained Dendritic Cells Enhance the Generation of Memory T Cells via ICAM1. *Cells*, *10*(9), 2267. https://doi.org/10.3390/cells10092267

Lineburg, K. E., Grant, E. J., Swaminathan, S., Chatzileontiadou, D. S. M., Szeto, C., Sloane, H., Panikkar, A., Raju, J., Crooks, P., Rehan, S., Nguyen, A. T., Lekieffre, L., Neller, M. A., Tong, Z. W. M., Jayasinghe, D., Chew, K. Y., Lobos, C. A., Halim, H., Burrows, J. M., … Gras, S. (2021). CD8+ T cells specific for an immunodominant SARS-CoV-2 nucleocapsid epitope cross-react with selective seasonal coronaviruses. *Immunity*, *54*(5), 1055-1065.e5. https://doi.org/10.1016/j.immuni.2021.04.006

Liu, D. X., Liang, J. Q., & Fung, T. S. (2021). Human Coronavirus-229E, -OC43, -NL63, and -HKU1 (Coronaviridae). *Encyclopedia of Virology*, 428–440. https://doi.org/10.1016/B978-0-12-809633-8.21501-X

Liu, H., Gao, X., Wang, G., Zhang, J., Zhou, J., Wei, T., Zhang, Y., Liu, Y., Piao, J., Zhang, Q., Wang, Y., Ma, X., Zhu, X., Rao, Y., Xia, W., Xie, H., & Zhang, W. (2021). *A Potential Novel COVID-19 Vaccine With RBD-HR1/HR2 Hexamer Structure* [Preprint]. Biochemistry. https://doi.org/10.1101/2021.10.28.465226

Liu, K.-Y., Shi, Y., Luo, P., Yu, S., Chen, L., Zhao, Z., Mao, X.-H., Guo, G., Wu, C., & Zou, Q.-M. (2011). Therapeutic efficacy of oral immunization with attenuated Salmonella typhimurium expressing *Helicobacter pylori* CagA, VacA and UreB fusion proteins in mice model. *Vaccine*, *29*(38), 6679–6685. https://doi.org/10.1016/j.vaccine.2011.06.099

Liu, L., Iketani, S., Guo, Y., Chan, J. F.-W., Wang, M., Liu, L., Luo, Y., Chu, H., Huang, Y., Nair, M. S., Yu, J., Chik, K. K.-H., Yuen, T. T.-T., Yoon, C., To, K. K.-W., Chen, H., Yin, M. T., Sobieszczyk, M. E., Huang, Y., … Ho, D. D. (2022). Striking antibody evasion manifested by the Omicron variant of SARS-CoV-2. *Nature*, *602*(7898), Article 7898. https://doi.org/10.1038/s41586-021-04388-0

Liu, P., Jiang, J.-Z., Wan, X.-F., Hua, Y., Li, L., Zhou, J., Wang, X., Hou, F., Chen, J., Zou, J., & Chen, J. (2020). Are pangolins the intermediate host of the 2019 novel

coronavirus (SARS-CoV-2)? *PLOS Pathogens*, *16*(5), e1008421. https://doi.org/10.1371/journal.ppat.1008421

Liu, X., Shaw, R. H., Stuart, A. S. V., Greenland, M., Aley, P. K., Andrews, N. J., Cameron, J. C., Charlton, S., Clutterbuck, E. A., Collins, A. M., Dinesh, T., England, A., Faust, S. N., Ferreira, D. M., Finn, A., Green, C. A., Hallis, B., Heath, P. T., Hill, H., … Com-COV Study Group. (2021). Safety and immunogenicity of heterologous versus homologous prime-boost schedules with an adenoviral vectored and mRNA COVID-19 vaccine (Com-COV): A single-blind, randomised, non-inferiority trial. *Lancet (London, England)*, *398*(10303), 856–869. https://doi.org/10.1016/S0140-6736(21)01694-9

Liu, Y., Wang, M., Cheng, A., Yang, Q., Wu, Y., Jia, R., Liu, M., Zhu, D., Chen, S., Zhang, S., Zhao, X.-X., Huang, J., Mao, S., Ou, X., Gao, Q., Wang, Y., Xu, Z., Chen, Z., Zhu, L., … Chen, X. (2020). The role of host eIF2α in viral infection. *Virology Journal*, *17*(1), 112. https://doi.org/10.1186/s12985-020-01362-6

Logunov, D. Y., Dolzhikova, I. V., Zubkova, O. V., Tukhvatulin, A. I., Shcheblyakov, D. V., Dzharullaeva, A. S., Grousova, D. M., Erokhova, A. S., Kovyrshina, A. V., Botikov, A. G., Izhaeva, F. M., Popova, O., Ozharovskaya, T. A., Esmagambetov, I. B., Favorskaya, I. A., Zrelkin, D. I., Voronina, D. V., Shcherbinin, D. N., Semikhin, A. S., … Gintsburg, A. L. (2020). Safety and immunogenicity of an rAd26 and rAd5 vector-based heterologous prime-boost COVID-19 vaccine in two formulations: Two open, non-randomised phase 1/2 studies from Russia. *Lancet (London, England)*, *396*(10255), 887–897. https://doi.org/10.1016/S0140-6736(20)31866-3

Lopez, J., Mommert, M., Mouton, W., Pizzorno, A., Brengel-Pesce, K., Mezidi, M., Villard, M., Lina, B., Richard, J.-C., Fassier, J.-B., Cheynet, V., Padey, B., Duliere, V., Julien, T., Paul, S., Bastard, P., Belot, A., Bal, A., Casanova, J.-L., … Trouillet-Assant, S. (2021). Early nasal type I IFN immunity against SARS-CoV-2 is compromised in patients with autoantibodies against type I IFNs. *The Journal of Experimental Medicine*, *218*(10), e20211211. https://doi.org/10.1084/jem.20211211

Lou, B., Li, T.-D., Zheng, S.-F., Su, Y.-Y., Li, Z.-Y., Liu, W., Yu, F., Ge, S.-X., Zou, Q.-D., Yuan, Q., Lin, S., Hong, C.-M., Yao, X.-Y., Zhang, X.-J., Wu, D.-H., Zhou, G.-L., Hou, W.-H., Li, T.-T., Zhang, Y.-L., … Chen, Y. (2020). Serology characteristics of SARS-CoV-2 infection since the exposure and post symptoms onset (p. 2020.03.23.20041707). medRxiv. https://doi.org/10.1101/2020.03.23.20041707

Loy, C., Irwig, L., Katelaris, P., & Talley, N. (1996). Do commercial serological kits for *Helicobacter pylori* infection differ in accuracy? A meta-analysis. *The American Journal of Gastroenterology, 91*(6). https://pubmed.ncbi.nlm.nih.gov/8651160/

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., Wang, W., Song, H., Huang, B., Zhu, N., Bi, Y., Ma, X., Zhan, F., Wang, L., Hu, T., Zhou, H., Hu, Z., Zhou, W., Zhao, L., … Tan, W. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: Implications for virus origins and receptor binding. *Lancet (London, England)*, *395*(10224), 565–574. https://doi.org/10.1016/S0140-6736(20)30251-8

Lucht, J. M. (2015). Public Acceptance of Plant Biotechnology and GM Crops. *Viruses*, *7*(8), 4254–4281. https://doi.org/10.3390/v7082819

Ma, B., Yang, X., Wang, H., Cao, H., Xu, P., Ding, M., & Liu, H. (2016). Characterization of a new *Lactobacillus salivarius* strain engineered to express IBV multi-epitope antigens by chromosomal integration. *Bioscience, Biotechnology, and Biochemistry*, *80*(3), 574–583. https://doi.org/10.1080/09168451.2015.1101330

Ma, D., Tian, S., Qin, Q., Yu, Y., Jiao, J., Xiong, X., Guo, Y., Zhang, X., & Ouyang, X. (2023). Construction of an inhalable recombinant M2e-FP-expressing *Bacillus subtilis* spores-based vaccine and evaluation of its protection efficacy against influenza in a mouse model. *Vaccine*, S0264-410X(23)00655-2. https://doi.org/10.1016/j.vaccine.2023.05.074

Ma, Q., Liu, J., Liu, Q., Kang, L., Liu, R., Jing, W., Wu, Y., & Liu, M. (2021). Global Percentage of Asymptomatic SARS-CoV-2 Infections Among the Tested Population and Individuals With Confirmed COVID-19 Diagnosis: A Systematic Review and Meta-analysis. *JAMA Network Open*, *4*(12), e2137257. https://doi.org/10.1001/jamanetworkopen.2021.37257

Ma, X., Zou, F., Yu, F., Li, R., Yuan, Y., Zhang, Y., Zhang, X., Deng, J., Chen, T., Song, Z., Qiao, Y., Zhan, Y., Liu, J., Zhang, J., Zhang, X., Peng, Z., Li, Y., Lin, Y., Liang, L., … Zhang, H. (2020). Nanoparticle Vaccines Based on the Receptor Binding Domain (RBD) and Heptad Repeat (HR) of SARS-CoV-2 Elicit Robust Protective Immune Responses. *Immunity*, *53*(6), 1315-1330.e9. https://doi.org/10.1016/j.immuni.2020.11.015

Machuca, M. A., Johnson, K. S., Liu, Y. C., Steer, D. L., Ottemann, K. M., & Roujeinikova, A. (2017). *Helicobacter pylori* chemoreceptor TlpC mediates chemotaxis to lactate. *Scientific Reports*, *7*(1), Article 1. https://doi.org/10.1038/s41598-017-14372-2

Madhi, S. A., Baillie, V., Cutland, C. L., Voysey, M., Koen, A. L., Fairlie, L., Padayachee, S. D., Dheda, K., Barnabas, S. L., Bhorat, Q. E., Briner, C., Kwatra, G., Ahmed, K., Aley, P., Bhikha, S., Bhiman, J. N., Bhorat, A. E., du Plessis, J., Esmail, A., … Wits-VIDA COVID Group. (2021). Efficacy of the ChAdOx1 nCoV-19 Covid-19 Vaccine against the B.1.351 Variant. *The New England Journal of Medicine*, *384*(20), 1885–1898. https://doi.org/10.1056/NEJMoa2102214

Maeda, S., Yoshida, H., Ogura, K., Mitsuno, Y., Hirata, H., Yamaji, Y., Akanuma, M., Shiratori, Y., & Omata, M. (2000). *H. pylori* activates NF-kappaB through a signaling pathway involving IkappaB kinases, NF-kappaB-inducing kinase, TRAF2, and TRAF6 in gastric cancer cells. *Gastroenterology*, *119*(1). https://doi.org/10.1053/gast.2000.8540

Mahase, E. (2021a). Covid-19: Pfizer's paxlovid is 89% effective in patients at risk of serious illness, company reports. *BMJ*, *375*, n2713. https://doi.org/10.1136/bmj.n2713

Mahase, E. (2021b). Covid-19: UK approves monoclonal antibody sotrovimab for over 12s at high risk. *BMJ*, *375*, n2990. https://doi.org/10.1136/bmj.n2990

Malfertheiner, P., Megraud, F., O'Morain, C., Bazzoli, F., El-Omar, E., Graham, D., Hunt, R., Rokkas, T., Vakil, N., & Kuipers, E. (2007). Current concepts in the management of *Helicobacter pylori* infection: The Maastricht III Consensus Report. *Gut*, *56*(6). https://doi.org/10.1136/gut.2006.101634

Mallidis, C. G., & Scholefield, J. (1987). Relation of the heat resistance of bacterial spores to chemical composition and structure I. Relation to core components. *Journal of Applied Bacteriology*, *62*(1), 65–69. https://doi.org/10.1111/j.1365-2672.1987.tb02381.x

Manik, M., & Singh, R. K. (2022). Role of toll-like receptors in modulation of cytokine storm signaling in SARS-CoV-2-induced COVID-19. *Journal of Medical Virology*, *94*(3), 869–877. https://doi.org/10.1002/jmv.27405

Mannar, D., Saville, J. W., Zhu, X., Srivastava, S. S., Berezuk, A. M., Zhou, S., Tuttle, K. S., Kim, A., Li, W., Dimitrov, D. S., & Subramaniam, S. (2021). Structural analysis of receptor binding domain mutations in SARS-CoV-2 variants of concern that modulate ACE2 and antibody binding. *Cell Reports*, *37*(12), 110156. https://doi.org/10.1016/j.celrep.2021.110156

Mantis, N. J., Rol, N., & Corthésy, B. (2011). Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunology*, *4*(6), 603–611. https://doi.org/10.1038/mi.2011.41

Markovska, R., Boyanova, L., Yordanov, D., Stankova, P., Gergova, G., & Mitov, I. (2018). Status of *Helicobacter pylori* cag pathogenicity island (cagPAI) integrity and significance of its individual genes. *Infection, Genetics and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases*, *59*. https://doi.org/10.1016/j.meegid.2018.02.009

Marshall, B. J., Armstrong, J. A., McGechie, D. B., & Clancy, R. J. (1985). Attempt to fulfil Koch's postulates for pyloric *Campylobacter*. *Medical Journal of Australia*, *142*(8), 436–439. https://doi.org/10.5694/j.1326-5377.1985.tb113443.x

Marshall, B., & Warren, J. (1984). Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet (London, England)*, *1*(8390). https://doi.org/10.1016/s0140-6736(84)91816-6

Martinsen, T. C., Bergh, K., & Waldum, H. L. (2005). Gastric juice: A barrier against infectious diseases. *Basic & Clinical Pharmacology & Toxicology*, *96*(2), 94–102. https://doi.org/10.1111/j.1742-7843.2005.pto960202.x

Marzi, M., Vakil, M. K., Bahmanyar, M., & Zarenezhad, E. (2022). Paxlovid: Mechanism of Action, Synthesis, and In Silico Study. *BioMed Research International, 2022*, 7341493. https://doi.org/10.1155/2022/7341493

Matchett, W. E., Joag, V., Stolley, J. M., Shepherd, F. K., Quarnstrom, C. F., Mickelson, C. K., Wijeyesinghe, S., Soerens, A. G., Becker, S., Thiede, J. M., Weyu, E., O'Flanagan, S. D., Walter, J. A., Vu, M. N., Menachery, V. D., Bold, T. D., Vezys, V., Jenkins, M. K., Langlois, R. A., & Masopust, D. (2021). Cutting Edge: Nucleocapsid Vaccine Elicits Spike-Independent SARS-CoV-2 Protective Immunity. *The Journal of Immunology*, *207*(2), 376–379. https://doi.org/10.4049/jimmunol.2100421

Mateus, J., Grifoni, A., Tarke, A., Sidney, J., Ramirez, S. I., Dan, J. M., Burger, Z. C., Rawlings, S. A., Smith, D. M., Phillips, E., Mallal, S., Lammers, M., Rubiro, P., Quiambao, L., Sutherland, A., Yu, E. D., da Silva Antunes, R., Greenbaum, J., Frazier, A., … Weiskopf, D. (2020). Selective and cross-reactive SARS-CoV-2 T cell epitopes in unexposed humans. *Science (New York, N.Y.)*, *370*(6512), 89–94. https://doi.org/10.1126/science.abd3871

Mathieu, E., Ritchie, H., Rodés-Guirao, L., Appel, C., Giattino, C., Hasell, J., Macdonald, B., Dattani, S., Beltekian, D., Ortiz-Ospina, E., & Roser, M. (2020). Coronavirus Pandemic (COVID-19). *Our World in Data*. https://ourworldindata.org/covid-vaccinations

Matsumoto, S., Washizuka, Y., Matsumoto, Y., Tawara, S., Ikeda, F., Yokota, Y., & Karita, M. (1997). Induction of ulceration and severe gastritis in Mongolian gerbil by *Helicobacter pylori* infection. *Journal of Medical Microbiology*, *46*(5). https://doi.org/10.1099/00222615-46-5-391

Mauro, M., Radovic, V., Wolfe, M., Kamath, M., Bercik, P., & Armstrong, D. (2006). 13C urea breath test for *Helicobacter pylori*: Evaluation of 10-minute breath collection. *Canadian Journal of Gastroenterology*, *20*(12), 775–778.

McClain, M. S., Cao, P., Iwamoto, H., Vinion-Dubiel, A. D., Szabo, G., Shao, Z., & Cover, T. L. (2001). A 12-amino-acid segment, present in type s2 but not type s1 *Helicobacter pylori* VacA proteins, abolishes cytotoxin activity and alters

membrane channel formation. *Journal of Bacteriology*, *183*(22), 6499–6508. https://doi.org/10.1128/JB.183.22.6499-6508.2001

McDonald, I. R., Riley, P. W., Sharp, R. J., & McCarthy, A. J. (1995). Factors affecting the electroporation of *Bacillus subtilis*. *The Journal of Applied Bacteriology*, *79*(2), 213–218. https://doi.org/10.1111/j.1365-2672.1995.tb00937.x

McFadden, J., & Huffman, W. (2017). Consumer Demand for Low-Acrylamide-Forming Potato Products: Evidence from Lab Auctions. *American Journal of Potato Research*, *94*(5), 465–480. https://doi.org/10.1007/s12230-017-9577-1

McGee, D. J., Langford, M. L., Watson, E. L., Carter, J. E., Chen, Y.-T., & Ottemann, K. M. (2005). Colonization and Inflammation Deficiencies in Mongolian Gerbils Infected by *Helicobacter pylori* Chemotaxis Mutants. *Infection and Immunity*, *73*(3), 1820–1827. https://doi.org/10.1128/IAI.73.3.1820-1827.2005

McKenney, P. T., Driks, A., & Eichenberger, patrick. (2013). The *Bacillus subtilis* endospore: Assembly and functions of the multilayered coat. *Nature Reviews. Microbiology*, *11*(1). https://doi.org/10.1038/nrmicro2921

McKenney, P. T., Driks, A., Eskandarian, H. A., Grabowski, P., Guberman, J., Wang, K. H., Gitai, Z., & Eichenberger, P. (2010). A Distance-Weighted Interaction Map Reveals a Previously Uncharacterized Layer of the *B. subtilis* Spore Coat. *Current Biology : CB*, *20*(10), 934. https://doi.org/10.1016/j.cub.2010.03.060

McKinn, S., Trinh, D. H., Drabarek, D., Trieu, T. T., Nguyen, P. T. L., Cao, T. H., Dang, A. D., Nguyen, T. A., Fox, G. J., & Bernays, S. (2021). Drivers of antibiotic use in Vietnam: Implications for designing community interventions. *BMJ Global Health*, *6*(7), e005875. https://doi.org/10.1136/bmjgh-2021-005875

McMahon, B. J., Bruce, M. G., Koch, A., Goodman, K. J., Tsukanov, V., Mulvad, G., Borresen, M. L., Sacco, F., Barrett, D., Westby, S., & Parkinson, A. J. (2016). The diagnosis and treatment of *Helicobacter pylori* infection in Arctic regions with a high prevalence of infection: Expert Commentary. *Epidemiology and Infection*, *144*(2), 225–233. https://doi.org/10.1017/S0950268815001181

Mera, R., Fontham, E. T. H., Bravo, L. E., Bravo, J. C., Piazuelo, M. B., Camargo, M. C., & Correa, P. (2005). Long term follow up of patients treated for *Helicobacter pylori* infection. *Gut*, *54*(11), 1536–1540. https://doi.org/10.1136/gut.2005.072009

Meseda, C. A., Stauft, C. B., Selvaraj, P., Lien, C. Z., Pedro, C., Nuñez, I. A., Woerner, A. M., Wang, T. T., & Weir, J. P. (2021). MVA vector expression of SARS-CoV-2 spike protein and protection of adult Syrian hamsters against SARS-CoV-2 challenge. *NPJ Vaccines*, *6*, 145. https://doi.org/10.1038/s41541-021-00410-8

Middleton, R., & Hofmeister, A. (2004). New shuttle vectors for ectopic insertion of genes into *Bacillus subtilis*. *Plasmid*, *51*(3), 238–245. https://doi.org/10.1016/j.plasmid.2004.01.006

Mignon, C., Sodoyer, R., & Werle, B. (2015). Antibiotic-Free Selection in Biotherapeutics: Now and Forever. *Pathogens*, *4*(2), 157–181. https://doi.org/10.3390/pathogens4020157

Min, Y.-Q., Huang, M., Sun, X., Deng, F., Wang, H., & Ning, Y.-J. (2021). Immune evasion of SARS-CoV-2 from interferon antiviral system. *Computational and Structural Biotechnology Journal*, *19*, 4217–4225. https://doi.org/10.1016/j.csbj.2021.07.023

Misiewicz, J. J., Harris, A. W., Bardhan, K. D., Levi, S., O'Morain, C., Cooper, B. T., Kerr, G. D., Dixon, M. F., Langworthy, H., Piper, D., & Group, L. H. S. (1997). One week triple therapy for *Helicobacter pylori*: A multicentre comparative study. *Gut*, *41*(6), 735–739. https://doi.org/10.1136/gut.41.6.735

Mobley, H. L. T. (2001). Urease. In *Helicobacter pylori: Physiology and Genetics*. ASM Press. https://www.ncbi.nlm.nih.gov/books/NBK2417/

Mohammadi, M., Shayestehpour, M., & Mirzaei, H. (2021). The impact of spike mutated variants of SARS-CoV2 [Alpha, Beta, Gamma, Delta, and Lambda] on the efficacy of subunit recombinant vaccines. *The Brazilian Journal of Infectious Diseases*, *25*(4), 101606. https://doi.org/10.1016/j.bjid.2021.101606

Möhlendick, B., Schönfelder, K., Breuckmann, K., Elsner, C., Babel, N., Balfanz, P., Dahl, E., Dreher, M., Fistera, D., Herbstreit, F., Hölzer, B., Koch, M., Kohnle, M.,

Marx, N., Risse, J., Schmidt, K., Skrzypczyk, S., Sutharsan, S., Taube, C., … Kribben, A. (2021). ACE2 polymorphism and susceptibility for SARS-CoV-2 infection and severity of COVID-19. *Pharmacogenetics and Genomics*, 10.1097/FPC.0000000000000436. https://doi.org/10.1097/FPC.0000000000000436

Moir, A. (1981). Germination properties of a spore coat-defective mutant of *Bacillus subtilis*. *Journal of Bacteriology*, *146*(3), 1106–1116.

Moldoveanu, Z., Clements, M. L., Prince, S. J., Murphy, B. R., & Mestecky, J. (1995). Human immune responses to influenza virus vaccines administered by systemic or mucosal routes. *Vaccine*, *13*(11), 1006–1012. https://doi.org/10.1016/0264-410x(95)00016-t

Monroe, A., & Setlow, P. (2006). Localization of the Transglutaminase Cross-Linking Sites in the *Bacillus subtilis* Spore Coat Protein GerQ. *Journal of Bacteriology*, *188*(21), 7609–7616. https://doi.org/10.1128/JB.01116-06

Montecucco, C., & Rappuoli, R. (2001). Living dangerously: How *Helicobacter pylori* survives in the human stomach. *Nature Reviews Molecular Cell Biology*, *2*(6), Article 6. https://doi.org/10.1038/35073084

Morgan, E., Arnold, M., Camargo, M. C., Gini, A., Kunzmann, A. T., Matsuda, T., Meheus, F., Verhoeven, R. H. A., Vignat, J., Laversanne, M., Ferlay, J., & Soerjomataram, I. (2022). The current and future incidence and mortality of gastric cancer in 185 countries, 2020–40: A population-based modelling study. *EClinicalMedicine*, *47*. https://doi.org/10.1016/j.eclinm.2022.101404

Morris, A., & Nicholson, G. (1987). Ingestion of *Campylobacter pyloridis* causes gastritis and raised fasting gastric pH. *The American Journal of Gastroenterology*, *82*(3), 192–199.

Moss, P. (2022). The T cell immune response against SARS-CoV-2. *Nature Immunology*, *23*(2), 186–193. https://doi.org/10.1038/s41590-021-01122-w

Moss, S. F., Moise, L., Lee, D. S., Kim, W., Zhang, S., Lee, J., Rogers, A. B., Martin, W., & De Groot, A. S. (2011). HelicoVax: Epitope-based therapeutic *H. pylori*

vaccination in a mouse model. *Vaccine*, *29*(11), 2085–2091. https://doi.org/10.1016/j.vaccine.2010.12.130

Mouro, V., & Fischer, A. (2022). Dealing with a mucosal viral pandemic: Lessons from COVID-19 vaccines. *Mucosal Immunology*, *15*(4), 584–594. https://doi.org/10.1038/s41385-022-00517-8

Mukhtar, H. & Ikram-ul-Haq. (2012). Concomitant production of two proteases and alpha-amylase by a novel strain of *Bacillus subtilis* in a microprocessor controlled bioreactor. *Brazilian Journal of Microbiology*, *43*(3), 1072–1079. https://doi.org/10.1590/S1517-83822012000300033

Mullard, A. (2021). FDA approves 100th monoclonal antibody product. *Nature Reviews Drug Discovery*, *20*(7), 491–495. https://doi.org/10.1038/d41573-021-00079-7

Mulrooney, S., & Hausinger, R. (1990). Sequence of the *Klebsiella aerogenes* urease genes and evidence for accessory proteins facilitating nickel incorporation. *Journal of Bacteriology*, *172*(10). https://doi.org/10.1128/jb.172.10.5837-5843.1990

Munoz, L., Sadaie, Y., & Doi, R. H. (1978). Spore coat protein of *Bacillus subtilis*. Structure and precursor synthesis. *The Journal of Biological Chemistry*, *253*(19), 6694–6701.

Muñoz-Fontela, C., Dowling, W. E., Funnell, S. G. P., Gsell, P.-S., Riveros-Balta, A. X., Albrecht, R. A., Andersen, H., Baric, R. S., Carroll, M. W., Cavaleri, M., Qin, C., Crozier, I., Dallmeier, K., de Waal, L., de Wit, E., Delang, L., Dohm, E., Duprex, W. P., Falzarano, D., … Barouch, D. H. (2020). Animal models for COVID-19. *Nature*, *586*(7830), 509–515. https://doi.org/10.1038/s41586-020-2787-6

Muraguchi, A., Kehrl, J., Longo, D., Volkman, D., Smith, K., & Fauci, A. (1985). Interleukin 2 receptors on human B cells. Implications for the role of interleukin 2 in human B cell function. *The Journal of Experimental Medicine*, *161*, 181–197. https://doi.org/10.1084/jem.161.1.181

Murphy, K. (2020). SARS CoV-2 Detection From Upper and Lower Respiratory Tract Specimens: Diagnostic and Infection Control Implications. *Chest*, *158*(5), 1804–1805. https://doi.org/10.1016/j.chest.2020.07.061

Mykytyn, A. Z., Breugem, T. I., Riesebosch, S., Schipper, D., van den Doel, P. B., Rottier, R. J., Lamers, M. M., & Haagmans, B. L. (2021). SARS-CoV-2 entry into human airway organoids is serine protease-mediated and facilitated by the multibasic cleavage site. *ELife*, *10*, e64508. https://doi.org/10.7554/eLife.64508

Naaber, P., Tserel, L., Kangro, K., Sepp, E., Jürjenson, V., Adamson, A., Haljasmägi, L., Rumm, A. P., Maruste, R., Kärner, J., Gerhold, J. M., Planken, A., Ustav, M., Kisand, K., & Peterson, P. (2021). Dynamics of antibody response to BNT162b2 vaccine after six months: A longitudinal prospective study. *The Lancet Regional Health - Europe*, *10*, 100208. https://doi.org/10.1016/j.lanepe.2021.100208

Nagy, P., Johansson, S., & Molloy-Bland, M. (2016). Systematic review of time trends in the prevalence of Helicobacter pylori infection in China and the USA. *Gut Pathogens*, *8*(1), 8. https://doi.org/10.1186/s13099-016-0091-7

Naqvi, A. A. T., Fatima, K., Mohammad, T., Fatima, U., Singh, I. K., Singh, A., Atif, S. M., Hariprasad, G., Hasan, G. M., & Hassan, Md. I. (2020). Insights into SARS-CoV-2 genome, structure, evolution, pathogenesis and therapies: Structural genomics approach. *Biochimica et Biophysica Acta. Molecular Basis of Disease*, *1866*(10), 165878. https://doi.org/10.1016/j.bbadis.2020.165878

Negri, A., Potocki, W., Iwanicki, A., Obuchowski, M., & Hinc, K. (2013). Expression and display of Clostridium difficile protein FliD on the surface of *Bacillus subtilis* spores. *Journal of Medical Microbiology*, *62*(Pt 9), 1379–1385. https://doi.org/10.1099/jmm.0.057372-0

Neuhard, J., Price, A. R., Schack, L., & Thomassen, E. (1978). Two thymidylate synthetases in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences*, *75*(3), 1194–1198. https://doi.org/10.1073/pnas.75.3.1194

Ng, K. T., Mohd-Ismail, N. K., & Tan, Y.-J. (2021). Spike S2 Subunit: The Dark Horse in the Race for Prophylactic and Therapeutic Interventions against SARS-CoV-2. *Vaccines*, *9*(2), Article 2. https://doi.org/10.3390/vaccines9020178

Nguyen, A. T. V., Pham, C. K., Pham, H. T. T., Pham, H. L., Nguyen, A. H., Dang, L. T., Huynh, H. A., Cutting, S. M., & Phan, T.-N. (2014). *Bacillus subtilis* spores expressing the VP28 antigen: A potential oral treatment to protect Litopenaeus

vannamei against white spot syndrome. *FEMS Microbiology Letters*, *358*(2), 202–208. https://doi.org/10.1111/1574-6968.12546

Nguyen, T. L., Uchida, T., Tsukamoto, Y., Trinh, D. T., Ta, L., Mai, B. H., Le, S. H., Thai, K. D., Ho, D. D., Hoang, H. H., Matsuhisa, T., Okimoto, T., Kodama, M., Murakami, K., Fujioka, T., Yamaoka, Y., & Moriyama, M. (2010). *Helicobacter pylori* infection and gastroduodenal diseases in Vietnam: A cross-sectional, hospital-based study. *BMC Gastroenterology*, *10*, 114. https://doi.org/10.1186/1471-230X-10-114

Nguyen, V. B., Nguyen, G. K., Phung, D. C., Okrainec, K., Raymond, J., Dupond, C., Kremp, O., Kalach, N., & Vidal-Trecan, G. (2006). Intra-familial Transmission of *Helicobacter pylori* Infection in Children of Households with Multiple Generations in Vietnam. *European Journal of Epidemiology*, *21*(6), 459–463. https://doi.org/10.1007/s10654-006-9016-y

Ni, L., Ye, F., Cheng, M.-L., Feng, Y., Deng, Y.-Q., Zhao, H., Wei, P., Ge, J., Gou, M., Li, X., Sun, L., Cao, T., Wang, P., Zhou, C., Zhang, R., Liang, P., Guo, H., Wang, X., Qin, C.-F., … Dong, C. (2020). Detection of SARS-CoV-2-Specific Humoral and Cellular Immunity in COVID-19 Convalescent Individuals. *Immunity*, *52*(6), 971-977.e3. https://doi.org/10.1016/j.immuni.2020.04.023

Nicholson, W. L. (2002). Roles of *Bacillus* endospores in the environment. *Cellular and Molecular Life Sciences: CMLS*, *59*(3), 410–416. https://doi.org/10.1007/s00018-002-8433-7

Nicholson, W. L., Fajardo-Cavazos, P., Rebeil, R., Slieman, T. A., Riesenman, P. J., Law, J. F., & Xue, Y. (2002). Bacterial endospores and their significance in stress resistance. *Antonie van Leeuwenhoek*, *81*(1), 27–32. https://doi.org/10.1023/A:1020561122764

Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J., & Setlow, P. (2000). Resistance of *Bacillus* Endospores to Extreme Terrestrial and Extraterrestrial Environments. *Microbiology and Molecular Biology Reviews*, *64*(3), 548–572.

Nim, Y. S., & Wong, K.-B. (2019). The Maturation Pathway of Nickel Urease. *Inorganics*, *7*(7), Article 7. https://doi.org/10.3390/inorganics7070085

Ning, D., Leng, X., Li, Q., & Xu, W. (2011). Surface-displayed VP28 on *Bacillus subtilis* spores induce protection against white spot syndrome virus in crayfish by oral administration. *Journal of Applied Microbiology*, *111*(6), 1327–1336. https://doi.org/10.1111/j.1365-2672.2011.05156.x

Nishiura, H., Kobayashi, T., Miyama, T., Suzuki, A., Jung, S., Hayashi, K., Kinoshita, R., Yang, Y., Yuan, B., Akhmetzhanov, A. R., & Linton, N. M. (2020). Estimation of the asymptomatic ratio of novel coronavirus infections (COVID-19). *International Journal of Infectious Diseases*, *94*, 154–155. https://doi.org/10.1016/j.ijid.2020.03.020

Noach, L. A., Bosma, N. B., Jansen, J., Hoek, F. J., van Deventer, S. J., & Tytgat, G. N. (1994). Mucosal tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 production in patients with *Helicobacter pylori* infection. *Scandinavian Journal of Gastroenterology*, *29*(5), 425–429. https://doi.org/10.3109/00365529409096833

Noto, J. M., Romero-Gallo, J., Piazuelo, M. B., & Peek, R. M. (2016). The Mongolian Gerbil: A Robust Model of *Helicobacter pylori*-Induced Gastric Inflammation and Cancer. *Methods in Molecular Biology (Clifton, N.J.)*, *1422*, 263–280. https://doi.org/10.1007/978-1-4939-3603-8_24

Odenbreit, S., Odenbreit, J., Sedlmaier, B., Gerland, E., Fischer, W., & Haas, R. (2000). Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science (New York, N.Y.)*, *287*(5457). https://doi.org/10.1126/science.287.5457.1497

O'Donovan, G. A., & Neuhard, J. (1970). Pyrimidine metabolism in microorganisms. *Bacteriological Reviews*, *34*(3), 278–343. https://doi.org/10.1128/br.34.3.278-343.1970

Ogando, N. S., Dalebout, T. J., Zevenhoven-Dobbe, J. C., Limpens, R. W. A. L., van der Meer, Y., Caly, L., Druce, J., de Vries, J. J. C., Kikkert, M., Bárcena, M., Sidorov, I., & Snijder, E. J. (2020). SARS-coronavirus-2 replication in Vero E6 cells: Replication kinetics, rapid adaptation and cytopathology. *The Journal of General Virology*, *101*(9), 925–940. https://doi.org/10.1099/jgv.0.001453

Ogata, S. K., Godoy, A. P. O., da Silva Patricio, F. R., & Kawakami, E. (2013). High *Helicobacter pylori* resistance to metronidazole and clarithromycin in Brazilian children and adolescents. *Journal of Pediatric Gastroenterology and Nutrition*, *56*(6), 645–648. https://doi.org/10.1097/MPG.0b013e31828b3669

Oggioni, M. R., Ciabattini, A., Cuppone, A. M., & Pozzi, G. (2003). *Bacillus* spores for vaccine delivery. *Vaccine*, *21*, S96–S101. https://doi.org/10.1016/S0264-410X(03)00207-X

Ogura, K., Maeda, S., Nakao, M., Watanabe, T., Tada, M., Kyutoku, T., Yoshida, H., Shiratori, Y., & Omata, M. (2000). Virulence Factors of *Helicobacter pylori* Responsible for Gastric Diseases in Mongolian Gerbil. *The Journal of Experimental Medicine*, *192*(11), 1601–1610.

Oh, H., & Ghosh, S. (2013). NF-κB: Roles and regulation in different CD4(+) T-cell subsets. *Immunological Reviews*, *252*(1). https://doi.org/10.1111/imr.12033

Oh, Y., Kim, J. A., Kim, C.-H., Choi, S.-K., & Pan, J.-G. (2020). *Bacillus subtilis* spore vaccines displaying protective antigen induce functional antibodies and protective potency. *BMC Veterinary Research*, *16*, 259. https://doi.org/10.1186/s12917-020-02468-3

Ohkusa, T., Okayasu, I., Miwa, H., Ohtaka, K., Endo, S., & Sato, N. (2003). *Helicobacter pylori* infection induces duodenitis and superficial duodenal ulcer in Mongolian gerbils. *Gut*, *52*(6). https://doi.org/10.1136/gut.52.6.797

Ohnishi, N., Yuasa, H., Tanaka, S., Sawa, H., Miura, M., Matsui, A., Higashi, H., Musashi, M., Iwabuchi, K., Suzuki, M., Yamada, G., Azuma, T., & Hatakeyama, M. (2008). Transgenic expression of *Helicobacter pylori* CagA induces gastrointestinal and hematopoietic neoplasms in mouse. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(3), 1003–1008. https://doi.org/10.1073/pnas.0711183105

Ohse, M., Takahashi, K., Kadowaki, Y., & Kusaoke, H. (1995). Effects of plasmid DNA sizes and several other factors on transformation of *Bacillus subtilis* ISW1214 with plasmid DNA by electroporation. *Bioscience, Biotechnology, and Biochemistry*, *59*(8), 1433–1437. https://doi.org/10.1271/bbb.59.1433

Okada, T., Yanagisawa, K., & Ryan, F. J. (1960). Elective Production of Thymine-less Mutants. *Nature*, *188*(4747), Article 4747. https://doi.org/10.1038/188340a0

Okuya, K., Yoshida, R., Manzoor, R., Saito, S., Suzuki, T., Sasaki, M., Saito, T., Kida, Y., Mori-Kajihara, A., Kondoh, T., Sato, M., Kajihara, M., Miyamoto, H., Ichii, O., Higashi, H., & Takada, A. (2020). Potential Role of Nonneutralizing IgA Antibodies in Cross-Protective Immunity against Influenza A Viruses of Multiple Hemagglutinin Subtypes. *Journal of Virology*, *94*(12), e00408-20. https://doi.org/10.1128/JVI.00408-20

Olbermann, P., Josenhans, C., Moodley, Y., Uhr, M., Stamer, C., Vauterin, M., Suerbaum, S., Achtman, M., & Linz, B. (2010). A Global Overview of the Genetic and Functional Diversity in the *Helicobacter pylori* cag Pathogenicity Island. *PLOS Genetics*, *6*(8), e1001069. https://doi.org/10.1371/journal.pgen.1001069

Oliveira, S. C., de Magalhães, M. T. Q., & Homan, E. J. (2020). Immunoinformatic Analysis of SARS-CoV-2 Nucleocapsid Protein and Identification of COVID-19 Vaccine Targets. *Frontiers in Immunology*, *11*. https://www.frontiersin.org/articles/10.3389/fimmu.2020.587615

Ong, E., Wong, M. U., Huffman, A., & He, Y. (2020). COVID-19 Coronavirus Vaccine Design Using Reverse Vaccinology and Machine Learning. *Frontiers in Immunology*, *11*. https://www.frontiersin.org/articles/10.3389/fimmu.2020.01581

Oran, D. P., & Topol, E. J. (2021). The Proportion of SARS-CoV-2 Infections That Are Asymptomatic: A Systematic Review. *Annals of Internal Medicine*, *174*(5), 655–662. https://doi.org/10.7326/M20-6976

Ozaydin, N., Turkyilmaz, S. A., & Cali, S. (2013). Prevalence and risk factors of *helicobacter pylori* in Turkey: A nationally-representative, cross-sectional, screening with the 13C-Urea breath test. *BMC Public Health*, *13*(1), 1215. https://doi.org/10.1186/1471-2458-13-1215

Pajon, R., Doria-Rose, N. A., Shen, X., Schmidt, S. D., O'Dell, S., McDanal, C., Feng, W., Tong, J., Eaton, A., Maglinao, M., Tang, H., Manning, K. E., Edara, V.-V., Lai, L., Ellis, M., Moore, K. M., Floyd, K., Foster, S. L., Posavad, C. M., … Montefiori, D. C. (2022). SARS-CoV-2 Omicron Variant Neutralization after mRNA-1273

Booster Vaccination. *The New England Journal of Medicine*, NEJMc2119912. https://doi.org/10.1056/NEJMc2119912

Palframan, S. L., Kwok, T., & Gabriel, K. (2012). Vacuolating cytotoxin A (VacA), a key toxin for *Helicobacter pylori* pathogenesis. *Frontiers in Cellular and Infection Microbiology*, *2*, 92. https://doi.org/10.3389/fcimb.2012.00092

Pang, W., Lu, Y., Zhao, Y.-B., Shen, F., Fan, C.-F., Wang, Q., He, W.-Q., He, X.-Y., Li, Z.-K., Chen, T.-T., Yang, C.-X., Li, Y.-Z., Xiao, S.-X., Zhao, Z.-J., Huang, X.-S., Luo, R.-H., Yang, L.-M., Zhang, M., Dong, X.-Q., … Zheng, Y.-T. (2022). A variant-proof SARS-CoV-2 vaccine targeting HR1 domain in S2 subunit of spike protein. *Cell Research*, *32*(12), Article 12. https://doi.org/10.1038/s41422-022-00746-3

Papastergiou, V., Georgopoulos, S., & Georgopoulos, S. (2014). Treatment of *Helicobacter pylori* infection: Meeting the challenge of antimicrobial resistance. *World Journal of Gastroenterology*, *20*(29). https://doi.org/10.3748/wjg.v20.i29.9898

Papini, E., de Bernard, M., Milia, E., Bugnoli, M., Zerial, M., Rappuoli, R., & Montecucco, C. (1994). Cellular vacuoles induced by *Helicobacter pylori* originate from late endosomal compartments. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(21), 9720–9724.

Park, I. S., Carr, M. B., & Hausinger, R. P. (1994). *In vitro* activation of urease apoprotein and role of UreD as a chaperone required for nickel metallocenter assembly. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(8), 3233. https://doi.org/10.1073/pnas.91.8.3233

Park, J. S., Jun, J. S., Seo, J.-H., Youn, H.-S., & Rhee, K.-H. (2020). Changing prevalence of *Helicobacter pylori* infection in children and adolescents. *Clinical and Experimental Pediatrics*, *64*(1), 21–25. https://doi.org/10.3345/cep.2019.01543

Park, S., Xu, Y., Stowell, X. F., Gai, F., Saven, J. G., & Boder, E. T. (2006). Limitations of yeast surface display in engineering proteins of high thermostability. *Protein Engineering, Design and Selection*, *19*(5), 211–217. https://doi.org/10.1093/protein/gzl003

Parsonnet, J. (2003). What is the *Helicobacter pylori* Global Reinfection Rate? *Canadian Journal of Gastroenterology and Hepatology*, *17*, 46B-48B. https://doi.org/10.1155/2003/567816

Parsonnet, J., Vandersteen, D., Goates, J., Sibley, R., Pritikin, J., & Chang, Y. (1991). *Helicobacter pylori* infection in intestinal- and diffuse-type gastric adenocarcinomas. *Journal of the National Cancer Institute*, *83*(9). https://doi.org/10.1093/jnci/83.9.640

Paules, C. I., Marston, H. D., & Fauci, A. S. (2020). Coronavirus Infections-More Than Just the Common Cold. *JAMA*, *323*(8), 707–708. https://doi.org/10.1001/jama.2020.0757

Peek Jr, R. M., & Crabtree, J. E. (2006). *Helicobacter* infection and gastric neoplasia. *The Journal of Pathology*, *208*(2), 233–248. https://doi.org/10.1002/path.1868

Peek, R. M., & Blaser, M. J. (2002). *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. *Nature Reviews Cancer*, *2*(1), Article 1. https://doi.org/10.1038/nrc703

Peiris, J. S. M., Lai, S. T., Poon, L. L. M., Guan, Y., Yam, L. Y. C., Lim, W., Nicholls, J., Yee, W. K. S., Yan, W. W., Cheung, M. T., Cheng, V. C. C., Chan, K. H., Tsang, D. N. C., Yung, R. W. H., Ng, T. K., & Yuen, K. Y. (2003). Coronavirus as a possible cause of severe acute respiratory syndrome. *The Lancet*, *361*(9366), 1319–1325. https://doi.org/10.1016/S0140-6736(03)13077-2

Pérez-Pérez, G. I., Sack, R. B., Reid, R., Santosham, M., Croll, J., & Blaser, M. J. (2003). Transient and Persistent *Helicobacter pylori* Colonization in Native American Children. *Journal of Clinical Microbiology*, *41*(6), 2401–2407. https://doi.org/10.1128/JCM.41.6.2401-2407.2003

Permpoonpattana, P., Hong, H. A., Phetcharaburanin, J., Huang, J.-M., Cook, J., Fairweather, N. F., & Cutting, S. M. (2011). Immunization with *Bacillus* Spores Expressing Toxin A Peptide Repeats Protects against Infection with *Clostridium difficile* Strains Producing Toxins A and B ▿. *Infection and Immunity*, *79*(6), 2295–2302. https://doi.org/10.1128/IAI.00130-11

Permpoonpattana, P., Phetcharaburanin, J., Mikelsone, A., Dembek, M., Tan, S., Brisson, M.-C., La Ragione, R., Brisson, A. R., Fairweather, N., Hong, H. A., & Cutting, S. M. (2013). Functional Characterization of *Clostridium difficile* Spore Coat Proteins. *Journal of Bacteriology*, *195*(7), 1492–1503. https://doi.org/10.1128/JB.02104-12

Petrillo, C., Castaldi, S., Lanzilli, M., Saggese, A., Donadio, G., Baccigalupi, L., Ricca, E., & Isticato, R. (2020). The temperature of growth and sporulation modulates the efficiency of spore-display in *Bacillus subtilis*. *Microbial Cell Factories*, *19*(1), 185. https://doi.org/10.1186/s12934-020-01446-6

Pfaender, S., Mar, K. B., Michailidis, E., Kratzel, A., Boys, I. N., V'kovski, P., Fan, W., Kelly, J. N., Hirt, D., Ebert, N., Stalder, H., Kleine-Weber, H., Hoffmann, M., Hoffmann, H.-H., Saeed, M., Dijkman, R., Steinmann, E., Wight-Carter, M., McDougal, M. B., … Thiel, V. (2020). LY6E impairs coronavirus fusion and confers immune control of viral disease. *Nature Microbiology*, *5*(11), 1330–1339. https://doi.org/10.1038/s41564-020-0769-y

Planas, D., Bruel, T., Grzelak, L., Guivel-Benhassine, F., Staropoli, I., Porrot, F., Planchais, C., Buchrieser, J., Rajah, M. M., Bishop, E., Albert, M., Donati, F., Prot, M., Behillil, S., Enouf, V., Maquart, M., Smati-Lafarge, M., Varon, E., Schortgen, F., … Schwartz, O. (2021). Sensitivity of infectious SARS-CoV-2 B.1.1.7 and B.1.351 variants to neutralizing antibodies. *Nature Medicine*, *27*(5), Article 5. https://doi.org/10.1038/s41591-021-01318-5

Polack, F. P., Thomas, S. J., Kitchin, N., Absalon, J., Gurtman, A., Lockhart, S., Perez, J. L., Pérez Marc, G., Moreira, E. D., Zerbini, C., Bailey, R., Swanson, K. A., Roychoudhury, S., Koury, K., Li, P., Kalina, W. V., Cooper, D., Frenck, R. W., Hammitt, L. L., … C4591001 Clinical Trial Group. (2020). Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *The New England Journal of Medicine*, *383*(27), 2603–2615. https://doi.org/10.1056/NEJMoa2034577

Potot, S., Serra, C. R., Henriques, A. O., & Schyns, G. (2010). Display of Recombinant Proteins on *Bacillus subtilis* Spores, Using a Coat-Associated Enzyme as the Carrier. *Applied and Environmental Microbiology*, *76*(17), 5926–5933. https://doi.org/10.1128/AEM.01103-10

Potrykus, I. (2001). The ‘Golden rice’ tale. *In Vitro Cellular & Developmental Biology - Plant*, *37*(2), 93–100. https://doi.org/10.1007/s11627-001-0019-9

Qiu, E., Li, Z., & Han, S. (2021). Methods for detection of *Helicobacter pylori* from stool sample: Current options and developments. *Brazilian Journal of Microbiology*, *52*(4), 2057–2062. https://doi.org/10.1007/s42770-021-00589-x

Rabilloud, T. (1996). Solubilization of proteins for electrophoretic analyses. *Electrophoresis*, *17*(5), 813–829. https://doi.org/10.1002/elps.1150170503

Raghavan, S., Svennerholm, A.-M., & Holmgren, J. (2002). Effects of oral vaccination and immunomodulation by cholera toxin on experimental *Helicobacter pylori* infection, reinfection, and gastritis. *Infection and Immunity*, *70*(8), 4621–4627. https://doi.org/10.1128/IAI.70.8.4621-4627.2002

Raleigh, V. (2022). *Deaths from Covid-19 (coronavirus):* The King’s Fund. https://www.kingsfund.org.uk/publications/deaths-covid-19

Ramsay, A. J., & Kohonen-Corish, M. (1993). Interleukin-5 expressed by a recombinant virus vector enhances specific mucosal IgA responses *in vivo*. *European Journal of Immunology*, *23*(12), 3141–3145. https://doi.org/10.1002/eji.1830231215

Rawla, P., & Barsouk, A. (2019). Epidemiology of gastric cancer: Global trends, risk factors and prevention. *Przegląd Gastroenterologiczny*, *14*(1), 26–38. https://doi.org/10.5114/pg.2018.80001

Reljic, R., Sibley, L., Huang, J.-M., Pepponi, I., Hoppe, A., Hong, H. A., & Cutting, S. M. (2013). Mucosal Vaccination against Tuberculosis Using Inert Bioparticles. *Infection and Immunity*, *81*(11), 4071–4080. https://doi.org/10.1128/IAI.00786-13

Rengarajan, J., Sassetti, C. M., Naroditskaya, V., Sloutsky, A., Bloom, B. R., & Rubin, E. J. (2004). The folate pathway is a target for resistance to the drug para-aminosalicylic acid (PAS) in mycobacteria. *Molecular Microbiology*, *53*(1), 275–282. https://doi.org/10.1111/j.1365-2958.2004.04120.x

Rhee, K.-J., Sethupathi, P., Driks, A., Lanning, D. K., & Knight, K. L. (2004). Role of commensal bacteria in development of gut-associated lymphoid tissues and

preimmune antibody repertoire. *Journal of Immunology (Baltimore, Md.: 1950)*, *172*(2), 1118–1124. https://doi.org/10.4049/jimmunol.172.2.1118

Ricca, E., & Cutting, S. M. (2003). Emerging Applications of Bacterial Spores in Nanobiotechnology. *Journal of Nanobiotechnology*, *1*(1). https://doi.org/10.1186/1477-3155-1-6

Riddell, S., Goldie, S., Hill, A., Eagles, D., & Drew, T. W. (2020). The effect of temperature on persistence of SARS-CoV-2 on common surfaces. *Virology Journal*, *17*(1), 145. https://doi.org/10.1186/s12985-020-01418-7

Riley, E. P., Lopez-Garrido, J., Sugie, J., Liu, R. B., & Pogliano, K. (2021). Metabolic differentiation and intercellular nurturing underpin bacterial endospore formation. *Science Advances*, *7*(4), eabd6385. https://doi.org/10.1126/sciadv.abd6385

Rizos, K., Lattemann, C. T., Bumann, D., Meyer, T. F., & Aebischer, T. (2003). Autodisplay. *Infection and Immunity*, *71*(11), 6320–6328. https://doi.org/10.1128/iai.71.11.6320-6328.2003

Rizwan, M., Alvi, A., & Ahmed, N. (2008). Novel Protein Antigen (JHP940) from the Genomic Plasticity Region of Helicobacter pylori Induces Tumor Necrosis Factor Alpha and Interleukin-8 Secretion by Human Macrophages. *Journal of Bacteriology*, *190*(3), 1146–1151. https://doi.org/10.1128/JB.01309-07

Robson, F., Khan, K. S., Le, T. K., Paris, C., Demirbag, S., Barfuss, P., Rocchi, P., & Ng, W.-L. (2020). Coronavirus RNA Proofreading: Molecular Basis and Therapeutic Targeting. *Molecular Cell*, *79*(5), 710–727. https://doi.org/10.1016/j.molcel.2020.07.027

Rodes, L., Coussa-Charley, M., Marinescu, D., Paul, A., Fakhoury, M., Abbasi, S., Khan, A., Tomaro-Duchesneau, C., & Prakash, S. (2013). Design of a novel gut bacterial adhesion model for probiotic applications. *Artificial Cells, Nanomedicine, and Biotechnology*, *41*(2), 116–124. https://doi.org/10.3109/10731199.2012.712047

Rolfe, R. (1967). On the mechanism of thymineless death in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences*, *57*(1), 114–121. https://doi.org/10.1073/pnas.57.1.114

Romano, M., Ruggiero, A., Squeglia, F., Maga, G., & Berisio, R. (2020). A Structural View of SARS-CoV-2 RNA Replication Machinery: RNA Synthesis, Proofreading and Final Capping. *Cells*, *9*(5), 1267. https://doi.org/10.3390/cells9051267

Rossi, A., Michelini, Z., Leone, P., Borghi, M., Blasi, M., Bona, R., Spada, M., Grasso, F., Gugliotta, A., Klotman, M. E., Cara, A., & Negri, D. (2014). Optimization of Mucosal Responses after Intramuscular Immunization with Integrase Defective Lentiviral Vector. *PLoS ONE*, *9*(9), e107377. https://doi.org/10.1371/journal.pone.0107377

Rothenbacher, D., Inceoglu, J., Bode, G., & Brenner, H. (2000). Acquisition of *Helicobacter pylori* infection in a high-risk population occurs within the first 2 years of life. *The Journal of Pediatrics*, *136*(6), 744–748.

Rowland, M., Daly, L., Vaughan, M., Higgins, A., Bourke, B., & Drumm, B. (2006). Age-specific incidence of *Helicobacter pylori*. *Gastroenterology*, *130*(1), 65–72; quiz 211. https://doi.org/10.1053/j.gastro.2005.11.004

Ruiz, B., Correa, P., Fontham, E. T., & Ramakrishnan, T. (1996). Antral atrophy, *Helicobacter pylori* colonization, and gastric pH. *American Journal of Clinical Pathology*, *105*(1), 96–101. https://doi.org/10.1093/ajcp/105.1.96

Rupnow, M. F., Shachter, R. D., Owens, D. K., & Parsonnet, J. (2001). Quantifying the population impact of a prophylactic *Helicobacter pylori* vaccine. *Vaccine*, *20*(5–6), 879–885. https://doi.org/10.1016/s0264-410x(01)00401-7

S, X., M, L., C, W., W, X., Q, L., S, F., F, Q., L, B., L, D., S, L., C, Q., F, S., Z, S., Y, Z., S, J., & L, L. (2020). Inhibition of SARS-CoV-2 (previously 2019-nCoV) infection by a highly potent pan-coronavirus fusion inhibitor targeting its spike protein that harbors a high capacity to mediate membrane fusion. *Cell Research*, *30*(4). https://doi.org/10.1038/s41422-020-0305-x

Sabbagh, P., Mohammadnia-Afrouzi, M., Javanian, M., Babazadeh, A., Koppolu, V., Vasigala, V. R., Nouri, H. R., & Ebrahimpour, S. (2019). Diagnostic methods for *Helicobacter pylori* infection: Ideals, options, and limitations. *European Journal of Clinical Microbiology & Infectious Diseases*, *38*(1), 55–66. https://doi.org/10.1007/s10096-018-3414-4

Sacco, M., Ricca, E., Losick, R., & Cutting, S. (1995). An additional GerE-controlled gene encoding an abundant spore coat protein from *Bacillus subtilis*. *Journal of Bacteriology*, *177*(2), 372–377.

Saleem, N., & Howden, C. W. (2020). Update on the Management of *Helicobacter pylori* Infection. *Current Treatment Options in Gastroenterology*, *18*(3), 476–487. https://doi.org/10.1007/s11938-020-00300-3

Sambrook, J. R., & Russell, D. W. (2001). *Molecular cloning: A laboratory manual.* (3rd ed., Vol. 1). Cold Spring Harbor Laboratory Press.

Sankarapandian, V., Venmathi Maran, B. A., Rajendran, R. L., Jogalekar, M. P., Gurunagarajan, S., Krishnamoorthy, R., Gangadaran, P., & Ahn, B.-C. (2022). An Update on the Effectiveness of Probiotics in the Prevention and Treatment of Cancer. *Life*, *12*(1), 59. https://doi.org/10.3390/life12010059

Sano, K., Bhavsar, D., Singh, G., Floda, D., Srivastava, K., Gleason, C., PARIS Study Group, Carreño, J. M., Simon, V., & Krammer, F. (2022). SARS-CoV-2 vaccination induces mucosal antibody responses in previously infected individuals. *Nature Communications*, *13*(1), 5135. https://doi.org/10.1038/s41467-022-32389-8

Sasaki, Y., Ito, Y., & Sasaki, T. (2004). ThyA as a Selection Marker in Construction of Food-Grade Host-Vector and Integration Systems for Streptococcus thermophilus. *Applied and Environmental Microbiology*, *70*(3), 1858. https://doi.org/10.1128/AEM.70.3.1858-1864.2004

Savarino, V., Vigneri, S., & Celle, G. (1999). The 13C urea breath test in the diagnosis of *Helicobacter pylori* infection. *Gut*, *45*(suppl 1), I18–I22. https://doi.org/10.1136/gut.45.2008.i18

Savoldi, A., Carrara, E., Graham, D. Y., Conti, M., & Tacconelli, E. (2018). Prevalence of Antibiotic Resistance in *Helicobacter pylori*: A Systematic Review and Meta-analysis in World Health Organization Regions. *Gastroenterology*, *155*(5), 1372-1382.e17. https://doi.org/10.1053/j.gastro.2018.07.007

Schmitt, W., & Haas, R. (1994). Genetic analysis of the *Helicobacter pylori* vacuolating cytotoxin: Structural similarities with the IgA protease type of exported protein. *Molecular Microbiology*, *12*(2), 307–319. https://doi.org/10.1111/j.1365-2958.1994.tb01019.x

Schubert, M. L. (2011). Gastric secretion. *Current Opinion in Gastroenterology*, *27*(6), 536. https://doi.org/10.1097/MOG.0b013e32834bd53f

Schweinitzer, T., Mizote, T., Ishikawa, N., Dudnik, A., Inatsu, S., Schreiber, S., Suerbaum, S., Aizawa, S., & Josenhans, C. (2008). Functional characterization and mutagenesis of the proposed behavioral sensor TlpD of *Helicobacter pylori*. *Journal of Bacteriology*, *190*(9), 3244–3255. https://doi.org/10.1128/JB.01940-07

Sciochetti, S. A., Piggot, P. J., & Blakely, G. W. (2001). Identification and Characterization of the dif Site from *Bacillus subtilis*. *Journal of Bacteriology*, *183*(3), 1058–1068. https://doi.org/10.1128/JB.183.3.1058-1068.2001

Scott Algood, H. M. (2020). T cell cytokines impact epithelial cell responses during *Helicobacter pylori* infection. *Journal of Immunology (Baltimore, Md.: 1950)*, *204*(6), 1421–1428. https://doi.org/10.4049/jimmunol.1901307

Sengupta, A., Azharuddin, M., Cardona, M. E., Devito, C., von Castelmur, E., Wehlin, A., Pietras, Z., Sunnerhagen, M., Selegård, R., Aili, D., Alamer, A., Hinkula, J., & Al-Otaibi, N. (2022). Intranasal Coronavirus SARS-CoV-2 Immunization with Lipid Adjuvants Provides Systemic and Mucosal Immune Response against SARS-CoV-2 S1 Spike and Nucleocapsid Protein. *Vaccines*, *10*(4), 504. https://doi.org/10.3390/vaccines10040504

Seo, J.-H., Youn, J. H., Kim, E. A., Jun, J.-S., Park, J.-S., Yeom, J. S., Lim, J.-Y., Woo, H.-O., Youn, H.-S., Ko, G.-H., Park, J.-S., Baik, S.-C., Lee, W.-K., Cho, M.-J., & Rhee, K.-H. (2017). *Helicobacter pylori* Antigens Inducing Early Immune Response in Infants. *Journal of Korean Medical Science*, *32*(7), 1139–1146. https://doi.org/10.3346/jkms.2017.32.7.1139

Serpa Neto, A., Cardoso, S. O., Manetta, J. A., Pereira, V. G. M., Espósito, D. C., Pasqualucci, M. de O. P., Damasceno, M. C. T., & Schultz, M. J. (2012). Association between use of lung-protective ventilation with lower tidal volumes

and clinical outcomes among patients without acute respiratory distress syndrome: A meta-analysis. *JAMA*, *308*(16), 1651–1659. https://doi.org/10.1001/jama.2012.13730

Sethuraman, N., Jeremiah, S. S., & Ryo, A. (2020). Interpreting Diagnostic Tests for SARS-CoV-2. *JAMA*, *323*(22), 2249–2251. https://doi.org/10.1001/jama.2020.8259

Setlow, B., & Setlow, P. (1987). Thymine-containing dimers as well as spore photoproducts are found in ultraviolet-irradiated *Bacillus subtilis* spores that lack small acid-soluble proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *84*(2), 421–423.

Setlow, B., & Setlow, P. (1993). Binding of small, acid-soluble spore proteins to DNA plays a significant role in the resistance of *Bacillus subtilis* spores to hydrogen peroxide. *Applied and Environmental Microbiology*, *59*(10), 3418–3423.

Setlow, P. (2006). Spores of Bacillus subtilis: Their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology*, *101*(3). https://doi.org/10.1111/j.1365-2672.2005.02736.x

Setlow, P., & Johnson, E. A. (2019). Spores and Their Significance. In *Food Microbiology* (pp. 23–63). John Wiley & Sons, Ltd. https://doi.org/10.1128/9781555819972.ch2

Sette, A., & Crotty, S. (2021). Adaptive immunity to SARS-CoV-2 and COVID-19. *Cell*, *184*(4), 861–880. https://doi.org/10.1016/j.cell.2021.01.007

Shaffer, B. T., & Lighthart, B. (1997). Survey of Culturable Airborne Bacteria at Four Diverse Locations in Oregon: Urban, Rural, Forest, and Coastal. *Microbial Ecology*, *34*(3), 167–177. https://doi.org/10.1007/s002489900046

Shahryari, A., Saghaeian Jazi, M., Mohammadi, S., Razavi Nikoo, H., Nazari, Z., Hosseini, E. S., Burtscher, I., Mowla, S. J., & Lickert, H. (2019). Development and Clinical Translation of Approved Gene Therapy Products for Genetic Disorders. *Frontiers in Genetics*, *10*. https://www.frontiersin.org/articles/10.3389/fgene.2019.00868

Shen, C., Yin, X.-C., Jiao, B.-Y., Li, J., Jia, P., Zhang, X.-W., Cheng, X.-H., Ren, J.-X., Lan, H.-D., Hou, W.-B., Fang, M., Li, X., Fei, Y.-T., Robinson, N., & Liu, J.-P. (2022). Evaluation of adverse effects/events of genetically modified food consumption: A systematic review of animal and human studies. *Environmental Sciences Europe*, *34*(1), 8. https://doi.org/10.1186/s12302-021-00578-9

Shimotsu, H., & Henner, D. J. (1986). Modulation of Bacillus subtilis levansucrase gene expression by sucrose and regulation of the steady-state mRNA level by sacU and sacQ genes. *Journal of Bacteriology*, *168*(1), 380–388. https://doi.org/10.1128/jb.168.1.380-388.1986

Shin, M.-K., Jun, J.-S., Kwon, S.-W., Lee, D.-H., Ha, J.-H., Park, J.-S., Song, D. H., Jung, M., Kang, H.-L., Baik, S.-C., Park, J., Youn, H.-S., Cho, M., Seo, J.-H., & Lee, W.-K. (2018). Characterization of Specific IgA Response to Antigenic Determinants of Helicobacter pylori Urease Encoded by ureA and ureB in Children. *Journal of Bacteriology and Virology*, *48*, 14. https://doi.org/10.4167/jbv.2018.48.1.14

Shrestha, A. B., Pokharel, P., Sapkota, U. H., Shrestha, S., Mohamed, S. A., Khanal, S., Jha, S. K., Mohanty, A., Padhi, B. K., Asija, A., Sedhai, Y. R., Rijal, R., Singh, K., Chattu, V. K., Rodriguez-Morales, A. J., Barboza, J. J., & Sah, R. (2023). Drug Resistance Patterns of Commonly Used Antibiotics for the Treatment of Helicobacter pylori Infection among South Asian Countries: A Systematic Review and Meta-Analysis. *Tropical Medicine and Infectious Disease*, *8*(3), Article 3. https://doi.org/10.3390/tropicalmed8030172

Shukla, A. A., Wolfe, L. S., Mostafa, S. S., & Norman, C. (2017). Evolving trends in mAb production processes. *Bioengineering & Translational Medicine*, *2*(1), 58–69. https://doi.org/10.1002/btm2.10061

Shuman, S., & Glickman, M. S. (2007). Bacterial DNA repair by non-homologous end joining. *Nature Reviews. Microbiology*, *5*(11), 852–861. https://doi.org/10.1038/nrmicro1768

Sia, S. F., Yan, L.-M., Chin, A. W. H., Fung, K., Choy, K.-T., Wong, A. Y. L., Kaewpreedee, P., Perera, R. A. P. M., Poon, L. L. M., Nicholls, J. M., Peiris, M., & Yen, H.-L. (2020). Pathogenesis and transmission of SARS-CoV-2 in golden

hamsters. *Nature*, *583*(7818), 834–838. https://doi.org/10.1038/s41586-020-2342-5

Sibley, L., Reljic, R., Radford, D. S., Huang, J.-M., Hong, H. A., Cranenburgh, R. M., & Cutting, S. M. (2014). Recombinant *Bacillus subtilis* spores expressing MPT64 evaluated as a vaccine against tuberculosis in the murine model. *FEMS Microbiology Letters*, *358*(2), 170–179. https://doi.org/10.1111/1574-6968.12525

Silva, M. S. da, Demoliner, M., Hansen, A. W., Gularte, J. S., Silveira, F., Heldt, F. H., Filippi, M., Pereira, V. M. de A. G., Silva, F. P. da, Mallmann, L., Fink, P., Silva, L. L. da, Weber, M. N., Almeida, P. R. de, Fleck, J. D., & Spilki, F. R. (2021). Early detection of SARS-CoV-2 P.1 variant in Southern Brazil and reinfection of the same patient by P.2. *Revista Do Instituto De Medicina Tropical De Sao Paulo*, *63*, e58. https://doi.org/10.1590/S1678-9946202163058

Sipponen, P., Kekki, M., Haapakoski, J., Ihamäki, T., & Siurala, M. (1985). Gastric cancer risk in chronic atrophic gastritis: Statistical calculations of cross-sectional data. *International Journal of Cancer*, *35*(2). https://doi.org/10.1002/ijc.2910350206

Smeekens, J. M., Johnson-Weaver, B. T., Hinton, A. L., Azcarate-Peril, M. A., Moran, T. P., Immormino, R. M., Kesselring, J. R., Steinbach, E. C., Orgel, K. A., Staats, H. F., Burks, A. W., Mucha, P. J., Ferris, M. T., & Kulis, M. D. (2020). Fecal IgA, Antigen Absorption, and Gut Microbiome Composition Are Associated With Food Antigen Sensitization in Genetically Susceptible Mice. *Frontiers in Immunology*, *11*, 599637. https://doi.org/10.3389/fimmu.2020.599637

Smith, G. P. (1985). Filamentous fusion phage: Novel expression vectors that display cloned antigens on the virion surface. *Science (New York, N.Y.)*, *228*(4705). https://doi.org/10.1126/science.4001944

Smith, S., Fowora, M., & Pellicano, R. (2019). Infections with Helicobacter pylori and challenges encountered in Africa. *World Journal of Gastroenterology*, *25*(25), 3183. https://doi.org/10.3748/wjg.v25.i25.3183

Smythies, L. E., Novak, M. J., Waites, K. B., Lindsey, J. R., Morrow, C. D., & Smith, P. D. (2005). Poliovirus replicons encoding the B subunit of *Helicobacter pylori*

urease protect mice against *H. pylori* infection. *Vaccine*, *23*(7), 901–909. https://doi.org/10.1016/j.vaccine.2004.07.037

Sobala, G. M., Crabtree, J. E., Dixon, M. F., Schorah, C. J., Taylor, J. D., Rathbone, B. J., Heatley, R. V., & Axon, A. T. (1991). Acute *Helicobacter pylori* infection: Clinical features, local and systemic immune response, gastric mucosal histology, and gastric juice ascorbic acid concentrations. *Gut*, *32*(11), 1415–1418.

Sommer, A., Hussaini, G., Tarwotjo, I., Susanto, D., & Soegiharto, T. (1981). Incidence, prevalence, and scale of blinding malnutrition. *The Lancet*, *317*(8235), 1407–1408.

Song, M., Hong, H. A., Huang, J.-M., Colenutt, C., Khang, D. D., Nguyen, T. V. A., Park, S.-M., Shim, B.-S., Song, H. H., Cheon, I. S., Jang, J. E., Choi, J., Choi, Y. K., Stadler, K., & Cutting, S. M. (2012). Killed *Bacillus subtilis* spores as a mucosal adjuvant for an H5N1 vaccine. *Vaccine*, *30*(22), 3266–3277. https://doi.org/10.1016/j.vaccine.2012.03.016

Sorci, G., Faivre, B., & Morand, S. (2020). Explaining among-country variation in COVID-19 case fatality rate. *Scientific Reports*, *10*(1), Article 1. https://doi.org/10.1038/s41598-020-75848-2

Sorokulova, I. B., Pinchuk, I. V., Denayrolles, M., Osipova, I. G., Huang, J. M., Cutting, S. M., & Urdaci, M. C. (2008). The safety of two *Bacillus* probiotic strains for human use. *Digestive Diseases and Sciences*, *53*(4), 954–963. https://doi.org/10.1007/s10620-007-9959-1

Spadiut, O., Capone, S., Krainer, F., Glieder, A., & Herwig, C. (2014). Microbials for the production of monoclonal antibodies and antibody fragments. *Trends in Biotechnology*, *32*(1), 54–60. https://doi.org/10.1016/j.tibtech.2013.10.002

Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B., Cox, E., Remon, J. P., & Remaut, E. (2003). Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. *Nature Biotechnology*, *21*(7), 785–789. https://doi.org/10.1038/nbt840

Sterlin, D., Mathian, A., Miyara, M., Mohr, A., Anna, F., Claër, L., Quentric, P., Fadlallah, J., Devilliers, H., Ghillani, P., Gunn, C., Hockett, R., Mudumba, S.,

Guihot, A., Luyt, C.-E., Mayaux, J., Beurton, A., Fourati, S., Bruel, T., … Gorochov, G. (2021). IgA dominates the early neutralizing antibody response to SARS-CoV-2. *Science Translational Medicine*, *13*(577), eabd2223. https://doi.org/10.1126/scitranslmed.abd2223

Stirling, F., & Silver, P. A. (2020). Controlling the Implementation of Transgenic Microbes: Are We Ready for What Synthetic Biology Has to Offer? *Molecular Cell*, *78*(4), 614–623. https://doi.org/10.1016/j.molcel.2020.03.034

Stowe, J., Andrews, N., Kirsebom, F., Ramsay, M., & Bernal, J. L. (2022). Effectiveness of COVID-19 vaccines against Omicron and Delta hospitalisation, a test negative case-control study. *Nature Communications*, *13*(1), Article 1. https://doi.org/10.1038/s41467-022-33378-7

Stragier, P., & Losick, R. (1996). Molecular genetics of sporulation in *Bacillus subtilis*. *Annual Review of Genetics*, *30*, 297–241. https://doi.org/10.1146/annurev.genet.30.1.297

Sugimoto, M., Furuta, T., & Yamaoka, Y. (2009). Influence of inflammatory cytokine polymorphisms on eradication rates of *Helicobacter pylori*. *Journal of Gastroenterology and Hepatology*, *24*(11), 1725–1732. https://doi.org/10.1111/j.1440-1746.2009.06047.x

Sugimoto, M., Watada, M., Jung, S. W., Graham, D. Y., & Yamaoka, Y. (2012). Role of *Helicobacter pylori* Plasticity Region Genes in Development of Gastroduodenal Diseases. *Journal of Clinical Microbiology*, *50*(2), 441. https://doi.org/10.1128/JCM.00906-11

Sugimoto, M., Zali, M. R., & Yamaoka, Y. (2009). The association of vacA genotypes and Helicobacter pylori-related gastroduodenal diseases in the Middle East. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology*, *28*(10), 1227–1236. https://doi.org/10.1007/s10096-009-0772-y

Sugimura, N., Li, Q., Chu, E. S. H., Lau, H. C. H., Fong, W., Liu, W., Liang, C., Nakatsu, G., Su, A. C. Y., Coker, O. O., Wu, W. K. K., Chan, F. K. L., & Yu, J. (2022). *Lactobacillus gallinarum* modulates the gut microbiota and produces anti-cancer

metabolites to protect against colorectal tumourigenesis. *Gut*, *71*(10), 2011–2021. https://doi.org/10.1136/gutjnl-2020-323951

Sui, L., Zhao, Y., Wang, W., Wu, P., Wang, Z., Yu, Y., Hou, Z., Tan, G., & Liu, Q. (2021). SARS-CoV-2 Membrane Protein Inhibits Type I Interferon Production Through Ubiquitin-Mediated Degradation of TBK1. *Frontiers in Immunology*, *12*, 662989. https://doi.org/10.3389/fimmu.2021.662989

Sun, Q., Sun, L., Liu, H.-H., Chen, X., Seth, R. B., Forman, J., & Chen, Z. J. (2006). The Specific and Essential Role of MAVS in Antiviral Innate Immune Responses. *Immunity*, *24*(5), 633–642. https://doi.org/10.1016/j.immuni.2006.04.004

Sung, J. C.-C., Liu, Y., Wu, K.-C., Choi, M.-C., Ma, C. H.-Y., Lin, J., He, E. I. C., Leung, D. Y.-M., Sze, E. T.-P., Hamied, Y. K., Lam, D. M.-K., & Kwong, K. W.-Y. (2022). Retraction: Sung et al. Expression of SARS-CoV-2 Spike Protein Receptor Binding Domain on Recombinant *B. subtilis* on Spore Surface: A Potential COVID-19 Oral Vaccine Candidate. Vaccines 2022, 10, 2. *Vaccines*, *10*(11), Article 11. https://doi.org/10.3390/vaccines10111852

Sungnak, W., Huang, N., Bécavin, C., Berg, M., Queen, R., Litvinukova, M., Talavera-López, C., Maatz, H., Reichart, D., Sampaziotis, F., Worlock, K. B., Yoshida, M., & Barnes, J. L. (2020). SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes. *Nature Medicine*, *26*(5), Article 5. https://doi.org/10.1038/s41591-020-0868-6

Supasa, P., Zhou, D., Dejnirattisai, W., Liu, C., Mentzer, A. J., Ginn, H. M., Zhao, Y., Duyvesteyn, H. M. E., Nutalai, R., Tuekprakhon, A., Wang, B., Paesen, G. C., Slon-Campos, J., López-Camacho, C., Hallis, B., Coombes, N., Bewley, K. R., Charlton, S., Walter, T. S., … Screaton, G. R. (2021). Reduced neutralization of SARS-CoV-2 B.1.1.7 variant by convalescent and vaccine sera. *Cell*, *184*(8), 2201-2211.e7. https://doi.org/10.1016/j.cell.2021.02.033

Suzuki, H., Masaoka, T., Nomura, S., Hoshino, Y., Kurabayashi, K., Minegishi, Y., Suzuki, M., & Ishii, H. (2003). Current consensus on the diagnosis and treatment of *H. pylori*-associated gastroduodenal disease. *The Keio Journal of Medicine*, *52*(3), 163–173. https://doi.org/10.2302/kjm.52.163

Svennerholm, A.-M., & Lundgren, A. (2007). Progress in vaccine development against Helicobacter pylori. *FEMS Immunology and Medical Microbiology*. https://doi.org/10.1111/j.1574-695X.2007.00237.x

Sýkora, J., Valecková, K., Amlerová, J., Siala, K., Dedek, P., Watkins, S., Varvarovská, J., Stožický, F., Pazdiora, P., & Schwarz, J. (2005). Effects of a Specially Designed Fermented Milk Product Containing Probiotic Lactobacillus casei DN-114 001 and the Eradication of *H. pylori* in Children: A Prospective Randomized Double-Blind Study. *Journal of Clinical Gastroenterology*, *39*(8), 692. https://doi.org/10.1097/01.mcg.0000173855.77191.44

Szostková, M., & Horáková, D. (1998). The effect of plasmid DNA sizes and other factors on electrotransformation of *Escherichia coli* JM109. *Bioelectrochemistry and Bioenergetics*, *47*(2), 319–323. https://doi.org/10.1016/S0302-4598(98)00203-7

Tabynov, K., Turebekov, N., Babayeva, M., Fomin, G., Yerubayev, T., Yespolov, T., Li, L., Renukaradhya, G. J., Petrovsky, N., & Tabynov, K. (2022). An adjuvanted subunit SARS-CoV-2 spike protein vaccine provides protection against Covid-19 infection and transmission. *NPJ Vaccines*, *7*(1), 24. https://doi.org/10.1038/s41541-022-00450-8

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D. L., Pulcini, C., Kahlmeter, G., Kluytmans, J., Carmeli, Y., Ouellette, M., Outterson, K., Patel, J., Cavaleri, M., Cox, E. M., Houchens, C. R., Grayson, M. L., Hansen, P., Singh, N., … Zorzet, A. (2018). Discovery, research, and development of new antibiotics: The WHO priority list of antibiotic-resistant bacteria and tuberculosis. *The Lancet Infectious Diseases*, *18*(3), 318–327. https://doi.org/10.1016/S1473-3099(17)30753-3

Tafakori, V., Ahmadian, G., & Amoozegar, M. A. (2012). Surface display of bacterial metallothioneins and a chitin binding domain on *Escherichia coli* increase cadmium adsorption and cell immobilization. *Applied Biochemistry and Biotechnology*, *167*(3), 462–473. https://doi.org/10.1007/s12010-012-9684-x

Takahashi-Kanemitsu, A., Knight, C. T., & Hatakeyama, M. (2020). Molecular anatomy and pathogenic actions of *Helicobacter pylori* CagA that underpin gastric

carcinogenesis. *Cellular and Molecular Immunology*, *17*(1), 50–63. https://doi.org/10.1038/s41423-019-0339-5

Tan, C. S., Collier, A. Y., Yu, J., Liu, J., Chandrashekar, A., McMahan, K., Jacob-Dolan, C., He, X., Roy, V., Hauser, B. M., Munt, J. E., Mallory, M. L., Mattocks, M., Powers, J. M., Meganck, R. M., Rowe, M., Hemond, R., Bondzie, E. A., Jaegle, K. H., … Barouch, D. H. (2022). Durability of Heterologous and Homologous COVID-19 Vaccine Boosts. *JAMA Network Open*, *5*(8), e2226335. https://doi.org/10.1001/jamanetworkopen.2022.26335

Tan, I. S., & Ramamurthi, K. S. (2014). Spore formation in *Bacillus subtilis*. *Environmental Microbiology Reports*, *6*(3), 212–225. https://doi.org/10.1111/1758-2229.12130

Tang, P., Hasan, M. R., Chemaitelly, H., Yassine, H. M., Benslimane, F. M., Al Khatib, H. A., AlMukdad, S., Coyle, P., Ayoub, H. H., Al Kanaani, Z., Al Kuwari, E., Jeremijenko, A., Kaleeckal, A. H., Latif, A. N., Shaik, R. M., Abdul Rahim, H. F., Nasrallah, G. K., Al Kuwari, M. G., Al Romaihi, H. E., … Abu-Raddad, L. J. (2021). BNT162b2 and mRNA-1273 COVID-19 vaccine effectiveness against the SARS-CoV-2 Delta variant in Qatar. *Nature Medicine*. https://doi.org/10.1038/s41591-021-01583-4

Tanne, J. H. (2022). Covid-19: CDC and FDA approve Pfizer and Moderna vaccines for under 5s. *BMJ*, *377*, o1507. https://doi.org/10.1136/bmj.o1507

Taus, E., Hofmann, C., Ibarrondo, F. J., Hausner, M. A., Fulcher, J. A., Krogstad, P., Ferbas, K. G., Tobin, N. H., Rimoin, A. W., Aldrovandi, G. M., & Yang, O. O. (2022). Dominant CD8+ T Cell Nucleocapsid Targeting in SARS-CoV-2 Infection and Broad Spike Targeting From Vaccination. *Frontiers in Immunology*, *13*. https://www.frontiersin.org/articles/10.3389/fimmu.2022.835830

Taylor, D. E., Ge, Z., Purych, D., Lo, T., & Hiratsuka, K. (1997). Cloning and sequence analysis of two copies of a 23S rRNA gene from *Helicobacter pylori* and association of clarithromycin resistance with 23S rRNA mutations. *Antimicrobial Agents and Chemotherapy*, *41*(12), 2621–2628. https://doi.org/10.1128/AAC.41.12.2621

Tegally, H., Wilkinson, E., Giovanetti, M., Iranzadeh, A., Fonseca, V., Giandhari, J., Doolabh, D., Pillay, S., San, E. J., Msomi, N., Mlisana, K., von Gottberg, A., Walaza, S., Allam, M., Ismail, A., Mohale, T., Glass, A. J., Engelbrecht, S., Van Zyl, G., … de Oliveira, T. (2021). Detection of a SARS-CoV-2 variant of concern in South Africa. *Nature*, *592*(7854), 438–443. https://doi.org/10.1038/s41586-021-03402-9

Telford, J. L., Ghiara, P., Dell'Orco, M., Comanducci, M., Burroni, D., Bugnoli, M., Tecce, M. F., Censini, S., Covacci, A., & Xiang, Z. (1994). Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. *The Journal of Experimental Medicine*, *179*(5), 1653–1658. https://doi.org/10.1084/jem.179.5.1653

Tenny, S., & Hoffman, M. R. (2022). Prevalence. In *StatPearls [Internet]*. StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK430867/

Terry, K., Williams, S. M., Connolly, L., & Ottemann, K. M. (2005). Chemotaxis Plays Multiple Roles during *Helicobacter pylori* Animal Infection. *Infection and Immunity*, *73*(2), 803. https://doi.org/10.1128/IAI.73.2.803-811.2005

The RECOVERY Collaborative Group. (2021). Dexamethasone in Hospitalized Patients with Covid-19. *New England Journal of Medicine*, *384*(8), 693–704. https://doi.org/10.1056/NEJMoa2021436

Thomas, J. E., Dale, A., Harding, M., Coward, W. A., Cole, T. J., & Weaver, L. T. (1999). Helicobacter pylori Colonization in Early Life. *Pediatric Research*, *45*(2), Article 2. https://doi.org/10.1203/00006450-199902000-00010

Thompson, M. R., Kaminski, J. J., Kurt-Jones, E. A., & Fitzgerald, K. A. (2011). Pattern Recognition Receptors and the Innate Immune Response to Viral Infection. *Viruses*, *3*(6), 920–940. https://doi.org/10.3390/v3060920

Thorne, L. G., Bouhaddou, M., Reuschl, A.-K., Zuliani-Alvarez, L., Polacco, B., Pelin, A., Batra, J., Whelan, M. V. X., Hosmillo, M., Fossati, A., Ragazzini, R., Jungreis, I., Ummadi, M., Rojc, A., Turner, J., Bischof, M. L., Obernier, K., Braberg, H., Soucheray, M., … Krogan, N. J. (2022). Evolution of enhanced innate immune

evasion by SARS-CoV-2. *Nature*, *602*(7897), Article 7897. https://doi.org/10.1038/s41586-021-04352-y

Tiboni, M., Casettari, L., & Illum, L. (2021). Nasal vaccination against SARS-CoV-2: Synergistic or alternative to intramuscular vaccines? *International Journal of Pharmaceutics*, *603*, 120686. https://doi.org/10.1016/j.ijpharm.2021.120686

Tjong, E., Dimri, M., & Mohiuddin, S. S. (2022). Biochemistry, Tetrahydrofolate. In *StatPearls [Internet]*. StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK539712/

Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E. F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., … Venter, J. C. (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature*, *388*(6642), 539–547. https://doi.org/10.1038/41483

Torok, A. M., Bouton, A. H., & Goldberg, J. B. (2005). Helicobacter pylori Induces Interleukin-8 Secretion by Toll-Like Receptor 2- and Toll-Like Receptor 5-Dependent and -Independent Pathways. *Infection and Immunity*, *73*(3), 1523–1531. https://doi.org/10.1128/IAI.73.3.1523-1531.2005

Tran, T. T., Mamo, G., Mattiasson, B., & Hatti-Kaul, R. (2010). A thermostable phytase from Bacillus sp. MD2: Cloning, expression and high-level production in *Escherichia coli*. *Journal of Industrial Microbiology & Biotechnology*, *37*(3), 279–287. https://doi.org/10.1007/s10295-009-0671-3

Tregoning, J. S., Flight, K. E., Higham, S. L., Wang, Z., & Pierce, B. F. (2021). Progress of the COVID-19 vaccine effort: Viruses, vaccines and variants versus efficacy, effectiveness and escape. *Nature Reviews. Immunology*, *21*(10), 626–636. https://doi.org/10.1038/s41577-021-00592-1

Tseng, H. F., Ackerson, B. K., Luo, Y., Sy, L. S., Talarico, C. A., Tian, Y., Bruxvoort, K. J., Tubert, J. E., Florea, A., Ku, J. H., Lee, G. S., Choi, S. K., Takhar, H. S., Aragones, M., & Qian, L. (2022). Effectiveness of mRNA-1273 against SARS-

CoV-2 Omicron and Delta variants. *Nature Medicine, 28*(5), Article 5. https://doi.org/10.1038/s41591-022-01753-y

Tzotzos, S. J., Fischer, B., Fischer, H., & Zeitlinger, M. (2020). Incidence of ARDS and outcomes in hospitalized patients with COVID-19: A global literature survey. *Critical Care, 24*, 516. https://doi.org/10.1186/s13054-020-03240-7

Uemura, N., Okamoto, S., Yamamoto, S., Matsumura, N., Yamaguchi, S., Yamakido, M., Taniyama, K., Sasaki, N., & Schlemper, R. J. (2001). Helicobacter pylori infection and the development of gastric cancer. *The New England Journal of Medicine, 345*(11), 784–789. https://doi.org/10.1056/NEJMoa001999

UK Health Security Agency. (2023, March 9). *COVID-19: The green book, chapter 14a*. GOV.UK. https://www.gov.uk/government/publications/covid-19-the-green-book-chapter-14a

Ulanowski, Z., & Ludlow, I. K. (1993). The influence of the cortex on protoplast dehydration in bacterial spores studied with light scattering. *Current Microbiology, 26*(1), 31–35. https://doi.org/10.1007/BF01577239

Vaira, D., Holton, J., Menegatti, M., Ricci, C., Gatta, L., Geminiani, A., & Miglioli, M. (2000). Invasive and non-invasive tests for *Helicobacter pylori* infection. *Alimentary Pharmacology & Therapeutics, 14*(s3), 13–22. https://doi.org/10.1046/j.1365-2036.2000.00096.x

Valdez, A., Yepiz-Plascencia, G., Ricca, E., & Olmos, J. (2014). First Litopenaeus vannamei WSSV 100% oral vaccination protection using CotC::Vp26 fusion protein displayed on *Bacillus subtilis* spores surface. *Journal of Applied Microbiology, 117*(2), 347–357. https://doi.org/10.1111/jam.12550

van Doremalen, N., Bushmaker, T., Morris, D. H., Holbrook, M. G., Gamble, A., Williamson, B. N., Tamin, A., Harcourt, J. L., Thornburg, N. J., Gerber, S. I., Lloyd-Smith, J. O., de Wit, E., & Munster, V. J. (2020). Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1. *The New England Journal of Medicine, 382*(16), 1564–1567. https://doi.org/10.1056/NEJMc2004973

Vanaparthy, R., Mohan, G., Vasireddy, D., & Atluri, P. (2021). Review of COVID-19 viral vector-based vaccines and COVID-19 variants. *Le Infezioni in Medicina*, *29*(3), 328–338. https://doi.org/10.53854/liim-2903-3

Vandermeulen, G., Marie, C., Scherman, D., & Préat, V. (2011). New Generation of Plasmid Backbones Devoid of Antibiotic Resistance Marker for Gene Therapy Trials. *Molecular Therapy*, *19*(11), 1942–1949. https://doi.org/10.1038/mt.2011.182

Vaughan, A. (2021). Omicron emerges. *New Scientist (1971)*, *252*(3363), 7. https://doi.org/10.1016/S0262-4079(21)02140-0

Vázquez-Cruz, C., Ochoa-Sánchez, J. C., & Olmedo-Alvarez, G. (1996). Pulse-Field gel-electrophoretic analysis of the amplification and copy-number stability of an integrational plasmid in *Bacillus subtilis*. *Applied Microbiology and Biotechnology*, *46*(1), 55–60. https://doi.org/10.1007/s002530050782

Vela Ramirez, J. E., Sharpe, L. A., & Peppas, N. A. (2017). Current state and challenges in developing oral vaccines. *Advanced Drug Delivery Reviews*, *114*, 116–131. https://doi.org/10.1016/j.addr.2017.04.008

Vermeire. (2015, May 4). *Opinion on Synthetic Biology II - Risk assessment methodologies and safety aspects* [Text]. sciensano.be; European Union. https://www.sciensano.be/en/biblio/opinion-synthetic-biology-ii-risk-assessment-methodologies-and-safety-aspects

Vilaichone, R. K., Mahachai, V., Shiota, S., Uchida, T., Ratanachu-ek, T., Tshering, L., Tung, N. L., Fujioka, T., Moriyama, M., & Yamaoka, Y. (2013). Extremely high prevalence of *Helicobacter pylori* infection in Bhutan. *World Journal of Gastroenterology : WJG*, *19*(18), 2806–2810. https://doi.org/10.3748/wjg.v19.i18.2806

Vilaichone, R. K., Quach, D. T., Yamaoka, Y., Sugano, K., & Mahachai, V. (2018). Prevalence and Pattern of Antibiotic Resistant Strains of *Helicobacter Pylori* Infection in ASEAN. *Asian Pacific Journal of Cancer Prevention : APJCP*, *19*(5), 1411–1413. https://doi.org/10.22034/APJCP.2018.19.5.1411

Voysey, M., Clemens, S. A. C., Madhi, S. A., Weckx, L. Y., Folegatti, P. M., Aley, P. K., Angus, B., Baillie, V. L., Barnabas, S. L., Bhorat, Q. E., Bibi, S., Briner, C., Cicconi, P., Collins, A. M., Colin-Jones, R., Cutland, C. L., Darton, T. C., Dheda, K., Duncan, C. J. A., … Oxford COVID Vaccine Trial Group. (2021). Safety and efficacy of the ChAdOx1 nCoV-19 vaccine (AZD1222) against SARS-CoV-2: An interim analysis of four randomised controlled trials in Brazil, South Africa, and the UK. *Lancet (London, England)*, *397*(10269), 99–111. https://doi.org/10.1016/S0140-6736(20)32661-1

Wan, E. Y. F., Mok, A. H. Y., Yan, V. K. C., Chan, C. I. Y., Wang, B., Lai, F. T. T., Chui, C. S. L., Li, X., Wong, C. K. H., Lau, C. S., Wong, I. C. K., & Chan, E. W. Y. (2022). Effectiveness of BNT162b2 and CoronaVac vaccinations against SARS-CoV-2 omicron infection in people aged 60 years or above: A case–control study. *Journal of Travel Medicine*, *29*(8), taac119. https://doi.org/10.1093/jtm/taac119

Wan, Y., Shang, J., Graham, R., Baric, R. S., & Li, F. (2020). Receptor Recognition by the Novel Coronavirus from Wuhan: An Analysis Based on Decade-Long Structural Studies of SARS Coronavirus. *Journal of Virology*, *94*(7), e00127-20. https://doi.org/10.1128/JVI.00127-20

Wang, H., Liu, Q., Hu, J., Zhou, M., Yu, M.-Q., Li, K.-Y., Xu, D., Xiao, Y., Yang, J.-Y., Lu, Y.-J., Wang, F., Yin, P., & Xu, S.-Y. (2020). Nasopharyngeal Swabs Are More Sensitive Than Oropharyngeal Swabs for COVID-19 Diagnosis and Monitoring the SARS-CoV-2 Load. *Frontiers in Medicine*, *7*, 334. https://doi.org/10.3389/fmed.2020.00334

Wang, J., Wang, Y., Zhang, E., Zhou, M., Lin, J., & Yang, Q. (2019). Intranasal administration with recombinant Bacillus subtilis induces strong mucosal immune responses against pseudorabies. *Microbial Cell Factories*, *18*(1), 103. https://doi.org/10.1186/s12934-019-1151-8

Wang, M., Cao, R., Zhang, L., Yang, X., Liu, J., Xu, M., Shi, Z., Hu, Z., Zhong, W., & Xiao, G. (2020). Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) *in vitro*. *Cell Research*, *30*(3), 269–271. https://doi.org/10.1038/s41422-020-0282-0

Wang, P., Casner, R. G., Nair, M. S., Wang, M., Yu, J., Cerutti, G., Liu, L., Kwong, P. D., Huang, Y., Shapiro, L., & Ho, D. D. (2021). Increased resistance of SARS-CoV-2 variant P.1 to antibody neutralization. *Cell Host & Microbe*, *29*(5), 747-751.e4. https://doi.org/10.1016/j.chom.2021.04.007

Wang, W., Xu, Y., Gao, R., Lu, R., Han, K., Wu, G., & Tan, W. (2020). Detection of SARS-CoV-2 in Different Types of Clinical Specimens. *JAMA*, *323*(18), 1843–1844. https://doi.org/10.1001/jama.2020.3786

Wang, Y., Rubtsov, A., Heiser, R., White, J., Crawford, F., Marrack, P., & Kappler, J. W. (2005). Using a baculovirus display library to identify MHC class I mimotopes. *Proceedings of the National Academy of Sciences*, *102*(7), 2476–2481. https://doi.org/10.1073/pnas.0409798102

Wang, Z., Lorenzi, J. C. C., Muecksch, F., Finkin, S., Viant, C., Gaebler, C., Cipolla, M., Hoffmann, H.-H., Oliveira, T. Y., Oren, D. A., Ramos, V., Nogueira, L., Michailidis, E., Robbiani, D. F., Gazumyan, A., Rice, C. M., Hatziioannou, T., Bieniasz, P. D., Caskey, M., & Nussenzweig, M. C. (2021). Enhanced SARS-CoV-2 neutralization by dimeric IgA. *Science Translational Medicine*, *13*(577), eabf1555. https://doi.org/10.1126/scitranslmed.abf1555

Watanabe, T., Tada, M., Nagai, H., Sasaki, S., & Nakao, M. (1998). *Helicobacter pylori* infection induces gastric cancer in mongolian gerbils. *Gastroenterology*, *115*(3). https://doi.org/10.1016/s0016-5085(98)70143-x

Watzlawick, H., & Altenbuchner, J. (2019). Multiple integration of the gene ganA into the *Bacillus subtilis* chromosome for enhanced β-galactosidase production using the CRISPR/Cas9 system. *AMB Express*, *9*(1), 158. https://doi.org/10.1186/s13568-019-0884-4

Weeks, D. L., Eskandari, S., Scott, D. R., & Sachs, G. (2000). A H+-gated urea channel: The link between *Helicobacter pylori* urease and gastric colonization. *Science (New York, N.Y.)*, *287*(5452), 482–485. https://doi.org/10.1126/science.287.5452.482

Weinreich, D. M., Sivapalasingam, S., Norton, T., Ali, S., Gao, H., Bhore, R., Musser, B. J., Soo, Y., Rofail, D., Im, J., Perry, C., Pan, C., Hosain, R., Mahmood, A., Davis, J. D., Turner, K. C., Hooper, A. T., Hamilton, J. D., Baum, A., … Trial

Investigators. (2021). REGN-COV2, a Neutralizing Antibody Cocktail, in Outpatients with Covid-19. *The New England Journal of Medicine*, *384*(3), 238–251. https://doi.org/10.1056/NEJMoa2035002

Westbrook, A. W., Moo-Young, M., & Chou, C. P. (2016). Development of a CRISPR-Cas9 Tool Kit for Comprehensive Engineering of. *Applied and Environmental Microbiology*, *82*(16), 4876–4895. https://doi.org/10.1128/AEM.01159-16

WHO. (2017). *WHO publishes list of bacteria for which new antibiotics are urgently needed*. https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

WHO. (2021a). *Interim recommendations for the use of the Janssen Ad26.COV2.S (COVID-19) vaccine: Interim guidance, first issued 17 March 2021, updated 15 June 2021, updated 9 December 2021* (WHO/2019-nCoV/vaccines/SAGE_recommendation/Ad26.COV2.S/2021.3). World Health Organization. https://apps.who.int/iris/handle/10665/350212

WHO. (2021b). *Interim recommendations for use of the ChAdOx1-S [recombinant] vaccine against COVID-19 (AstraZeneca COVID-19 vaccine AZD1222 Vaxzevria™, SII COVISHIELD™)*. https://www.who.int/publications-detail-redirect/WHO-2019-nCoV-vaccines-SAGE_recommendation-AZD1222-2021.1

WHO. (2021c). *Interim recommendations for use of the inactivated COVID-19 vaccine BIBP developed by China National Biotec Group (CNBG), Sinopharm*. https://www.who.int/publications-detail-redirect/WHO-2019-nCoV-vaccines-SAGE-recommendation-BIBP

WHO. (2021d). *Interim recommendations for use of the Moderna mRNA-1273 vaccine against COVID-19: Interim guidance, 25 January 2021* (WHO/2019-nCoV/vaccines/SAGE_recommendation/mRNA-1273/2021.1). World Health Organization. https://apps.who.int/iris/handle/10665/338862

WHO. (2021e). *Interim recommendations for use of the Pfizer–BioNTech COVID-19 vaccine, BNT162b2, under emergency use listing: Interim guidance, 8 January 2021* (WHO/2019-nCoV/vaccines/SAGE_recommendation/BNT162b2/2021.1). World Health Organization. https://apps.who.int/iris/handle/10665/338484

WHO. (2021f). *The Sinovac-CoronaVac COVID-19 vaccine: What you need to know*. https://www.who.int/news-room/feature-stories/detail/the-sinovac-covid-19-vaccine-what-you-need-to-know

WHO. (2021g, February 15). *COVISHIELD*. WHO - Prequalification of Medical Products (IVDs, Medicines, Vaccines and Immunization Devices, Vector Control). https://extranet.who.int/pqweb/vaccines/covid-19-vaccine-chadox1-s-recombinant-covishield

WHO. (2021h, November 3). *COVID-19 Vaccines with WHO Emergency Use Listing*. WHO - Prequalification of Medical Products (IVDs, Medicines, Vaccines and Immunization Devices, Vector Control). https://extranet.who.int/pqweb/vaccines/vaccinescovid-19-vaccine-eul-issued

WHO. (2022). *The Bharat Biotech BBV152 COVAXIN vaccine against COVID-19: What you need to know*. https://www.who.int/news-room/feature-stories/detail/the-bharat-biotech-bbv152-covaxin-vaccine-against-covid-19-what-you-need-to-know

WHO. (2023). *WHO* [WHO]. COVID-19 Vaccine Tracker and Landscape. https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines

Wiersinga, W. J., Rhodes, A., Cheng, A. C., Peacock, S. J., & Prescott, H. C. (2020). Pathophysiology, Transmission, Diagnosis, and Treatment of Coronavirus Disease 2019 (COVID-19): A Review. *JAMA*, *324*(8), 782–793. https://doi.org/10.1001/jama.2020.12839

Williams, S. M., Chen, Y.-T., Andermann, T. M., Carter, J. E., McGee, D. J., & Ottemann, K. M. (2007). Helicobacter pylori Chemotaxis Modulates Inflammation and Bacterium-Gastric Epithelium Interactions in Infected Mice. *Infection and Immunity*, *75*(8), 3747. https://doi.org/10.1128/IAI.00082-07

Wong, S. L. (1995). Advances in the use of *Bacillus subtilis* for the expression and secretion of heterologous proteins. *Current Opinion in Biotechnology*, *6*(5), 517–522. https://doi.org/10.1016/0958-1669(95)80085-9

Woodrow, K. A., Bennett, K. M., & Lo, D. D. (2012). Mucosal vaccine design and delivery. *Annual Review of Biomedical Engineering*, *14*, 17–46. https://doi.org/10.1146/annurev-bioeng-071811-150054

Wray, L. V., Ferson, A. E., & Fisher, S. H. (1997). Expression of the *Bacillus subtilis* ureABC operon is controlled by multiple regulatory factors including CodY, GlnR, TnrA, and Spo0H. *Journal of Bacteriology*, *179*(17), 5494–5501. https://doi.org/10.1128/jb.179.17.5494-5501.1997

Wroblewski, L. E., Peek, R. M., & Wilson, K. T. (2010). *Helicobacter pylori* and Gastric Cancer: Factors That Modulate Disease Risk. *Clinical Microbiology Reviews*, *23*(4), 713–739. https://doi.org/10.1128/CMR.00011-10

Wu, A., Peng, Y., Huang, B., Ding, X., Wang, X., Niu, P., Meng, J., Zhu, Z., Zhang, Z., Wang, J., Sheng, J., Quan, L., Xia, Z., Tan, W., Cheng, G., & Jiang, T. (2020). Genome Composition and Divergence of the Novel Coronavirus (2019-nCoV) Originating in China. *Cell Host & Microbe*, *27*(3), 325–328. https://doi.org/10.1016/j.chom.2020.02.001

Wu, F., Zhao, S., Yu, B., Chen, Y.-M., Wang, W., Song, Z.-G., Hu, Y., Tao, Z.-W., Tian, J.-H., Pei, Y.-Y., Yuan, M.-L., Zhang, Y.-L., Dai, F.-H., Liu, Y., Wang, Q.-M., Zheng, J.-J., Xu, L., Holmes, E. C., & Zhang, Y.-Z. (2020). A new coronavirus associated with human respiratory disease in China. *Nature*, *579*(7798), Article 7798. https://doi.org/10.1038/s41586-020-2008-3

Wu, Q., Dudley, M. Z., Chen, X., Bai, X., Dong, K., Zhuang, T., Salmon, D., & Yu, H. (2021). Evaluation of the safety profile of COVID-19 vaccines: A rapid review. *BMC Medicine*, *19*(1), 173. https://doi.org/10.1186/s12916-021-02059-5

Wu, S., Huang, J., Zhang, Z., Wu, J., Zhu, T., Zhang, J., Luo, L., Fan, P., Wang, B., Chen, C., Chen, Y., Song, X., Wang, Y., Si, W., Sun, T., Wang, X., Hou, L., & Chen, W. (2021). Safety, tolerability, and immunogenicity of an aerosolised adenovirus type-5 vector-based COVID-19 vaccine (Ad5-nCoV) in adults: Preliminary report of an open-label and randomised phase 1 clinical trial. *The Lancet. Infectious Diseases*, *21*(12), 1654–1664. https://doi.org/10.1016/S1473-3099(21)00396-0

Xia, S., Zhu, Y., Liu, M., Lan, Q., Xu, W., Wu, Y., Ying, T., Liu, S., Shi, Z., Jiang, S., & Lu, L. (2020). Fusion mechanism of 2019-nCoV and fusion inhibitors targeting HR1 domain in spike protein. *Cellular & Molecular Immunology*, *17*(7), Article 7. https://doi.org/10.1038/s41423-020-0374-2

Xiao, K., Zhai, J., Feng, Y., Zhou, N., Zhang, X., Zou, J.-J., Li, N., Guo, Y., Li, X., Shen, X., Zhang, Z., Shu, F., Huang, W., Li, Y., Zhang, Z., Chen, R.-A., Wu, Y.-J., Peng, S.-M., Huang, M., … Shen, Y. (2020). Isolation of SARS-CoV-2-related coronavirus from Malayan pangolins. *Nature*, *583*(7815), Article 7815. https://doi.org/10.1038/s41586-020-2313-x

Xie, J., Wen, J., Chen, C., Luo, M., Hu, B., Wu, D., Ye, J., Lin, Y., Ning, L., Ning, Y., & Li, Y. (2020). Notch 1 Is Involved in CD4+ T Cell Differentiation Into Th1 Subtype During *Helicobacter pylori* Infection. *Frontiers in Cellular and Infection Microbiology*, *10*. https://www.frontiersin.org/articles/10.3389/fcimb.2020.575271

Xie, X., Liu, Y., Liu, J., Zhang, X., Zou, J., Fontes-Garfias, C. R., Xia, H., Swanson, K. A., Cutler, M., Cooper, D., Menachery, V. D., Weaver, S. C., Dormitzer, P. R., & Shi, P.-Y. (2021). Neutralization of SARS-CoV-2 spike 69/70 deletion, E484K and N501Y variants by BNT162b2 vaccine-elicited sera. *Nature Medicine*, *27*(4), 620–621. https://doi.org/10.1038/s41591-021-01270-4

Xue, J., Zheng, J., Shang, X., Qin, E., Zhao, P., He, Y., Liu, M., Zhang, J., Liu, H., & Bai, C. (2020). Risk factors for prolonged viral clearance in adult patients with COVID-19 in Beijing, China: A prospective observational study. *International Immunopharmacology*, *89*, 107031. https://doi.org/10.1016/j.intimp.2020.107031

Yan, F., & Polk, D. B. (2011). Probiotics and immune health. *Current Opinion in Gastroenterology*, *27*(6), 496–501. https://doi.org/10.1097/MOG.0b013e32834baa4d

Yan, X., Yu, H.-J., Hong, Q., & Li, S.-P. (2008). Cre/lox System and PCR-Based Genome Engineering in *Bacillus subtilis*. *Applied and Environmental Microbiology*, *74*(17), 5556. https://doi.org/10.1128/AEM.01156-08

Yang, J., Dai, L., Pan, X., Wang, H., Li, B., Zhu, J., Li, M., Shi, X.-L., & Wang, B. (2015). Protection against *Helicobacter pylori* infection in BALB/c mice by oral

administration of multi-epitope vaccine of CTB-UreI-UreB. *Pathogens and Disease*, *73*(5), ftv026. https://doi.org/10.1093/femspd/ftv026

Yang, X., Yu, Y., Xu, J., Shu, H., Xia, J., Liu, H., Wu, Y., Zhang, L., Yu, Z., Fang, M., Yu, T., Wang, Y., Pan, S., Zou, X., Yuan, S., & Shang, Y. (2020). Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: A single-centered, retrospective, observational study. *The Lancet. Respiratory Medicine*, *8*(5), 475–481. https://doi.org/10.1016/S2213-2600(20)30079-5

Yang, Y., Jing, Y., Yang, J., & Yang, Q. (2018). Effects of intranasal administration with *Bacillus subtilis* on immune cells in the nasal mucosa and tonsils of piglets. *Experimental and Therapeutic Medicine*, *15*(6), 5189–5198. https://doi.org/10.3892/etm.2018.6093

Yang, Y., Yang, M., Shen, C., Wang, F., Yuan, J., Li, J., Zhang, M., Wang, Z., Xing, L., Wei, J., Peng, L., Wong, G., Zheng, H., Wu, W., Liao, M., Feng, K., Li, J., Yang, Q., Zhao, J., … Liu, Y. (2020). Evaluating the accuracy of different respiratory specimens in the laboratory diagnosis and monitoring the viral shedding of 2019-nCoV infections (p. 2020.02.11.20021493). medRxiv. https://doi.org/10.1101/2020.02.11.20021493

Ye, F., Lin, X., Chen, Z., Yang, F., Lin, S., Yang, J., Chen, H., Sun, H., Wang, L., Wen, A., Zhang, X., Dai, Y., Cao, Y., Yang, J., Shen, G., Yang, L., Li, J., Wang, Z., Wang, W., … Lu, G. (2021). S19W, T27W, and N330Y mutations in ACE2 enhance SARS-CoV-2 S-RBD binding toward both wild-type and antibody-resistant viruses and its molecular basis. *Signal Transduction and Targeted Therapy*, *6*(1), Article 1. https://doi.org/10.1038/s41392-021-00756-4

Ye, X., Al-Babili, S., Klöti, A., Zhang, J., Lucca, P., Beyer, P., & Potrykus, I. (2000). Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science (New York, N.Y.)*, *287*(5451), 303–305. https://doi.org/10.1126/science.287.5451.303

Yeung, M.-L., Yao, Y., Jia, L., Chan, J. F. W., Chan, K.-H., Cheung, K.-F., Chen, H., Poon, V. K. M., Tsang, A. K. L., To, K. K. W., Yiu, M.-K., Teng, J. L. L., Chu, H.,

Zhou, J., Zhang, Q., Deng, W., Lau, S. K. P., Lau, J. Y. N., Woo, P. C. Y., … Yuen, K.-Y. (2016). MERS coronavirus induces apoptosis in kidney and lung by upregulating Smad7 and FGF2. *Nature Microbiology*, *1*(3), Article 3. https://doi.org/10.1038/nmicrobiol.2016.4

Yin, X., Riva, L., Pu, Y., Martin-Sancho, L., Kanamune, J., Yamamoto, Y., Sakai, K., Gotoh, S., Miorin, L., De Jesus, P. D., Yang, C.-C., Herbert, K. M., Yoh, S., Hultquist, J. F., García-Sastre, A., & Chanda, S. K. (2021). MDA5 Governs the Innate Immune Response to SARS-CoV-2 in Lung Epithelial Cells. *Cell Reports*, *34*(2), 108628. https://doi.org/10.1016/j.celrep.2020.108628

Yokota, K., Kurebayashi, Y., Takayama, Y., Hayashi, S., Isogai, H., Isogai, E., Imai, K., Yabana, T., Yachi, A., & Oguma, K. (1991). Colonization of *Helicobacter pylori* in the gastric mucosa of Mongolian gerbils. *Microbiology and Immunology*, *35*(6), 475–480. https://doi.org/10.1111/j.1348-0421.1991.tb01577.x

Yoshikura, H. (2012). On case-fatality rate: Review and hypothesis. *Japanese Journal of Infectious Diseases*, *65*(4), 279–288. https://doi.org/10.7883/yoken.65.279

Young, T. (2004). *Genetically Modified Organisms and Biosafety*. IUCN, Gland, Switzerland, and Cambridge, UK. https://doi.org/10.2305/IUCN.CH.2004.PGC.1.en

Zabala Torrres, B., Lucero, Y., Lagomarcino, A. J., Orellana-Manzano, A., George, S., Torres, J. P., & O'Ryan, M. (2017). Review: Prevalence and dynamics of *Helicobacter pylori* infection during childhood. *Helicobacter*, *22*(5). https://doi.org/10.1111/hel.12399

Zeng, M., Mao, X.-H., Li, J.-X., Tong, W.-D., Wang, B., Zhang, Y.-J., Guo, G., Zhao, Z.-J., Li, L., Wu, D.-L., Lu, D.-S., Tan, Z.-M., Liang, H.-Y., Wu, C., Li, D.-H., Luo, P., Zeng, H., Zhang, W.-J., Zhang, J.-Y., … Zou, Q.-M. (2015). Efficacy, safety, and immunogenicity of an oral recombinant *Helicobacter pylori* vaccine in children in China: A randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet (London, England)*, *386*(10002), 1457–1464. https://doi.org/10.1016/S0140-6736(15)60310-5

Zhang, J., Fitz-James, P. C., & Aronson, A. I. (1993). Cloning and characterization of a cluster of genes encoding polypeptides present in the insoluble fraction of the spore coat of *Bacillus subtilis*. *Journal of Bacteriology*, *175*(12), 3757–3766. https://doi.org/10.1128/jb.175.12.3757-3766.1993

Zhang, J.-Y., Liu, T., Guo, H., Liu, X.-F., Zhuang, Y., Yu, S., Chen, L., Wu, C., Zhao, Z., Tang, B., Luo, P., Mao, X.-H., Guo, G., Shi, Y., & Zou, Q.-M. (2011). Induction of a Th17 cell response by *Helicobacter pylori* Urease subunit B. *Immunobiology*, *216*(7), 803–810. https://doi.org/10.1016/j.imbio.2010.12.006

Zhao, Y., Zhao, Z., Wang, Y., Zhou, Y., Ma, Y., & Zuo, W. (2020). Single-Cell RNA Expression Profiling of ACE2, the Receptor of SARS-CoV-2. *American Journal of Respiratory and Critical Care Medicine*, *202*(5), 756–759. https://doi.org/10.1164/rccm.202001-0179LE

Zheng, L. B., Donovan, W. P., Fitz-James, P. C., & Losick, R. (1988). Gene encoding a morphogenic protein required in the assembly of the outer coat of the *Bacillus subtilis* endospore. *Genes & Development*, *2*(8), 1047–1054. https://doi.org/10.1101/gad.2.8.1047

Zhou, D., Dejnirattisai, W., Supasa, P., Liu, C., Mentzer, A. J., Ginn, H. M., Zhao, Y., Duyvesteyn, H. M. E., Tuekprakhon, A., Nutalai, R., Wang, B., Paesen, G. C., Lopez-Camacho, C., Slon-Campos, J., Hallis, B., Coombes, N., Bewley, K., Charlton, S., Walter, T. S., … Screaton, G. R. (2021). Evidence of escape of SARS-CoV-2 variant B.1.351 from natural and vaccine-induced sera. *Cell*, *184*(9), 2348-2361.e6. https://doi.org/10.1016/j.cell.2021.02.037

Zhou, F., Yu, T., Du, R., Fan, G., Liu, Y., Liu, Z., Xiang, J., Wang, Y., Song, B., Gu, X., Guan, L., Wei, Y., Li, H., Wu, X., Xu, J., Tu, S., Zhang, Y., Chen, H., & Cao, B. (2020). Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: A retrospective cohort study. *The Lancet*, *395*(10229), 1054–1062. https://doi.org/10.1016/S0140-6736(20)30566-3

Zhou, H., Li, X., Wang, Z., Yin, J., Tan, H., Qiao, X., Jiang, Y., Cui, W., Liu, M., Li, Y., Xu, Y., & Tang, L. (2018). Construction and characterization of thymidine auxotrophic (ΔthyA) recombinant *Lactobacillus casei* expressing bovine

lactoferricin. *BMC Veterinary Research*, *14*, 206. https://doi.org/10.1186/s12917-018-1516-y

Zhou, H., Yang, J., Zhou, C., Chen, B., Fang, H., Chen, S., Zhang, X., Wang, L., & Zhang, L. (2021). A Review of SARS-CoV2: Compared With SARS-CoV and MERS-CoV. *Frontiers in Medicine*, *8*. https://www.frontiersin.org/articles/10.3389/fmed.2021.628370

Zhou, P., Yang, X.-L., Wang, X.-G., Hu, B., Zhang, L., Zhang, W., Si, H.-R., Zhu, Y., Li, B., Huang, C.-L., Chen, H.-D., Chen, J., Luo, Y., Guo, H., Jiang, R.-D., Liu, M.-Q., Chen, Y., Shen, X.-R., Wang, X., … Shi, Z.-L. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*, *579*(7798), 270–273. https://doi.org/10.1038/s41586-020-2012-7

Zhou, R., Wang, P., Wong, Y.-C., Xu, H., Lau, S.-Y., Liu, L., Mok, B. W.-Y., Peng, Q., Liu, N., Woo, K.-F., Deng, S., Tam, R. C.-Y., Huang, H., Zhang, A. J., Zhou, D., Zhou, B., Chan, C.-Y., Du, Z., Yang, D., … Chen, Z. (2022). Nasal prevention of SARS-CoV-2 infection by intranasal influenza-based boost vaccination in mouse models. *EBioMedicine*, *75*, 103762. https://doi.org/10.1016/j.ebiom.2021.103762

Zhou, W.-Y., Shi, Y., Wu, C., Zhang, W.-J., Mao, X.-H., Guo, G., Li, H.-X., & Zou, Q.-M. (2009). Therapeutic efficacy of a multi-epitope vaccine against Helicobacter pylori infection in BALB/c mice model. *Vaccine*, *27*(36), 5013–5019. https://doi.org/10.1016/j.vaccine.2009.05.009

Zhou, Z., Dong, H., Huang, Y., Yao, S., Liang, B., Xie, Y., Long, Y., Mai, J., & Gong, S. (2017a). Recombinant *Bacillus subtilis* spores expressing cholera toxin B subunit and Helicobacter pylori urease B confer protection against *H. pylori* in mice. *Journal of Medical Microbiology*, *66*(1), 83–89. https://doi.org/10.1099/jmm.0.000404

Zhou, Z., Gong, S., Li, X.-M., Yang, Y., Guan, R., Zhou, S., Yao, S., Xie, Y., Ou, Z., Zhao, J., & Liu, Z. (2015a). Expression of *Helicobacter pylori* urease B on the surface of *Bacillus subtilis* spores. *Journal of Medical Microbiology*, *64*(Pt 1), 104–110. https://doi.org/10.1099/jmm.0.076430-0

Zhu, J., Yamane, H., & Paul, W. E. (2010). Differentiation of Effector CD4 T Cell Populations. *Annual Review of Immunology*, *28*, 445–489. https://doi.org/10.1146/annurev-immunol-030409-101212

Zilberman, D., Holland, T. G., & Trilnick, I. (2018). Agricultural GMOs—What We Know and Where Scientists Disagree. *Sustainability*, *10*(5), Article 5. https://doi.org/10.3390/su10051514

Zilhão, R., Isticato, R., Martins, L. O., Steil, L., Völker, U., Ricca, E., Moran, C. P., & Henriques, A. O. (2005). Assembly and Function of a Spore Coat-Associated Transglutaminase of *Bacillus subtilis*. *Journal of Bacteriology*, *187*(22), 7753–7764. https://doi.org/10.1128/JB.187.22.7753-7764.2005

Zost, S. J., Gilchuk, P., Case, J. B., Binshtein, E., Chen, R. E., Nkolola, J. P., Schäfer, A., Reidy, J. X., Trivette, A., Nargi, R. S., Sutton, R. E., Suryadevara, N., Martinez, D. R., Williamson, L. E., Chen, E. C., Jones, T., Day, S., Myers, L., Hassan, A. O., … Crowe, J. E. (2020). Potently neutralizing and protective human antibodies against SARS-CoV-2. *Nature*, *584*(7821), Article 7821. https://doi.org/10.1038/s41586-020-2548-6

Appendix A: *thyA:cotB* and *thyA::cotC* sequences cloned in pThyA

Plasmid	Sequence[1]
pThyA::CotB	CAGATCATATAAGGAATGAACCGCTGCCAAATATCATAAAAAAGTTGTTAATG ATCAAATGAATGAAATTAGAGAGAATTTATTTTAAAGAAAGCCCAATTGCACA TGGACAAATGACAATTGATATTGAGGTAAAATACAATGCATTAAATCAGAAAA AGCTATTGGAGGATTTAATGTGTTTAGACAATTTCCAATTTGGTATACACAAAC ACCTGACTATTTGAATTTTTATGTACCGCAATATCAAACCATTTCGTATAATCC TCAACAATGTTATCAACGGTGTATGTACCAAACTGGCGGTAACTATGAGCTAT GTGACAGACTATGTTATGGAGAAATACAGGTGTAAAAGAGGGGGATTAACTCC TCTTTAAACACACAGTGAGTGGAATAAGATCCTCACTTTATCTGCAAGTGCTTA GTATTTGCGATAATATTGCATTCGTAATAAATTATGCTTAGCAACTGAAAATGA AAGAAGGATATGAATAGTC**ATG**ACGCAATTCGATAAACAATACAATTCAATTA TAAAGGATATTATCAATAATGGAATCTCAGACGAAGAGTTTGATGTAAGAACC AAGTGGGACTCAGATGGAACACCGGCACATACTCTAAGTGTAATCAGTAAGCA AATGAGATTCGACAACTCAGAGGTTCCGATTTTAACGACAAAAAAGGTTGCCT GGAAAACAGCCATTAAAGAGTTGCTCTGGATTTGGCAGCTGAAATCTAATGAT GTTAATGATTTAAACATGATGGGCGTCCATATTTGGGATCAGTGGAAACAAGA AGACGGAACCATCGGACATGCATATGCGAAAATCATGGCGATGTATGAACGG ATTAGGCCGTTTGTCCTCATGGACCCGTATAAAAAGAATGATATTGAGCGTTTT GACCGTGAGCCGGATGTGATCTGCGAGTATATTAAAAACCGTTCACAATACCT CAAAGATCATTTAAGTATTTTATGAATGCGTGAAAATGGGTATTCGCGGAAAA AGCGACAATTAGGCTATTGAATTAGTTCAACAAATAAATGTGACACGTATATA TGCAGTATGTTTATCATCTATGTATAAGTGACTAGGAGGAATTTGA**ATG**AGCA AGAGGAGAATGAAATATCATTCAAATAATGAAATATCGTATTATAACTTTTTG CACTCAATGAAAGATAAAATTGTTACTGTATATCGTGGAGGTCCGGAATCTAA AAAAGGAAAATTAACAGCTGTAAAATCAGATTATATAGCTTTACAAGCTGAAA AAAAAATAATTTATTATCAGTTGGAGCATGTGAAAAGTATTACTGAGGATACC AATAATAGCACCACAACAATTGAGACTGAGGAAATGCTCGATGCTGATGATTT TCATAGCTTAATCGGACATTTAATAAACCAATCAGTTCAATTTAACCAAGGGG GTCCGGAATCTAAAAAAGGAAGATTGGTCTGGCTGGGAGATGATTACGCTGCG TTAAACACAAATGAGGATGGGGTAGTGTATTTTAATATCCATCACATCAAAAG TATAAGTAAACACGAGCCTGATTTGAAAATAGAAGAGCAGACGCCAGTTGGA GTTTTGGAAGCTGATGATTTAAGCGAGGTTTTTAAGAGTCTGACTCATAAATGG GTTTCAATTAATCGTGGAGGTCCGGAAGCCATTGAGGGTATCCTTGTAGATAAT GCCGACGGCCATTATACTATAGTGAAAAATCAAGAGGTGCTTCGCATCTATCC TTTTCACATAAAAAGCATCAGCTTAGGTCCAAAAGGGTCGTACAAAAAAGAGG ATCAAAAAAATGAACAAAACCAGGAAGACAATAATGATAAGGACAGCAATTC GTTCATTTCTTCAAAATCATATAGCTCATCAAAATCATCTAAACGATCACTAAA ATCTTCAGATGATCAATCATCCAAATCTGGTCGTTCGTCACGTTCAAAAAGTTC TTCAAAATCATCTAAACGATCACTAAAATCTTCGGATTATCAATCATCCAAATC TGGCCGTTCGTCACGTTCAAAAAGTTCTTCAAAATCATCTAAACGATCATTAAA ATCTTCAGATTATCAATCATCAAAATCATCTAAACGATCACCAAGATCT*GGATC* *CCCCGGGGGTACCGAGCTCGAATTC*TGAAGAACAATCCATCTTCACGCAGACAC ATTACAATGCTGTGGAATCCTGATGAATTAGACGCAATGGCCTTAACGCCATG TGTATACGAGACACAATGGTACGTTAAACATGGGAAACTCCACCTTGAGGTAA GAGCACGGAGCAATGATATGGCATTGGGAAATCCATTCAATGTATTCCAGTAT AATGTGTTGCAGCGCATGATTGCTCAAGTGACTGGTTATGAGCTTGGTGAATAT ATCTTTAACATTGGGGATTGCCATGTGTACACACGTCATATAGACAATTTGAAA ATTCAAATGGAAAGAGAACAGTTTGAAGCACCTGAACTATGGATCAATCCTGA AGTGAAAGATTTTTATGACTTTACCATTGATGATTTCAAGTTAATCAACTATAA ACATGGGGACAAGCTTTTATTTGAGGTAGCGGTTTAATGCTGCCTTTTTATTGT GCAGTGAATAGATAGCAGGTATCCTAATTTCATTAAGCAATCTGGAAGATGAA TAAAAATTGAAGGACAAACACGTATAATACATAAAAAAGATTAACTCTACAGT TAATCTTTTTTATTCAGAAGAAAATATCCTAACTTTGAAACTAAATACAAAGTA AAAGCAATCATTACAGTTCTAGATATTACAATTCCATGAATAGCTAGATCATAT CCAGCAGGTATCAACGCATTTGTATTACACATAAAATATATAGATATTAGAAG TGCTACAATAACTAAAATCATTCCAAAAAGACTTGTTTTTTCATATTTCATACC AATTTCCACCCTTATTAAAGTTAGGTTTAAACAAAAGAGCTGAAGAAACGAAC TATGACCAGTATGCTCCAAGGAAAACCGCCAGACAATGCTGGCGGCTTTTTGC TGCTTCGTTTATTTATTAACAGAGATCGTAACGTTATTTCCTGCAACTGAAACC TTTGCGAAATCC-3’

pThyA::CotC	CAGATCATATAAGGAATGAACCGCTGCCAAATATCATAAAAAAGTTGTTAATG ATCAAATGAATGAAATTAGAGAGAATTTATTTTAAAGAAAGCCCAATTGCACA TGGACAAATGACAATTGATATTGAGGTAAAATACAATGCATTAAATCAGAAAA AGCTATTGGAGGATTTAATGTGTTTAGACAATTTCCAATTTGGTATACACAAAC ACCTGACTATTTGAATTTTTATGTACCGCAATATCAAACCATTTCGTATAATCC TCAACAATGTTATCAACGGTGTATGTACCAAACTGGCGGTAACTATGAGCTAT GTGACAGACTATGTTATGGAGAAATACAGGTGTAAAAGAGGGGATTAACTCC TCTTTAAACACACAGTGAGTGGAATAAGATCCTCACTTTATCTGCAAGTGCTTA GTATTTGCGATAATATTGCATTCGTAATAAATTATGCTTAGCAACTGAAAATGA AAGAAGGATATGAATAGTC**ATG**ACGCAATTCGATAAACAATACAATTCAATTA TAAAGGATATTATCAATAATGGAATCTCAGACGAAGAGTTTGATGTAAGAACC AAGTGGGACTCAGATGGAACACCGGCACATACTCTAAGTGTAATCAGTAAGCA AATGAGATTCGACAACTCAGAGGTTCCGATTTTAACGACAAAAAAGGTTGCCT GGAAAACAGCCATTAAAGAGTTGCTCTGGATTTGGCAGCTGAAATCTAATGAT GTTAATGATTTAAACATGATGGGCGTCCATATTTGGGATCAGTGGAAACAAGA AGACGGAACCATCGGACATGCATATGATAAACTGCCGGCGCTTGGCCGTGCAG CAGGAAAAGCCTTATCAGAATTTAAACAAGCAACAAGCGGACTGACTCAGGAT ATCAGAAAAAATGACTCAGAAAACAAAGAAGACAAACAAATGTAGGATAAAT CGTTTGGGCCGATGAAAAATCGGCTCTTTATTTTGATTTGTTTTTGTGTCATCTG TCTTTTTCTATCATTTGGACAGCCCTTTTTTCCTTCTATGATTTTAACTGTCCAA GCCGCAAAATCTACTCGCCGTATAATAAAGCGTAGTAAAAATAAAGGAGGAGT ATAT**ATG**GGTTATTACAAAAAATACAAAGAAGAGTATTATACGGTCAAAAAA ACGTATTATAAGAAGTATTACGAATATGATAAAAAAGATTATGACTGTGATTA CGACAAAAAATATGATGACTATGATAAAAAATATTATGATCACGATAAAAAAG ACTATGATTATGTTGTAGAGTATAAAAAGCATAAAAAACACTAC*GGATCCCCC* *GGGGGTACCGAGCTCGAATTC*TGAAGAACAATCCATCTTCACGCAGACACATTA CAATGCTGTGGAATCCTGATGAATTAGACGCAATGGCCTTAACGCCATGTGTA TACGAGACACAATGGTACGTTAAACATGGGAAACTCCACCTTGAGGTAAGAGC ACGGAGCAATGATATGGCATTGGGAAATCCATTCAATGTATTCCAGTATAATG TGTTGCAGCGCATGATTGCTCAAGTGACTGGTTATGAGCTTGGTGAATATATCT TTAACATTGGGGATTGCCATGTGTACACACGTCATATAGACAATTTGAAAATTC AAATGGAAAGAGAACAGTTTGAAGCACCTGAACTATGGATCAATCCTGAAGTG AAAGATTTTTATGACTTTACCATTGATGATTTCAAGTTAATCAACTATAAACAT GGGGACAAGCTTTTATTTGAGGTAGCGGTTTAATGCTGCCTTTTTATTGTGCAG TGAATAGATAGCAGGTATCCTAATTTCATTAAGCAATCTGGAAGATGAATAAA AATTGAAGGACAAACACGTATAATACATAAAAAAGATTAACTCTACAGTTAAT CTTTTTTATTCAGAAGAAAATATCCTAACTTTGAAACTAAATACAAAGTAAAA GCAATCATTACAGTTCTAGATATTACAATTCCATGAATAGCTAGATCATATCCA GCAGGTATCAACGCATTTGTATTACACATAAAATATATAGATATTAGAAGTGC TACAATAACTAAAATCATTCCAAAAAGACTTGTTTTTTCATATTTCATACCAAT TTCCACCCTTATTAAAGTTAGGTTTAAACAAAAGAGCTGAAGAAACGAACTAT GACCAGTATGCTCCAAGGAAAACCGCCAGACAATGCTGGCGGCTTTTTGCTGC TTCGTTTATTTATTAACAGAGATCGTAACGTTATTTCCTGCAACTGAAACCTTT GCGAAATCC-3’
pThyB	CCAAATCTGCCGCTCAGTGTTTGCATGGAGAATGTAGAAAAAGTCCTGAACAA ACGTGAAATTATTCATGCTGTTTTGACAGGCCTTGCACTCGATCAGCTTGCAGA ACAGAAACTTCTCCCCGAACCGCTGCAGCACCTTGTTGAAACGGATGAACCGC TTTACGGCATAGATGAAATTATCCCGCTTTCAATCGTTAATGTGTACGGGTCGA TCGGTTTGACCAATTTCGGTTATTTGGATAAAGAGAAGATTGGAATTATTAAGG AACTTGATGAAAGTCCAGACGGTATTCACACCTTTTTGGATGATATTGTGGCAG CTCTTGCTGCAGCAGCGGCGAGCAGAATTGCACATACGCATCAGGATCTGCAA GATGAAGAAAAAGAACAGGATGAAAAGCCTGTCGTCAGCTGACTATAAAAAA ATCATTTCTGGGTTCAGAAATGATTTTTTATTGTGTTACACTACTAGAAGACTA CTTTTAAAGGATGAAAAAAA**ATG**AAACAGTATAAGGATTTCTGCAGACATGTTT TAGAGCATGGTGAGAAAAAGGGAGACCGGACTGGGACCGGAACAATCAGCAC TTTCGGATATCAAATGAGATTTAATTTACGGGAAGGCTTTCCGATGCTCACCAC TAAAAAACTCCACTTTAAATCAATTGCGCATGAACTGCTGTGGTTCTTAAAAGG AGATACGAATGTACGCTATCTGCAGGAAAACGGAGTGCGAATCTGGAATGAGT GGGCTGATGAAAACGGTGAACTTGGACCTGTATATGGCTCCCAATGGCGTTCT TGGCGGGGAGCTGATGGAGAAACCATTGATCAAATTTCCCGTCTTATTGAAGA TATTAAAACAAATCCGAACTCCAGACGCTTAATCGTCAGCGCCTGGA*AAGCTTG* *CATGCCTGCAGGTCGACTCTAGAGGATCCCCCGGGGGTACCGAGCTCGAATTC*TGA TGTTGGTGAAATTGATAAAATGGCGTTGCCGCCGTGCCATTGCCTGTTCCAATT

	CTATGTGTCTGACGGCAAGCTGTCCTGTCAGCTGTATCAGCGCTCTGCCGATGT TTTCTTAGGTGTGCCGTTTAATATTGCATCTTATGCCCTCCTAACCATGATCATT GCTCATGTGACTGGGCTTGAACCGGGCGAGTTCATCCATACGTTTGGTGATGTT CATATTTACCAAAATCATATTGAACAAGTCAATTTGCAGCTGGAAAGAGATGT TAGACCGCTTCCGCAGCTTCGTTTCGCCAGAAAGGTTGATTCTATTTTTAACTTT GCATTTGAGGACTTTATCATCGAGGATTATGATCCGCATCCTCATATAAAAGGG GCGGTCAGCGTATGATTTCATTCATTTTTGCGATGGATGCCAACAGGCTTATCG GCAAAGACAATGATTTGCCGTGGCATTTGCCCAATGATCTTGCATACTTTAAGA AAATAACATCGGGCCATTCAATCATTATGGGCCGGAAAACATTTGAATCGATC GGACGTCCGCTTCCAAATCGGAAAAATATTGTCGTTACCTCAGCGCCGGATTC AGAATTTCAGGGATGCACGGTTGTCAGTTCATTAAAGGATGTACTGGACATTT GTTCAGGCCCTGAAGAATGCTTTGTGATCGGAGGGGCTCAGCTCTATACGGAC CTGTTCCCTTATGCGGACAGACTGTATATGACGAAAATTCATCACGAGTTTGAG GGTGACCGTCACTTTCCTGAATTTGATGAATCCAATTGGAAGCTGGTTTCTTCT GAGCAGGGGACCAAAGACGAAAAAAACCCGTATGATTACGAATTTCTAATGTA TGAAAAAAAGAAATCTTCTAAAGCGGGAGGATTTTAATTGGTTCGCTACAGCC TTCTAGTGGTTTATATTGTGTATATGCTGTTAAAAAATATGAAACAATTATTTA ATCAAACAATGCTCGATCCCCGTCTGTCATACAAAAAACAGATGGCTCTTGTGT ACGAACAGCCAAAGGCGTTTTTAGAAGGCTGTATCGGCATCTCCGGTTCAGTT GTGAC GATCCATCAGCCAGA-3’

[1] The thy ORF (5’-3’) is shown in grey shading with the start codon in bold and flanking DNA (unshaded). The MCS is in italics. Primer annealing sites (forward and reverse) used to verify insertion are underlined. The promoter sequence of CotB and CotC is shown in orange and the CotB and CotC sequences are shown in green.

Appendix B: Amino acid sequences of the fusion genes[1]

Fusion protein	Sequence[1]
CotB-RBD$^{Wuh(319-530)}$	MSKRRMKYHSNNEISYYNFLHSMKDKIVTVYRGGPESKKGKLTAVKSD YIALQAEKKIIYYQLEHVKSITEDTNNSTTTIETEEMLDADDFHSLIGHLIN QSVQFNQGGPESKKGRLVWLGDDYAALNTNEDGVVYFNIHHIKSISKHE PDLKIEEQTPVGVLEADDLSEVFKSLTHKWVSINRGGPEAIEGILVDNAD GHYTIVKNQEVLRIYPFHIKSISLGPKGSYKKEDQKNEQNQEDNNDKDSN SFISSKSYSSSKSSKRSLKSSDDQSSKSGRSSRSKSSSKSSKRSLKSSDYQSS KSGRSSRSKSSSKSSKRSLKSSDYQSSKSSKRSPRSRVQPTESIVRFPNITNL CPFGEVFNATRFASVYAWNRKRISNCVADYSVLYNSASFSTFKCYGVSPT KLNDLCFTNVYADSFVIRGDEVRQIAPGQTGKIADYNYKLPDDFTGCVIA WNSNNLDSKVGGNYNYLYRLFRKSNLKPFERDISTEIYQAGSTPCNGVE GFNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELLHAPATVCGPKKS
CotB-RBD$^{Omi(316-538)}$	MSKRRMKYHSNNEISYYNFLHSMKDKIVTVYRGGPESKKGKLTAVKSD YIALQAEKKIIYYQLEHVKSITEDTNNSTTTIETEEMLDADDFHSLIGHLIN QSVQFNQGGPESKKGRLVWLGDDYAALNTNEDGVVYFNIHHIKSISKHE PDLKIEEQTPVGVLEADDLSEVFKSLTHKWVSINRGGPEAIEGILVDNAD GHYTIVKNQEVLRIYPFHIKSISLGPKGSYKKEDQKNEQNQEDNNDKDSN SFISSKSYSSSKSSKRSLKSSDDQSSKSGRSSRSKSSSKSSKRSLKSSDYQSS KSGRSSRSKSSSKSSKRSLKSSDYQSSKSSKRSPRSRVQPTESIVRFPNITNL CPFDEVFNATRFASVYAWNRKRISNCVADYSVLYNLAPFFTFKCYGVSPT KLNDLCFTNVYADSFVIRGDEVRQIAPGQTGNIADYNYKLPDDFTGCVIA WNSNKLDSKVSGNYNYLYRLFRKSNLKPFERDISTEIYQAGNKPCNGVA GFNCYFPLRSYSFRPTYGVGHQPYRVVVLSFELLHAPATVCGPKKSTNLV KNKCVNF
CotC-HR1-HR2$^{Wuh(920-1201)}$	MGYYKKYKEEYYTVKKTYYKKYYEYDKKDYDCDYDKKYDDYDKKYY DHDKKDYDYVVEYKKHKKHYQKLIANQFNSAIGKIQDSLSSTASALGKL QDVVNQNAQALNTLVKQLSSNFGAISSVLNDILSRLDKVEAEVQIDRLIT GRLQSLQTYVTQQLIRAAEIRASANLAATKMSECVLGQSKRVDFCGKGY HLMSFPQSAPHGVVFLHVTYVPAQEKNFTTAPAICHDGKAHFPREGVFV SNGTHWFVTQRNFYEPQIITTDNTFVSGNCDVVIGIVNNTVYDPLQPELDS FKEELDKYFKNHTSPDVDLGDISGINASVVNIQKEIDRLNEVAKNLNESLI DLQ
CotB-NC$^{(1-422)}$	MSKRRMKYHSNNEISYYNFLHSMKDKIVTVYRGGPESKKGKLTAVKSD YIALQAEKKIIYYQLEHVKSITEDTNNSTTTIETEEMLDADDFHSLIGHLIN QSVQFNQGGPESKKGRLVWLGDDYAALNTNEDGVVYFNIHHIKSISKHE PDLKIEEQTPVGVLEADDLSEVFKSLTHKWVSINRGGPEAIEGILVDNAD GHYTIVKNQEVLRIYPFHIKSISLGPKGSYKKEDQKNEQNQEDNNDKDSN SFISSKSYSSSKSSKRSLKSSDDQSSKSGRSSRSKSSSKSSKRSLKSSDYQSS KSGRSSRSKSSSKSSKRSLKSSDYQSSKSSKRSPRSMSDNGPQNQRNAPRI TFGGPSDSTGSNQNGERSGARSKQRRPQGLPNNTASWFTALTQHGKEDL KFPRGQGVPINTNSSPDDQIGYYRRATRRIRGGDGKMKDLSPRWYFYYL GTGPEAGLPYGANKDGIIWVATEGALNTPKDHIGTRNPANNAAIVLQLPQ GTTLPKGFYAEGSRGGSQASSRSSSRSRNSSRNSTPGSSMGTSPARMAGN GCDAALALLLLDRLNQLESKMSGKGQQQQGQTVTKKSAAEASKKPRQK RTATKAYNVTQAFGRRGPEQTQGNFGDQELIRQGTDYKHWPQIAQFAPS ASAFFGMSRIGMEVTPSGTWLTYTGAIKLDDKDPNFKDQVILLN
CotB-UreA$^{(1-237)}$	MSKRRMKYHSNNEISYYNFLHSMKDKIVTVYRGGPESKKGKLTAVKSD YIALQAEKKIIYYQLEHVKSITEDTNNSTTTIETEEMLDADDFHSLIGHLIN QSVQFNQGGPESKKGRLVWLGDDYAALNTNEDGVVYFNIHHIKSISKHE PDLKIEEQTPVGVLEADDLSEVFKSLTHKWVSINRGGPEAIEGILVDNAD GHYTIVKNQEVLRIYPFHIKSISLGPKGSYKKEDQKNEQNQEDNNDKDSN SFISSKSYSSSKSSKRSLKSSDDQSSKSGRSSRSKSSSKSSKRSLKSSDYQSS KSGRSSRSKSSSKSSKRSLKSSDYQSSKSSKRSPRSMKLTPKELDKLMLHY AGELAKKRKEKGIKLNYVEAVALISAHIMEEARAGKKTAAELMQEGRTL LKPDDVMDGVASMIHEVGIEAMFPDGTKLVTVHTPIEANGKLVPGELFL

	KNEDITINEGKKAVSVKVKNVGDRPVQIGSHFHFFEVNRCLDFDREKTFG KRLDIASGTAVRFEPGEEKSVELIDIGGNRRIFGFNALVDRQADNESKKIA LHRAKERGFHGAKSDDNYVKTIKE*
CotB-UreB$^{(365\text{-}568)}$	MSKRRMKYHSNNEISYYNFLHSMKDKIVTVYRGGPESKKGKLTAVKSD YIALQAEKKIIYYQLEHVKSITEDTNNSTTTIETEEMLDADDFHSLIGHLIN QSVQFNQGGPESKKGRLVWLGDDYAALNTNEDGVVYFNIHHIKSISKHE PDLKIEEQTPVGVLEADDLSEVFKSLTHKWVSINRGGPEAIEGILVDNAD GHYTIVKNQEVLRIYPFHIKSISLGPKGSYKKEDQKNEQNQEDNNDKDSN SFISSKSYSSSKSSKRSLKSSDDQSSKSGRSSRSKSSSKSSKRSLKSSDYQSS KSGRSSRSKSSSKSSKRSLKSSDYQSSKSSKRSPRSAMGRVGEVITRTWQT ADKNKKEFGRLKEEKGDNDNFRIKRYLSKYTINPAIAHGISEYVGSVEVG KVADLVLWSPAFFGVKPNMIIKGGFIALSQMGDANASIPTPQPVYYREMF AHHGKAKYDANITFVSQAAYDKGIKEELGLERQVLPVKNCRNITKKDM QFNDTTAHIEVNPETYHVFVDGKEVTSKPANKVSLAQLFSIF*

[1] Amino acid sequences of the fusion genes

List of Publication

Katsande, P. M., Fernández-Bastit, L., Ferreira, W. T., Vergara-Alert, J., Hess, M., Lloyd-Jones, K., Hong, H. A., Segales, J., & Cutting, S. M. (2022). Heterologous Systemic Prime–Intranasal Boosting Using a Spore SARS-CoV-2 Vaccine Confers Mucosal Immunity and Cross-Reactive Antibodies in Mice as well as Protection in Hamsters. *Vaccines*, *10*(11), 1900. https://doi.org/10.3390/vaccines10111900

James, J., Meyer, S. M., Hong, H. A., Dang, C., Linh, H. T. Y., Ferreira, W., **Katsande, P. M**., Vo, L., Hynes, D., Love, W., Banyard, A. C., & Cutting, S. M. (2022). Intranasal Treatment of Ferrets with Inert Bacterial Spores Reduces Disease Caused by a Challenging H7N9 Avian Influenza Virus. *Vaccines*, *10*(9), Article 9. https://doi.org/10.3390/vaccines10091559

Katsande, P. M., Nguyen, V. D., Nguyen, T. L. P., Nguyen, T. K. C., Mills, G., Bailey, D. M. D., Christie, G., Hong, H. A., & Cutting, S. M. (2023). Prophylactic immunization to *Helicobacter pylori* infection using spore vectored vaccines. *Helicobacter*, e12997. https://doi.org/10.1111/hel.12997

Thi Kim Cuc Nguyen1, Hoang Dang Khoa Do, Thi Lan Phuong Nguyen, Thu Thuy Pham, Bao Ngoc Mach, **Paidamoyo M. Katsande**, Huynh Anh Hong, Huu Thai Duong, Anh N. Phan, Minh Thiet Vu, Simon M. Cutting, Van Duy Nguyen. Genomic and vaccine preclinical studies reveal a novel mouse-adapted *Helicobacter pylori* model for hpEastAsia genotype in Southeast Asia. Manuscript has been submitted to the journal, Frontiers in Cellular and Infection Microbiology,

Alfawaz, D., **Katsande, P**., Jordan, H., Kuehne, S., Ferrero, R., & Rossiter, A. (2019). The 'missing' gastric microbe; the impact of gastric cancer-associated microbiota on helicobacter pylori growth *in vitro* and its implications in gastric carcinogenesis. *Access Microbiology*, *1*(1A). https://doi.org/10.1099/acmi.ac2019.po0334