Development of vaccines against Dengue virus: Use of *Lactococcus lactis* as a mucosal vaccine delivery vehicle

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
JOINT MASTER OF SCIENCE (INFECTIOUS DISEASES,
VACCINOLOGY AND DRUG DISCOVERY)
DEPARTMENT OF MICROBIOLOGY
NATIONAL UNIVERSITY OF SINGAPORE
2007

Acknowledgements

I would like to express my sincere thanks and utmost gratitude to :

Associate Professor Vincent Chow,
For his constant guidance and patience during the course of my project. Finally, I would like to thank him for giving me a chance to work on this interesting and enriching project.

Dr. Sylvie Alonso,
For giving her kind advice, the constant encouragement and most importantly cracking her head to troubleshoot the project. The experiences gained in her laboratory are truly invaluable.

Prof Guy Cornelis,
For being my link between Basel and Singapore.

NITD, STI, University of Basel and NUS,
For making this Joint Masters possible and making it such a wonderful experience.

Kelly,
For her constant help in viral and plaque assays aspects of my work. And also for all her help in other aspects of the project, which I am grateful for.

Wenwei, Siying, Lirui, Lili, Joe, Shiqian, Magenta - my fellow lab mates.
For the help they gave in various aspects of the project and for making the lab an enjoyable place to work in. Wenwei for starting the *L.lactis* project and all who had helped me in one way or another.

Damian, Eng Lee, King and the rest of my friends,
Thanks for the wonderful Wala sessions, chalets and meals. Stress levels were definitely much lower after spending time with you guys!

God,
For His eternal guidance and patience with me. And being there in my times of need. For through Him all things are truly possible.

Last but not least, I would like to thank my parents, my brother and Ivette for their constant love, concern, understanding and support throughout the entire project, without which this accomplishment would not have been possible.

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Summary

Mucosal vaccines, which are administered by oral or intranasal route, are more convenient than the usual parenteral vaccines due to their ease of administration and low cost. Both are priorities for developing countries plagued by infectious diseases when considering vaccination for public health policy. Moreover, mucosal vaccines are able to elicit serum-IgG and mucosal-IgA antibodies to neutralize toxins and viruses and induce cytotoxic T lymphocytes (CTL) activities .

In this context, we have embarked on the study of the use of *Lactococcus lactis* as a possible vaccine vector targeting dengue virus. This is a further study from previous work by Lin, W. (2006) who constructed a recombinant *L. lactis* strain producing in its cytoplasm the E domain III (EDIII) antigen from DEN2 virus, Singapore strain. *L. lactis* is a noninvasive, nonpathogenic, gram-positive bacterium which has a long history of widespread use in the food industry for the production of fermented milk products, thus it has a generally-regarded as safe (GRAS) status. Its GRAS status coupled to its inability to colonize the digestive and the respiratory tracts of both humans and mice, except gnotobiotic mice, make *L. lactis* a safe and attractive vaccine delivery vehicle for human use.

This study aims to study the immunization efficacy, via measuring the systemic anti-EDIII antibody response generated in two different mouse strains, BALB/c and C57BL/6, after nasal or oral administration of the EDIII-producing *L. lactis* strain (LLWE-EDIII). The systemic specific anti-EDIII IgG responses were compared. Our data indicate that EDIII-producing *L. lactis* bacteria are able to trigger

a strong and sustained antibody response against EDIII antigen in mice. Of the two strains and two routes of inoculation, it was observed that C57BL/6 mice inoculated via the nasal route were found to be the best responders. With the preliminary results of plaque reduction neutralization test (PRNT), the higher ELISA readings of anti-EDIII IgG might not necessary translates to higher neutralizing ability against a homotypic dengue virus with 3 amino acid mutation in the region targeted. However, more PRNT needs to be done to validate this observation or otherwise. But the ability of the sera raised in mice inoculated with LLWE-EDIII to neutralize dengue virus seems promising of using it as a mucosal vaccine targeting dengue virus.

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Abbreviations

ADE	antibody-dependent enhancement
AST	aspartate aminotransferase
ALT	alanine aminotransferase
BHK	baby hamster kidney
bp	base pair
cDNA	complementary DNA
Den	dengue
DF	dengue fever
DHF	dengue haemorrhagic fever
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside-5'-triphosphate
dsRNA	double stranded ribonucleic acids
DSS	dengue shock syndrome
E	envelope
ED III	E domain III
EDTA	ethylenedintrilo tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAE	Follicle associated epithelium
FCS	Fetal calf serum
g	gram

hr	hour
IFN	interferon
IL	interleukin
JEV	Japanese Encephalitis Virus
kDa	kilo daltons
l	Litre
µg	microgram
µl	microliter
µM	micromole
M	mole
mA	milliampere
mg	milligram
MHC	Major histocompatability complex
min	minute
ml	millilitre
mM	millimole
mRNA	messenger ribonucleic acid
MW	molecular weight
NOD	Non obese diabetic
nt	nucleotide
NS	non structural
OD	optical density
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PDCK	primary dog kidney cell
PDVI	Pediatric Dengue Vaccine Initiative
preM	premembrane
RC	replication complex
RDRP	RNA-dependent RNA polymerase
RNA	ribonucleic acid
SCID	Severe combined immunodeficiency
ssRNA	single stranded ribonucleic acid
TBEV	Tick borne encephalitis virus
TNF	tumour necrosis factor
U	units of enzyme activity
VP	vesicle packets
YF	Yellow fever

Chapter 1: Introduction

Dengue virus is the causative agent for dengue fever, dengue haemorrhagic fever and dengue shock syndrome. Dengue infection is considered to be one of the most important arthropod-borne disease causing up to 25 000 deaths annually. The disease is endemic in subtropical and tropical countries in most of which proper care of the patients and proper vector control are lacking (Gubler, 2002, Burke *et al.*, 2001). Thus, the need for a vaccine that is cheap and easy to administer is urgent.

This project aims as a proof-of-principle for *Lactococcus lactis* to be used as an effective dengue vaccine delivery vehicle through the oral or nasal route. *L. lactis* is a lactic bacterium whose GRAS (Generally Recognized As Safe) status represents an important advantage for its potential use as a live vehicle in humans. Moreover the use of lactic bacteria for vaccine delivery through the oral or nasal routes represents a very attractive means for vaccination in poor countries that can not afford parenteral injections. *L. lactis* has been previously shown to efficiently express heterologous proteins from various origins, and to trigger specific immune responses against the vaccine candidate (Steidler *et al*., 2000; Riberio *et al*., 2002; Xin *et al*., 2003 *et al*.,; Bermudez-Humaran *et al*., 2004; Pei *et al*., 2005; Perez *et al*., 2005; Zhang *et al*., 2005).

The dengue antigen E domain III has been selected for this project which had been shown to elicit protection in various vaccine delivery systems (Simmons *et al*, 1998; Zhang *et al*., 1988; Bray *et al*., 1989; Lai *et al*., 1990). This antigen has been

cloned and expressed into the cytoplasm of *L. lactis* and the recombinant strain has been administered to BALB/c and C57BL/6 mice via the nasal or the oral route. The colonization efficacy and the specific systemic antibody responses have then been analysed.

Chapter 2: SURVEY OF LITERATURE

2.1 Dengue virus

2.1.1 Classification

Dengue virus (DEN) is a member of the genus *flavivirius* of the *Flaviviridae* family. Flaviviruses are separated into groups by serology and genome sequence relatedness (Calisher *et al*., 1989; Blok *et al*., 1992). Other major viruses in this genus include Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), yellow fever virus (YFV) and West Nile virus (WNV). They are usually arthropod-borne and are transmitted via infected tick or mosquito vectors. These viruses are of major global concern as they cause significant morbidity and mortality worldwide (Monath and Heinz, 1996).

2.1.2 Structure of virions

Flaviviruses consist of spherical enveloped virions (diameter 40-60 nm) with host-derived lipid bilayer. The lipid envelope consists of 180 copies of 2 viral-derived type I membrane proteins, E (envelope) and M (membrane-like) (Kuhn *et al*., 2002). Dengue virus contains 7nm ring-shaped structures on the surface of its virus particles unlike most flaviviruses which do not contain regular surface projections (Smith *et al*.,1970). The viral RNA genome is associated with several copies of the basic capsid (C) protein (Chambers *et al*., 1990a) resulting in an electron-dense structure of approximately 30nm in diameter.

2.1.3 Organization of the dengue genome and translational process

The genome of flaviviruses is a positive single-stranded RNA of approximately 11kb (Chambers *et al*., 1990a). Its 5' terminus has a type 1 cap (m^7GpppAmp) followed by the conserved dinucleotide sequence AG and its 3' terminus consists of the conserved dinucleotide CU. The flaviviral RNA genome contains a large open reading frame of over 10,000 nucleotides encoding a single polyprotein precursor flanked by 5' and 3' untranslated regions. These regions contain conserved RNA elements had distinct conserved sequences are also found near the 5' and 3' terminus of mosquito-borne flaviviruses (Chambers *et al*., 1990a).

The polyprotein precursor is co-translationally and post-translationally processed by host proteases (such as furin) and viral serine protease (such as NS2B-3 protease) to produce ten mature viral proteins: pre-M (prM)/ membrane (M)- Envelope (E)-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3 (Chambers *et al*., 1990a). prM, M and E proteins constitute the structural proteins of the virus. Amongst these ten viral proteins, prM, E and NS1 are considered to elicit protective immunity as passive transfer of antibodies against each of these proteins had protected lethally challenged mice (Kaufman *et al*., 1987, Henchal *et al*., 1988, Kaufman *et al*.,1989,).

2.1.4 Proteins encoded by the viral RNA

2.1.4.1 Pre-M (prM) and Envelope (E) proteins

The prM and E proteins have been shown to be involved in various aspects of the viral infection including pathogenicity (Leitmeyer *et al*., 1999), viral attenuation (Blok *et al.,* 1992; Pryor *et al.,* 2001), cell fusion properties (Lee *et al*., 1997),

neurovirulence (Sanchez and Ruiz, 1996) and virus-induced cell apoptosis (Duarte dos Santos *et al.,* 2000).

The flaviviral envelope contains two structural glycoproteins, namely envelope E (MW 53-54 kDa) and membrane-like M (MW 8 kDa). However, the dengue virus envelope contains a mixture of pre-M (prM, MW 26 kDa) and M proteins with a predominance of prM proteins (Rice, 1996; Wang et al., 1999). Virion assembly occurs in association with rough ER membranes where the prM and E proteins associate with each other to form a stable heterodimer (Wengler and Wengler, 1989, Allison *et al.,* 1995b). This heterodimer is incorporated into immature virions during budding from the lumen (Mackenzie and Westaway, 2001). This association may be vital for the maintenance of E protein in a stable, fusion-inactive conformation before viral release (Konishi and Mason, 1993). It protects immature virions against inactivation during transport in acidic vesicles by stabilization of pH-sensitive epitopes on the E protein (Guirakhoo *et al.,* 1992; Heinz *et al.,* 1994; Allison *et al.,* 1995a). The immature virions are transported via the secretion pathway and, shortly before or coincident with their release, are converted to mature virions upon cleavage of prM protein to M proteins by cellular furin (Stadler *et al.,* 1997).

The flaviviral E protein is the major envelope protein of the virion (Rice, 1996) and is mostly glycosylated (Winkler *et al*., 1987; Chambers *et al*., 1990a). This protein is involved in receptor binding (Anderson *et al*.., 1992; Chen *et al*., 1996; Wang *et al*., 1999), membrane fusion (Schalich *et al.,* 1996; Rice,1996), virion assembly (Stiasny *et al.,* 2002) and is the primary target for neutralizing antibodies (Heinz, 1996).

The X-ray crystallographic structure of the E protein from TBEV and dengue-2 virus has been resolved (Rey *et al*., 1995, Modis *et al*., 2003). The ectodomain of the protein folds into three distinct domains (I-III). The Domain I is the central structure in which the other two domains flank with on either side. Domain II is the elongated dimerization domain with the putative fusion peptide involved in virus-mediated cell fusion (Rey *et al.,* 1995; Roehrig *et al.,* 1998; Allison *et al.,* 2001). At the interface of these two domains is contained an *N*-octyl-β-D-glucoside molecule. The flexibility of this interface might be vital for the conformational changes required during maturation and fusion (Modis *et al.,* 2003). The immunoglobulin-like domain III has been postulated to contain the receptor binding motifs (Crill *et al*., 2001) and is also an antigenic domain which is dependent on the integrity of a single disulphide bridge (Mandl *et al*., 1989).

2.1.4.1 NS1 Protein

Flaviviral NS1 is a 40-50 kDa detergent stable glycoprotein that exists as three discrete forms: membrane-associated, cell-surface associated and secreted form (Chambers *et al*., 1990a). The dimer is the major form of NS1 protein although a hexameric form of the secreted dengue virus type 1 NS1 protein was reported (Flamand *et al*., 1999). NS1 is secreted from infected mammalian cells but not from infected mosquito cells (Mason *et al*., 1989).

Although the functions of NS1 protein have yet to be fully elucidated, several lines of evidence have suggested that NS1 protein is involved in replication of viral RNA. Mutations in the glycosylation sites of NS1 have been shown to affect its

dimerization and subsequently impact virulence (Pryor *et al*., 1998). However, NS1 dimerization is not an absolute requirement for its function (Hall *et al.*, 1999). The NS1 protein has been shown to co-sediment with heavy membrane fractions containing RNA-dependent RNA polymerase (RDRP) activity from Kunjin virus-infected cells (Chu and Westaway, 1992). Using mutagenesis of NS1 protein, a temperature sensitive mutant of NS1 protein was found which blocked accumulation of viral RNA (Muylaert *et al.,* 1997). A yellow fever YF17D virus genome in which NS1 protein was deleted resulted in a defect in synthesis of minus-strand viral RNA compared to wild-type virus. This defect was complemented by supplying the NS1 protein in trans (Lindenbach and Rice, 1997). The immunogenicity depends on the structure and form of NS1 where soluble dimers are more immunogenic and give higher protection than monomers and membrane-associated NS1 (Falconar *et al.,* 1991).

Finally, using immunolocalisation techniques, dengue and Kunjin NS1 proteins have been shown to co-localize with NS3 protein, a component of the flaviviral replication complex (RC) and double stranded (ds) RNA in virus-induced membrane structures comprising vesicle packets (VP) of smooth membranes (Mackenzie and Young, 1996).

2.2 The dengue threat

With an annual estimate of 100 million cases of dengue fever, half a million cases of dengue haemorrhagic fever occurring in the world (Halstead, 1999) and a 30-fold increase of cases for the past 50 years, dengue ranks as the most important

mosquito borne viral disease in the world (Pinheiro, 1997). This emergence is closely tied to population growth, rapid urbanization, ineffective control of *Aedes aegypti* and modern transportation (Gubler, 2002). The dengue situation is exacerbated by the lack of specific treatment, vaccine and proper animal models. Various vaccine strategies are being investigated to develop dengue vaccine candidates, but so far none has been approved for human use yet (Halstead *et al*., 2002, Stephenson, 2005).

2.2.1 Dengue pathogenesis

Dengue virus consists of four serotypes and is the aetiological agent of dengue fever which may progress to dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). The main classical dengue fever features are biphasic fever which last for 2-7 days and rash. It is an acute febrile illness with other characteristics like abrupt onset of high fever, frontal headache, retro-orbital pain, myalgia, anorexia, abdominal discomfort, lymphoadenopathy and leucopenia. Hemorrhage and positive tourniquet test have also been reported in a few cases (Ahmed *et al*., 2001, Narayanan *et al.*, 2002). The disease usually subsides after an average of 5 days with the disappearance of the virus from the blood. Infection of one serotype would induce life-long immunity against homologous but not heterologous serotype of the virus (Sabin, 1952).

Dengue hemorrhagic fevers usually follow secondary dengue infections, although primary infections are still possible, especially in infants. This could be due to maternally acquired dengue antibodies (Halstead *et al*., 2002). Dengue hemorrhagic fever is distinguished from DF by its acute vascular permeability with

abnormalities in haemostasis. Its severity is divided into four grades for ease of management (Table 2.1). Grade III and IV are clinical definitions of dengue shock syndrome (DSS).

The clinical features are plasma leakage, bleeding tendency and hepatic alteration. Capillary leakage develops rapidly over a period of hours when the symptoms of classic DF resolve. Pleural effusion, ascites and haemoconcentration are indicative of such leakage (Bhamarapravati *et al*., 1967). This can quickly progress to shock if volumic loss is not remedied with proper fluid therapy. The hemorrhagic manifestations range from a positive tourniquet test to spontaneous bleeding from the gastrointestinal tract or any body orifice. Haemoconcentration (haematocrit increased by more than 20%) and marked thrombocytopenia (platelet count $<100 \times 10^9$/L) are two major characteristic features of DHF/DSS. Liver involvement in such infection would result in elevation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). As such three organ systems, hematological, vascular and hepatic, are involved in the pathological changes in DHF/DSS. Dysfunction of these systems would either directly or indirectly, cause the manifestations of DHF/DSS (Burke *et al*., 1988). Dengue viral infections leading to neurological complications have also been reported (Garcia-Rivera *et al*., 2002).

2.2.2 Hypotheses of dengue clinical features

The main hypothesis to explain the clinical features of DHF/DSS is the antibody-dependent enhancement (ADE) while other hypothesis being conceptualized as ADE could not explain the phenomena of DHF/DSS fully. Other hypothesis

Grading of Dengue Haemorrhagic Fever
Grade I: Fever accompanied by non-specific constituitional symptoms. The only haemorrhagic manifestation is a positive Hess test. Grade II: Spontaneous bleeding usually skin with or without bleeding from other orifices. This is in addition to manifestation of grade I. Grade III: Cirulatory failure (rapid weak pulse with pulse pressure < 20mm Hg) but systolic pressure still normal. Grade IV: Profound shock with unmeasurable blood pressure and or pulse.

Table 2.1: Grading of Dengue Haemorrhagic Fever. Adapted from WHO (1997).

includes a unifying hypothesis between ADE and T-cell activation and dengue viral virulence.

The concept of ADE of dengue viral replication in human mononuclear cells was formulated to explain the severe manifestations of DHF/DSS occurring in Thai children (Halstead *et al*., 1970). These children suffered from secondary dengue infection of a heterologous serotype. The ADE hypothesis postulates that the antibodies raised against one dengue serotype cannot neutralize but instead could enhance a secondary infection by another dengue serotype. The infectious complexes of virions and IgG antibodies would be internalized into monocytic cells via their Fcγ receptors, thereby increasing the number of infected monocytes. Subsequent lysis or immune clearance of such infected cells may lead to the release of vasoactive mediators and pro-coagulants (Rosen, 1986) (Fig 2.1). Sera obtained before infection from children who later developed DHF/DSS were also much more likely to demonstrate ADE *in vitro* (in human monocyte cells) than those who had only DF (Kliks *et al*., 1989). Babies less than 1 year old who acquired maternal anti-dengue antibodies are also susceptible to develop DHF/DSS following their first infection (Kilks *et al*., 1988). The association of DHF/DSS with secondary dengue virus infection is supported with a higher percentile of severe disease than primary infections. However, only 2-4% of such secondary infections progress to DHF/DSS (Guzman *et al*., 2002). Moreover, epidemiological studies in Peru, where over a period of 4 years (1993-1994), active surveillance for DF cases revealed that, in spite of secondary infection rates of up to 75%, no DHF cases have been detected (Watts *et al*., 1999). Therefore, ADE could not adequately explain the cases of DHF/DSS.

Neither does ADE explain the molecular mechanism of DHF/DSS clinical manifestations. It is not known how the increase of dengue virus infection by enhancing antibodies leads to DHF/DSS and its effects remain to be elucidated. The causal relationship between ADE and DHF/DSS remains unverified due to the lack of proper animal model although higher viral counts had been observed in secondary infected non-human primates (Bielefeldt-Ohmann, 1997).

Immunopathogenesis in DHF has been proposed by Kurane and Ennis which unifies ADE with T-cell activation (Kurane *et al.*, 1992; Rothman *et al*., 1999). Cross-reactive antibodies from the previous infection bind to virions without neutralization activity and enhance the entry of virus into monocytes. Thus, the number of viral infected monocytes increases. The level of T-cell activation is increased, due to the recognition of viral antigens via MHC class I and class II molecules by cross-reactive memory CD4 and CD8 T cells. These activated T cells produce pro-inflammatory cytokines such as IFN-γ, IL-2, TNFα and TNFβ, leading to the killing of the virus-infected monocytes. TNFα is also produced by activated monocytes due to viral infection and interaction with the T cells. The complement cascade is activated by the virus-antibody complexes (classical

pathway of activation) as well as by several cytokines to release C3a and C5a proteins which also affect directly vascular permeability. The synergistic effects of IFN-γ, TNFα and activated complement proteins trigger plasma leakage of endothelial cells in secondary dengue virus infection (Fig 2.2). However, not all DHF/DSS cases are secondary infections and no observable sequelae are usually found which is not easily

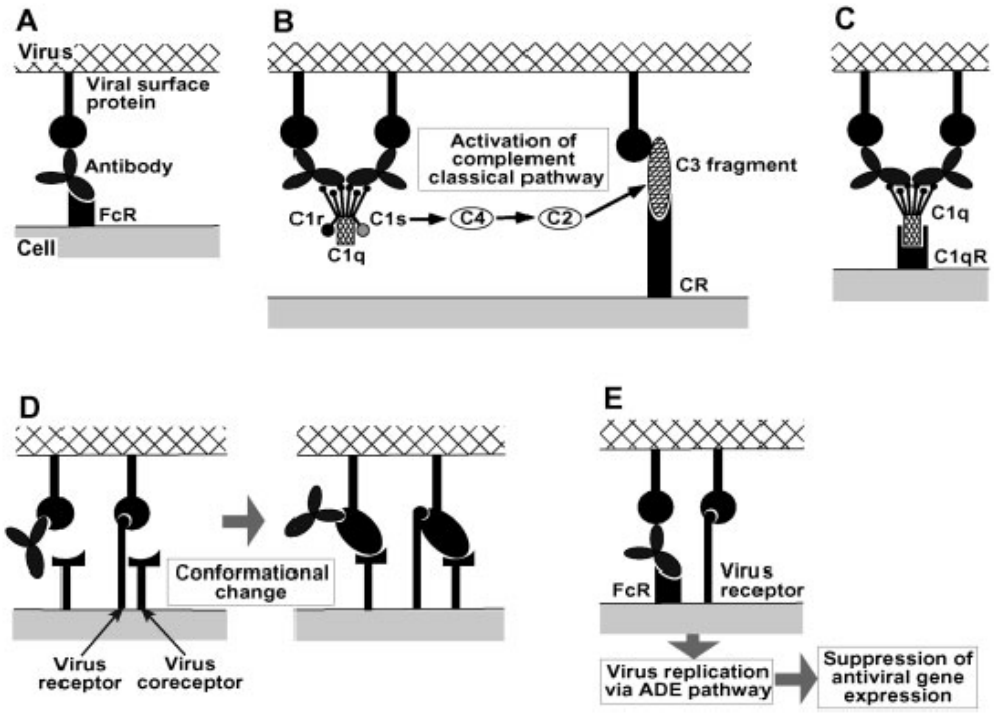


Figure 2.1. Proposed mechanism for ADE of viral infection

Interaction between antibody and FcR (A), complement C3 fragment and CR (B) or C1q and C1qR (C) promotes virus attachment to cells. Antibody bound to a receptor-binding site of the viral protein induces a conformational change, which facilitates membrane fusion (D). Viral replication via ADE entry suppresses cellular antiviral gene responses (E). (Adapted from Takada *et al*., 2003)

reconciled due to the known tissue destructive effects of inflammatory cytokines (Rothman *et al.* 1999).

Virus virulence is the capacity of a virus to produce disease in a host. This is an alternative hypothesis to that postulated by ADE. The differential manifestations of DF, DHF and DSS may be due to the variants of dengue virus with different degrees of virulence. The risk of DHF/DSS is higher in secondary infections with dengue serotype 2 compared to the other serotypes (Rico-Hesse *et. al*, 1998, 1997). Structural differences have also been observed among viral isolates from DF and DHF patients in four viral proteins such as prM, E, NS4b and NS5 (Leitmeyer *et al*., 1999). Of these four regions, E domain III has been shown to be one of the virulence determinants in mice-adapted dengue virus (Zulueta *et al*., 2006).

It was also reported that high dengue viremia titre is related with disease severity. Higher viral titers were observed in patients with DSS than those with DF in dengue-infected Thai children. Apparently, viral load is also a contributing factor in the development of DHF/DSS (Vaugh *et al*., 2000).

In 1953, Francis and co-workers proposed the doctrine of original antigenic sin to explain a phenomena they observed in influenza A infected individuals. These individuals showed a higher titer of antibodies against the original infecting virus when they were infected with a new or another subtype of influenza A virus (Francis, 1953) (Fig 2.3). Halstead *et al.*, (1983) suggested the existence of original antigenic sin due to observations in eight dengue infected Thai children. These children showed higher neutralizing antibody titers against the initial dengue serotype after they were infected with a different serotype in their secondary infection. The original antigenic

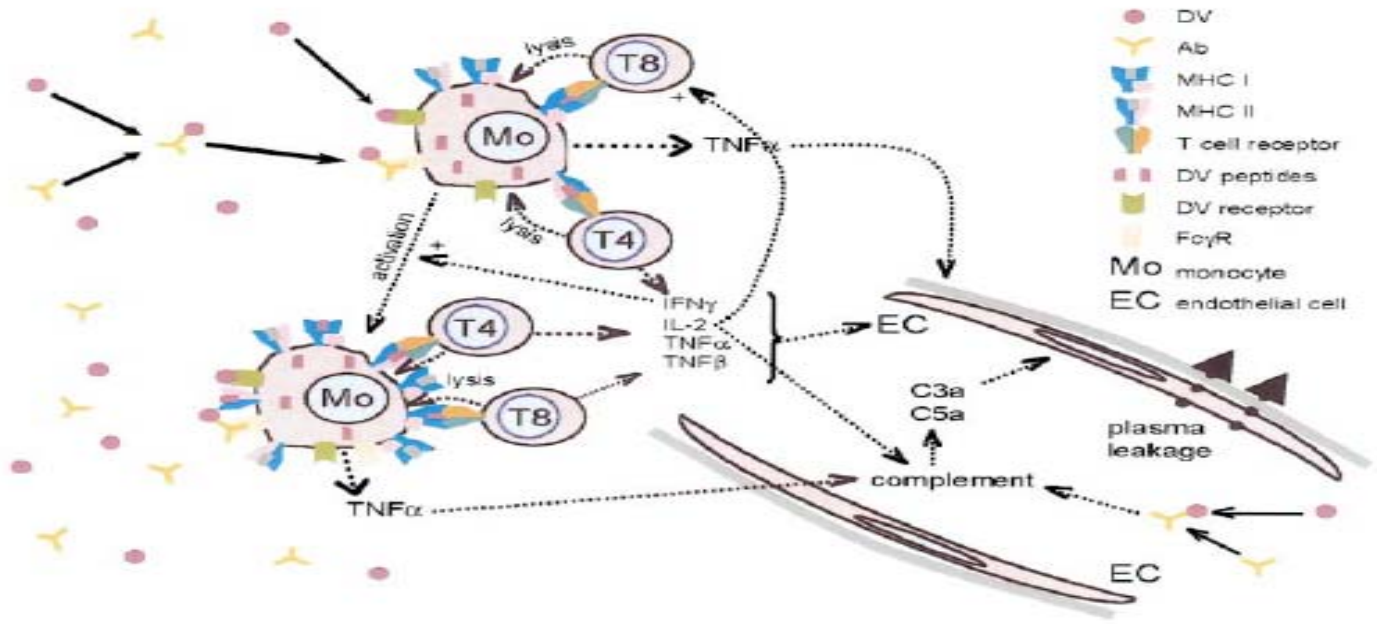


Fig 2.2. Immunopathogenesis of plasma leakage in DHF (Adapted from Rothman *et al.*, 1999)

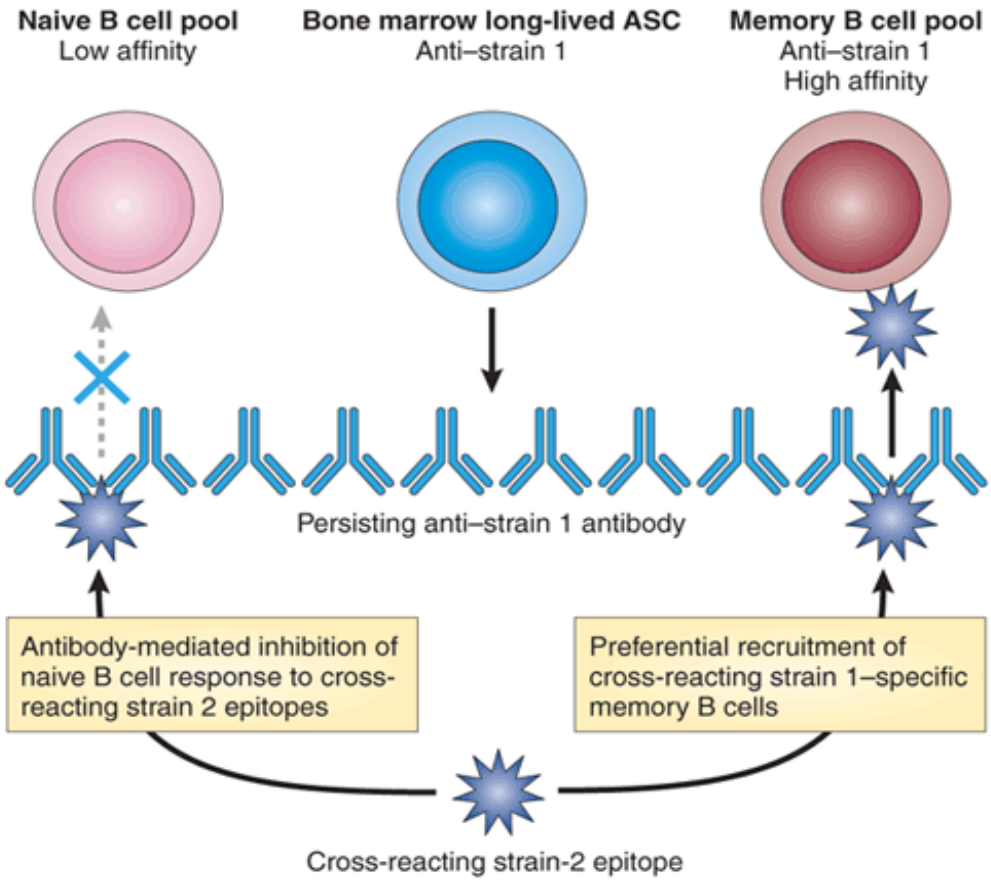


Figure 2.3. Phenomena of the original antigenic sin at the B cell level.
Exposure to a cross-reacting influenza strain (strain 2) is more likely to recruit memory B cells that have been induced against the original influenza strain (strain 1) rather than naïve B cells specific for strain 2. The latter are more likely to be inhibited by persisting anti-strain 1 antibodies. ASC: antibody secreting cells. (Adapted from Lambert *et al.*, 2005).

sin was also observed in dengue virus-specific CD8+ T-cell responses in Thai children (Mongkolsapaya *et al*., 2003). This process is where the host's immune system preferentially uses the memory response from the previous infection when a similar pathogen is encountered. However, this response might be suboptimal to a newly mounted immune response and prevents the mounting of a more suitable optimal response.

In these children, most of the dengue virus-specific T-cells were of low affinity to the infecting dengue serotype. They postulated that such T-cells were probably targeted against the initial dengue serotype and not the current infecting dengue serotype. The memory cells of the initial infection have a lower threshold for activation than naïve cells but are less effective in clearing the current infecting dengue serotype (Veiga-Fernandes, *et al*., 2000).Most of these T-cells were observed to be undergoing apoptosis and could contribute to the delay in viral elimination and increased immunopathology (Mongkolsapaya *et al*., 2003) .

2.2.3 Treatment of dengue fever and dengue hemorrhagic fever

Currently, there are no effective drugs against the dengue virus and the management of dengue infections is mainly supportive. Early detection, proper management and hospitalization could reduce the rate of case fatalities (WHO, 1997).

The only antipyretic drug used to control fever is paracetamol as other such drugs causes gastric irritation which might result in gastric bleeding. For fluid replacement therapy, the WHO recommends using crystalloid solutions although initial resuscitation using colloids (dextran 70 or 3% gelatin) restores patient's vitals

sooner than crystalloid solutions (Halliday *et al*., 1957; Wills, 2001). Antiviral drugs such as ribavirin and amantadine had been effective in inhibiting dengue virus replication *in vitro*. But these drugs have shown limited and contrasting results in dengue infected patients (Huang *et al*., 2001). Thus, there is an urgent need for dengue drug development.

2.3 Flavivirus vaccines

2.3.1 Licensed flavivirus vaccines

Today, only three flavivirus diseases have approved vaccines available. They are for yellow fever, Japanese encephalitis and tick-borne encephalitis. These three vaccines are produced by traditional methods of empirical attenuation of wild-type virus and formalin-inactivation to produce killed vaccines.

Yellow fever (YF) is a fatal haemorrhagic fever transmitted to humans by Aedes mosquitoes (Monath, 1999). The wild type Asibi strain of YF virus was attenuated by multiple passage in chick embryo tissue, resulting in the current 17D yellow fever vaccine (Stokes *et al.,* 1928). The 17D vaccine is the most successful flavivirus vaccine and had been administered to more than 400 million people, resulting in significant decrease in YF cases worldwide. A single dose of 17D vaccine could stimulate life long immunity and efficacy in 99% of the subjects (Monath, 1999). Most of the adverse effects were observed in infants before age restriction was instituted. After which, the 17D YF vaccine was considered safe to be used as a vector (ChimeriVax™) for engineering new vaccines for flavivirus, cancer (McAllister *et al*., 2000) and malaria (Bonaldo *et al*., 2002).

The most important cause of viral encephalitis in the Asia-Pacific region is the Japanese encephalitis virus (JEV) (Burke *et al*.,2001). The case-fatality rate ranges between 5 to 40% (Tsai, 2000). Currently, there are 3 vaccines against JEV used by the Asia-Pacific region. BIKEN (Osaka, Japan) produces one vaccine based on the wild-type Nakayama or Beijing-1 strains grown in adult mouse brains and China produces one vaccine based on the P3 strain grown in primary hamster kidney cells (WHO, 1998). These formalin-inactivated vaccines require two primary doses and a booster dose at 1 year, with subsequent booster every 3-4 years. The third vaccine against JEV is an empirically derived live attenuated vaccine designated SA14-14-2 and is licensed to be used in China only. The immunization schedule consists of two doses at 1 and 2 years of age, with immunization efficacy greater than 95% after the first dose (Tsai *et al.*, 1999). This vaccine is licensed to Glovax (Korea) for further development for the global market.

The two main subtypes of tick-borne encephalitis (TBE) are the Central European encephalitis and Russian spring-summer encephalitis. The Russian spring-summer strain is the more virulent strain causing most of the severe neurological problems, with a case-fatality rate of 20%. Both are antigenically related with up to 96% in amino acid homology for the E protein. They are able to induce cross-reactive and highly-protective immune responses (Burket *et al*., 2001). The vaccine used for TBE virus is a formalin-inactivated form of Austrian Central European encephalitis virus isolate grown in chick embryo cells by Baxter BioScience (Orth/Donau). Another vaccine is made from the European TBE virus by Behringwerke/Chiron

(Germany). Similar with all killed vaccines, multiple inoculation regimens are needed.

2.3.2 Dengue vaccines

According to Pediatric Dengue Vaccine Initiative (PDVI), the disease burden of dengue in South East Asia is 0.42 DALYs (disability adjusted life years) per 1000 population. Premature mortality accounts for 52% of this calculation and 48% to acute morbidity. Despite causing 6% of clinical cases, DHF represents 68% of the disease burden and 67% of the treatment costs (http://www.pdvi.org). Thus, governments consider the feasibility of a dengue vaccine to be more cost-effective.

The design of a safe dengue vaccine must address the problem of ADE due to pre-existing heterotypic dengue virus antibodies and the possible immune potentiation of the disease (Cardosa, 1998). This vaccine must also confer protection against all four serotypes and ideally should last throughout life (Trent *et al*., 1997). Advances in molecular biology and biotechnology, has led to many new strategies of vaccine design, such as chimerization of flaviviruses, specific mutagenesis of viral determinants of virulence. However, the traditional methods of formalin-inactivation and empirical attenuation are still being researched upon.

2.3.2.1 Inactivated vaccine

Formalin-inactivated dengue vaccine was able to raise antibody in both Swiss ICR and BALB/c mice to neutralize the dengue virus serotype 2 (Putnak *et al.,* 1996a). Despite being attempted for more than 60 years, the inactivated virus is

plagued by poor yield due to production methods. This was overcome by growing these viruses in either fetal rhesus lung diploid or Vero cell culture which makes such vaccine economically viable again (Putnal *et al*., 1996a; 1996b).

2.3.2.2 Live attenuated vaccine

The development of live attenuated tetravalent dengue vaccine is one of the strategies to obtain a vaccine against dengue viruses. Production of such vaccine is by using natural or chemical mutagens and serially passages them in cell cultures to attenuate the virus (Eckels *et al*., 1984). Currently, only two formulations of live-attenuated vaccine candidates are in various stages of clinical trials.

The first was developed by Mahidol University, Bangkok, Thailand and licensed to Aventis Pasteur, Lyon, France. The attenuation process is by serial passaging of Dengue serotype 1, 2 and 4 in primary dog kidney cell (PDKC) cultures while serotype 3 underwent the same process in primary African green monkey kidney cells (Bhamarapravati *et al*., 2000). Sero-conversion of the ten volunteers who received a single dose of tetravalent vaccine ranged from 30 to 70% for the four serotypes. Three of four volunteers showed a high titre of neutralizing antibody response against all four serotypes after the third dose (Chaturvedi *et al*., 1994). Despite the lack of serious adverse effects and high seroconversion rates, there were 16 breakthrough infections by dengue virus serotype 1, 2 and 4 resulting in mild illness (Halstead *et al*., 2005).

The second formulation of live attenuated dengue vaccine was developed at the Walter Reed Army Institute of Research (Silver Spring, MD, USA) and is

licensed to GlaxoSmithKline, Rixensart, Belgium. All four dengue viruses were serially passaged in PDKC culture and the final formulation produced in fetal lung cells of the rhesus monkey. After 2 doses, 80-90% of the adult volunteers developed neutralizing antibodies against all four serotypes of the virus (Halstead *et al*., 2002).

For both the Mahidol/Aventis and Walter Reed Army Institute of Research/GlaxoSmithKline attenuated virus, immnuo-interference by each of the attenuated virus hampers the raising of equivalent levels of immunity against each of the 4 serotypes. Optimization of dose-ratio and vaccination schedule must be carried out to improve immunity against all four serotypes of dengue virus (Sun *et al*., 2006).

2.3.2.3 Chimeric virus vaccine

The potential of creating chimeric flaviviruses was put forward by Rice *et al.* (1999) when they were able to produce a viable YF 17D (YF17D) virus strain from its full-length cDNA. Thus, chimeric flavivirus vaccine can be created by incorporating attenuated mutations or deletions of viral protein genes for the target antigen on the backbone of the YF17D vaccine strain (Rice *et al*., 1999). Using the YF17D vaccine virus, the ChimeriVax™ platform was created and was able to express the prM and E proteins of dengue in a chimeric yellow fever/dengue virus (ChimeriVax-DEN). The ChimeriVax-DEN was also able to protect immunized BALB/c mice from lethal dengue encephalitis (van der Most *et al*., 2000). Another ChimeriVax-DEN targeting 4 serotypes of dengue was constructed by Acambis, Inc., Cambridge, Massachusetts, USA (Guirakhoo *et al*., 2002). This chimeric virus vaccine showed poor infectivity in mosquitoes but was still protective in non human

primates (Guirakhoo *et al*., 2004). Initial phase I trials reported 100% neutralizing antibody seroconversion following the administration of the chimeric virus vaccine candidate in 48 adult volunteers (Halstead *et al*., 2005).

The four attenuated dengue virus vaccine strains developed by Mahidol University, Bangkok, Thailand also showed potential as a platform for chimeric virus vaccine candidates. Using the attenuated dengue virus PDK-53 strain of serotype 2 (CDC, USA) and Mahidol University were able to develop a vaccine targeting dengue serotype 1 (DEN-2/DEN-1). It was shown to be protective against dengue serotype 1 in outbred mice ICR when they were intracranially challenged (Huang *et al*., 2000). Chimeric virus against serotype 3 and 4 has also been synthesized using such the PDK-53 platform. Both mice and rhesus monkey have raised neutralizing antibodies following immunization with a tetravalent mixture of the above mentioned PDK-53 chimeric vaccines (Huang *et al.*, 2003). This vaccine candidate is licensed to Aventis Pasteur, Lyon, France and is undergoing further test in non human primates.

2.3.2.4 DNA vaccine

DNA vaccination is the direct injection of pure plasmid DNA to raise the immune response against the antigen expressed (Wolff *et al.,* 1990; Tang *et al*., 1992). This discovery had been directly translated to attempt to raise immune response against dengue 2 virus by Kochel *et al*. (1997). They have shown that BALB/c mice intradermally inoculated with prM and E expressing plasmid where able to develop antibodies against dengue serotype 2 virus. But only 60% were able to survive a lethal intracerebral challenge (Kochel *et al*., 1997). A similar experiment

was carried out targeting dengue serotype 1 in non human primates. The Aotus monkeys were observed to be protected from viraemia for up to six months and the highest antibody response was observed to be those co-injected with granulocyte macrophage colony stimulating factor (GM-CSF) (Raviprakash *et al*., 2001).

2.3.2.5 Recombinant subunit vaccine

With the rapid advancement in molecular biology techniques, the development of recombinant subunit vaccines for different viruses has become more prevalent. Majority of the focus in dengue virus had been on recombinant E and NS1 proteins, while some have tried other immunogenic proteins of dengue virus. These immunogens of dengue can be expressed in various expression systems (Table 2) such as bacterial (Srivastava *et al*., 1995; Sugrue *et al*., 1997; Simmons *et al*., 1998; Jaiswal *et al*., 2004), yeast (Sugrue *et al*., 1997; Bisht *et al*., 2001; 2002), mammalian (Konishi *et al*., 2002), viral (Jaiswal *et al*., 2003) and insect systems (Lai *et al*., 1989; Feighny *et al*., 1992; Men *et al*., 1991; Putnak *et al*., 1991; Eckels *et al*.,1994; Staropoli *et al*., 1996; 1997; Kelly *et al*., 2000). These systems have shown varying degrees of protection in mice with the recombinant proteins, and optimization is required especially with regards to the folding of proteins such as domain III of E protein (Jaiswal *et al*., 2004) (Table 2.2).

The flaviviral E protein is the only viral protein to elicit neutralizing antibodies against flaviviral infection and is sufficient to protect against infection (Brinton et al., 1998; Kliks *et al*., 1988). The neutralization activity of IgG2a subclass consistently showed the greatest ability to neutralize dengue viruses (Smuchny *et al*.,

Recombinant dengue vaccine				
Expression system	Antigen	Antibody response	Animal protected	Reference
Baculovirus/Sf9	NS1	NA	Mice	Lai *et al*., 1989
Baculovirus/Sf9	NS1	ab	No protection in mice	Feighny *et al*.,1992
Baculovirus/Sf9	E	Nab in mice	Partial in mice	Feighny *et al*.,1992
Baculovirus/Sf9	E(secreted monomer)	Nab in mice; NHP	Mice	Men *et al*., 1991
Baculovirus/Sf9	E truncated at the C terminus	Nab in mice	Mice	Putnak *et al*., 1991
Baculovirus/Sf9	E; C-M-E-NS1	Nab in mice	Mice	Eckels *et al*.,1994
Baculovirus/Sf9	E (fusion with poly-His)	Nab in mice	Mice	Staropoli *et al*., 1996,1997
Baculovirus/Sf9	E(intracellular particles)	Nab in mice	Mice	Kelly *et al*., 2000
Replication-defective adenovirus	E (ectodomain)	Nab in mice, T-cell response	Mice	Jaiswal *et al*., 2003
CHO cells	prM-E (extracellular particles)	Nab in mice	NA	Konishi *et al*., 2002
Yeast	C-prM-E (extracellular particles)	Nab in rabbits	NA	Sugrue *et al*., 1997
Yeast	E-HBsAg	Non-neutralizing ab in mice	NA	Bisht *et al*., 2001,2002
Escherichia coli	C terminal E-NS1	Nab in mice	Mice	Srivastava *et al*., 1995
E. coli	E	ab in rabbits	NA	Sugrue *et al*., 1997
E. coli	E domain III	Nab in mice,NHP	Mice	Simmons *et al*., 1998
E. coli	E domain III	NA	NA	Jaiswal *et al*., 2004

ab, antibody; C, structural C protein; CHO, Chinese hamster kidney; E, envelope glycoprotein; M, structural membrane protein; Nab,neutralizing antibody; NA, not known; NHP, nonhuman primate; prM, premembrane protein; sf9, Spodoptera frugiperda cells; NS, nonstructural protein

Table 2.2: Recombinant dengue vaccine. Adapted from Chaturvedi *et al*.,2005.

1995). With the elucidation of the 3D structure of E protein in TBE and dengue virus (Rey *et al*., 1998; Modis *et al*., 2003), mapping of neutralizing epitopes of flavivirus could be carried out. Using monoclonal antibodies, phage display techniques and synthetic peptides, the neutralizing epitopes were found to be located within the domain III (Crill *et al.*, 2001; Beasley *et al.,* 2002) and domain II (Roehrig *et al*., 1998) of E protein. With pan-dengue neutralizing monoclonal antibody 4E11, Thullier *et al* (2001) was able to identify the conserved regions amongst the four serotypes to be residue 310-314 of glycoprotein E. Other than B-cell epitopes, E protein also contains helper- and cytotoxic-T cell epitopes (Mathews *et al*., 1991; 1992; Rothman *et a*l., 1996). Thus, the E protein is able to elicit both cellular and humoral immune responses, making it a candidate for dengue vaccine development. Recent studies in non human primates have also shown that immunization with domain III of the E protein is protective against viral challenge (Hermmida *et al*., 2006).

The NS1 protein is highly immunogenic and can induce protection in experimental animals against flaviviral infections (Schlesinger et *al.*, 1987; Brinton *et al*., 1998). The anti-NS1 antibodies are non-neutralizing and are postulated to have complement-fixing activity, which enables them to kill infected cells (Iacon-Connors *et al*., 1996; Schlesinger *et al*., 1987; Falgout *et al.,* 1990). Both cell culture and *in vivo* experiments indicated that protection by anti-NS1 antibodies was provided via an Fc receptor-dependent mechanism which could only be stimulated by the Fc portion of IgG2a and IgG2b antibodies (Schlesinger *et al*., 1993; 1995). B-cell epitopes have been mapped on dengue virus NS1 to amino acids residues 25-33, 33-

50, 61-69, 111-121, 135-145, 173-177, 299-309 and 320-345 (Falconar *et al*., 1994; Garcia *et al*., 1997; Huang *et al*., 1999).T cell epitopes have also been found in the NS1 protein (Rothman *et al*., 1996). Antibodies against NS1 have been shown to be protective from viral infection (Mellado-Sanchez *et al*., 2005) and NS1 DNA vaccine can protect against intracerebral challenge in mice (Costa *et al*., 2006).

2.4 *Lactococcus lactis* - Classification

Lactococcus lactis is a Gram-positive non-sporulating, non-motile, facultative anaerobe bacterium. They are cocci in pairs and short chains, typically 0.5 - 1.5 μm in length. They belong to a group of bacteria known as the lactic acid bacteria (LAB) (Nester *et al*., 2004). Three different categories of LAB are widely studied in details for use as vaccine carriers, *Lactococcus lactis* MG1363, *Streptococcus gordonii* and members of *Lactobacillus* eg. *Lactobacillus plantarum* (Mercenier *et al*., 2001).

LAB are a phylogenetically heterogeneous group of Gram-positive cocci or bacilli. These bacteria are widely used traditionally in the food industry for production and preservation of fermented products. Thus, they are considered 'safe' with a GRAS (Generally Recognized As Safe) status (Pouwels *et al*., 1998). This is in contrast to other live vaccine delivery vehicle such as attenuated *Salmonella* and *Escherichia coli* (Norton *et al*., 1994). Unlike the rest of the LAB, *Lactococcus lactis* is non-colonizing and non-invasive (Iwaki *et al*., 1990; Dutot *et al*., 1993; Norton *et al*., 1994). The innocuous nature and the low intrinsic immunogenicity of *L. lactis* ensures its ability to be used repeatedly for vaccination (Norton *et al*., 1994).

2.5 *Lactococcus lactis* as a mucosal vaccine delivery vehicle

2.5.1 Mucosal vaccines

The main aim of mucosal vaccination is to prevent the initial colonization and infection by infectious pathogens. This is accomplished by the local mucosal immune response, including the production of IgA antibodies. IgA antibody promotes the entrapment of antigens or pathogens in the mucus, preventing direct contact of pathogens with the mucosal surface. This is known as 'immune exclusion' (Phalipon *et al*., 2002). Moreover, the antigen specific IgA antibody response elicited by mucosal immunization not only protects the immunized host from the pathogen but can also eliminate the healthy carrier condition. The healthy carrier condition may result in the subsequent transmission of infections to non-immunized individuals (De Magistris, 2006).

In the mucosal-associated lymphoid tissues (MALT), the lymphoid tissues are normally in aggregated and non-encapsulated forms. They are located in lamina propria and submucosal layer of the gastrointestinal, respiratory and genitourinary tracts (Roitt *et al*., 2001). The intestine contains lymphoid elements such as Peyer's Patches (PP), enriched with multifold (M) cells. The deep invagination of the basolateral plasma membrane of the M cells forms pockets that concentrate B cells, T cells, dendritic cells (DCs) and macrophages in the submucosal layer (Langridge, 2000). M cells are able to endocytose antigens from the mucosal surfaces, and transport them into the underlying follicle where the antigen presenting cells (APCs) are located (Roitt *et al*., 2001). Another mechanism of antigen sampling is by the

DCs which are able to migrate into spaces between epithelial cells and to the outer limit of the epithelium. Foreign antigens are directly sampled from the luminal compartment (Holt *et al*., 1990; Miller *et al*., 1992). The immunization of one mucosal site results in the secretion of the same specific IgA antibodies at other distal mucosal sites. This phenomenon is known as 'common mucosal immune system' (Mestecky, 1987). This is due to the expression and up regulation of 'homing receptors' and redundant counter-receptors which guide the activated lymphocytes back to the mucosa (Kunkel *et al*., 2003). For example, mucosa-activated IgA secreting B cells express CCR10 which is the receptor of the CCL28 chemokine. CCL28 is secreted by epithelial cells of the small and large intestines, salivary glands, tonsils, respiratory tract and lactating mammary glands. Thus, the B cells can be attracted to these tissues (Kunkel *et al*., 2003).

In addition to eliciting IgA antibody responses, mucosal vaccination is able to trigger systemic IgG responses against the antigen (Service, 1994) (Table 2.3). Induction of systemic IgG can be due to two mechanisms. Upon antigen uptake, the activated mucosal DCs are able to migrate to the lymph nodes and spleen where they present the processed antigen to naïve T cell and trigger the adaptive immunity (MacPherson *et al.,* 1999). The second mechanism involves a portion of the B cells being activated in the mucosa and expressing the peripheral homing receptors α4β1-integrin and leukocyte(L)-selectin allowing them to migrate to the regional lymph nodes (Kunkel *et al.,* 2003). Other than humoral immunity, cellular mediated response could be stimulated in the form of helper CD4+ T cells and CD8+ cytotoxic T lymphocytes which is important in combating intracellular pathogens like viruses.

Systemic IgG and local IgA response following mucosal immunization								
Immunogen	Route	Specific serum IgG	Responses of specific IgA antibodies*					Reference
			Small intestine	Large intestine	Cervix/ vagina	Salivary glands	Nasal cavity	
Cholera toxin B subunit	Nasal	+++++	ND	++	+++	-	+++	Kozlowski *et al*.,1997 Wassen *et al*., 1998
	Oral	+++	++++	++	+/-	+	-	Rudin *et al.* 1998
	Rectal	+++	ND	++++	-	+/-	ND	
	Vaginal	++	ND	+/-	+++	+/-	ND	
Live attenuated *Salmonella typhi* Ty21a	Oral	++	+++	-	+/-	+/-	-	Kantele *et al*.,1998 Forrest *et al*.,1990
	Rectal	+	+/-	++	-	-	+/-	
Poliovirus vaccine	Oral	++++	+++	++	-	ND	+	Ogra *et al.*,1969,1973
	Colonic	++++	ND	+++	ND	ND	-	
	Vaginal	-	ND	ND	+++	ND	-	
*Responses are based on geometric mean post-vaccination increases in specific antibody corresponding to: +++++,>50-fold; ++++,25-49.9 fold; +++, 10-24-fold; ++, 5-9.9-fold; +,2.5-4.9-fold; +/-,.2.5-fold in a minority of vaccine recipients;-,<2.5-fold in all vaccine recipients. ND, not determined								

Table 2.3: Systemic IgG and local IgA response following mucosal immunization. Adapted from Neutra *et al*.,2006

Thus, mucosal vaccines can activate both arms of the adaptive immune system (De Magistris, 2006).

The administration of mucosal vaccines provides some practical advantage like being non-invasive and needle-free. This would ensure compliance due to ease of distribution and administration without the need for trained personnel. It can also avoid blood transmissible infections due to needle re-use or needle stick injury (Levine, 2003). Due to limited understanding of mucosal immunity, complex measurement of mucosal response to vaccine dose ratio, there are only a few mucosal vaccines approved for human use. The approved vaccines include vaccines against poliovirus (Modlin, 2004), *Salmonella typhi* (Levine, 2000), *Vibrio cholera* (Levine, 2000), rotavirus (Kapikian , 1996) and influenza virus (Belshe, 1998).

2.5.2 *Lactococcus lactis* as antigen delivery vehicle

Formerly known as *Streptococcus lactis*, *L. lactis* has been widely studied as an antigenic or therapeutic protein delivery vehicle to the mucosal surface. Coupled with a GRAS status, this vehicle could potentially be used to elicit specific mucosal and systemic immune responses (Wells *et al*., 1993). Unlike other LAB, *L. lactis* does not colonise and has a low innate immunogenicity enabling its repeated use for booster effect without tolerance (Norton *et al*., 1994). The low proteolytic activity against self-produced heterologous protein makes it analogous to inert microparticles or antigen-loaded liposomes. Another advantage *L. lactis* has over another widely studied LAB - *Lactobacillus* sp., is its ease to genetic manipulation (Wells *et al*., 1993).

Expression systems were designed to direct synthesized proteins to the cytoplasm, membrane anchored (with a LPXTG motif) or secreted (using signal peptide of Usp45) out of the cell (Reveneau *et al*., 2002; Raha *et al*., 2005). Various inducible (nisin-inducible) and constitutive lactococcal promoters have also been used to increase the yield of the heterologous protein produced (Wells *et al.,* 1995). In order to circumvent the need for use for antibiotics the two system using threonine- and pyridmidine-auxotrophs *L. lactis* were also introduced (Sorensen *et al*., 2000; Glenting *et al.,* 2002). *L. lactis* was also reported to be an efficient vehicle for tetanus toxin fragment C via both nasal and oral routes of inoculation. The nasal route was observed to be more efficient in protection against lethal challenge against tetanus toxin (Norton *et al*., 1995). To date, many studies have described the use of *L. lactis* as a vehicle to deliver several antigens of pathogenic organisms such as SARS-coronavirus (Pei *et al*., 2005), Rotavirus vp7 antigen (Perez *et al*., 2005), human papillomavirus type 16 E7 antigen (Bermudez-Humaran *et al*., 2004), Human immunodeficiency virus Env (Xin *et al*., 2003), Enterovirus 71 VP1 (Raha *et al*., 2005), *Helicobacter pylori* urease subunit B (Lee *et al.,* 2001), *Brucella abortus* L7/L12 antigen (Riberio *et al*., 2002) and malarial MSP-1 (Zhang *et al*., 2005). Therapeutic proteins such as interleukin-10 have been used to treat murine colitis (Steidler *et al*., 2000) or in combination with antigens (Steidler *et al*., 1998). Thus, *L. lactis* represents a suitable and promising mucosal vaccine delivery vehicle.

2.5.3 LAB as immunomodulators

LAB strains have been shown to be able to interact with the host immune system. The main immune response induced by oral administration of LAB is hyporesponsiveness which is also known as oral tolerance. This tolerance is due to the induction of cytokines such as IL-10 or transforming growth factor β (TGF β) or by clonal deletion (Elson *et al*., 1996). Such tolerance is usually induced by T-cell dependent antigens with the presence of $CD8^+$ T cells required for this process (Mowatt, 1987; Challacombe *et al*.,1980). Dosage determines the ability of LAB to result in tolerance.

LAB have also been shown to potentiate some immune responses. *L. plantarum* has been found to induce a delayed type hypersensitivity when administered intraperitoneally (Bloksma *et al*., 1979). Intraperitoneally administered *Lactobacillus casei* was demonstrated to increase the phagocytic activity of macrophages and monocytes (Saito *et al*, 1983; Kato *et al*., 1983). This was also observed in mice orally administered with *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* and *Streptococcus thermophilus* (Perdigon *et al*., 1986). But unlike *Propionibacterium acnes* and *Mycobacterium bovis, Bacillus Calmette-Guerin* (BCG), there was no hepatomegaly or splenomegaly which is common side effects of these immunomodulators (Yasutake *et al*., 1984). Kupffer cells, the immune cells of spleen and lungs and peritoneal macrophages were activated by administration of *Lactobacillus casei* (Hashimoto *et al*., 1985). Similarly, *L. lactis* has been shown to increase the number of immune cells responsible for inflammatory response such as macrophages, neutrophils and eosinophil (Perdigon *et al*., 2001). The authors

postulated that *L. latis* interacts with the epithelial cells of the small intestine or the follicle associated epithelium in Peyer's patches.

2.6 Animal models

2.6.1 Mouse models for dengue virus

The Dengue virus is an intriguing virus and the immune mechanisms involved in the pathogenesis of DHF/DSS remains an enigma. Despite the dengue-infected non-human primates result in viremia, they do no become ill like humans (Halstead *et al*., 1971; Marchette ,*et al*., 1972; Schiavetta *et al.,* 2003). With the limited use of non-human primates due to ethical and financial reasons, the development of small animal models has become necessary for vaccine and antiviral studies (Atrasheuskaya *et al*., 2003; Shresta *et al*., 2004). An overview of some of the murine models such as inbred, knockout and humanized SCID strains for dengue virus study is presented below.

2.6.1.1 Inbred mouse strains

The inbred strains used as dengue murine models are BALB/c, C57BL/6 and A/J mice. BALB/c mice peritoneally infected with Dengue virus type 2 were observed to have significant hepatic injuries and elevated alanine aminotransferase and aspartate aminotransferase. These observations mimic the liver alterations in dengue infected humans (Paes *et al*., 2005). Atrasheukay and co-workers (2003) infected 4-weeks old BALB/c mice with mouse brain-adapted Dengue virus which develop severe anaemia, thrombocytopenia, non-overt haemorrhage and shock. They

were also able to reduce mortality in fatally infected mice with anti-TNF-α antibodies (Atrasheukay *et al*., 2003).

A/J mice inoculated intravenously with non-adapted dengue virus presented a transient thrombocytopenia and produced anti-platelet antibodies. These antibodies were postulated to contribute to DHF in human patients. Thus, the A/J mice are valuable model for immunopathogenicity study of dengue infection. The BALB/c or C57BL/6 mice were less sensitive to the strain of virus inoculated (Huang *et al*, 2000a; 2000b). The higher susceptibility of A/J mice could be due to a lower lymphoid, granuloid, monocytoid and NK cell count as compared to C57BL/6 mice (Miller *et al.*, 1998; Whyte *et al*., 1998). However, dengue virus was not able to replicate to detectable levels in blood serum of any of those inbred strains.

2.6.1.2 Knockout strains

Recently, the AG129 mouse strain was studied by several groups for its use as a potential dengue virus animal model. This strain lacks both α/β-interferon and γ-interferon receptor genes (Johnson *et al*., 1999; Shresta *et al*., 2004; 2006). Unlike inbred strains, dengue infected AG129 showed detectable virus titres in the serum, brain and spleen (Johnson *et al*., 1999). Shresta and co-workers (2006) demonstrated that infected AG129 mice had increased vascular permeability. This observation is a significant hallmark of DHF/DSS in humans. Paralysis due to neuronal abnormalities was observed in infected mice, which has been described in rare cases of DHF/DSS in humans (Solomon *et al*., 2000).

2.6.1.3 Humanized SCID strains

Severe combined immunodeficiency mice grafted with human cells are susceptible to dengue viral infection as the virus is able to replicate more efficiently. The main cell targets for viral infection and replication are DC, macrophages and hepatic cells (Kwan *et al*., 2005; Wu *et al*., 2000). Thus, reconstituting such cells in SCID mice would enable initial replication which results in higher viraemia. The two basic models were the human erythroleukemia cell line (K562) and human hepatocarcinoma cell line (HepG2) engrafted SCID mice (An *et al*., 1999; Lin *et al.*, 1998). These engrafted cells have been shown to be permissive for infection and replication of dengue virus (Knowles *et al*., 1984). Dengue clinical symptoms like thrombocytopenia, prolong thromboplastin time and increased hematocrit, were observed in paralyzed dengue-2 infected HepG2-SCID mice but were not related to the immune responses to viral infection (An *et al*., 1999). K562-SCID mice inoculated with dengue type 2 developed paralysis and die (Lin *et al.*, 1998). However, the lack of an immunocompetent background makes these two models unsuitable for vaccine and antiviral studies.

In a recent report, Bente and co workers were able to engraft human cord blood haematopoietic progenitor cell (CD34+ cells) in the immunodeficient environment of non-obese diabetic (NOD)-SCID mice. With the ability to mount a primary and secondary immune response similar to humans, the humanized NOD-SCID mice were able to develop clinical signs of DF as in humans when infected by virus-infected mosquitoes (Bente *et al*., 2005).

2.6.2 Mouse models for the study of *Lactococcus lactis* as vaccine vehicle

In the immunity against infectious pathogens, Th1 cells are involved in eradicating intracellular micro-organisms like viruses, while Th2 cells protects the host from extracellular pathogens such as helminthes. Thus, Th1-prone C57BL/6 (H-2^b) and Th2-prone BALB/c (H-2^d) mice are widely used for vaccine studies (Gorham *et al.*, 1996; Reiner *et al*., 1995). It is also observed that for protection experiments BALB/c and C57BL/6 are better to be used in parallel (Zhang *et al*., 2005).

Enteric anti-lactococcal secretory IgA antibodies have been detected in control, non-infected mice with BALB/c showing a higher titer than C57BL/6, indicating that these animals either have been exposed naturally to *L. lactis* or produce antibodies against other LAB which cross-react with *L. lactis* (Norton *et.al*., 1994). Moreover, an increase in anti-lactococcal class switching from IgM to IgG as the mice get older might be due to the continual exposure to environmental *L. lactis* or cross reactive LAB. In this study, *L. lactis* was also reported to be a suitable vaccine vehicle with optimal dosage of 1 x 10^8 bacteria for oral inoculation in eliciting the optimal amount of anti-lactococcal antibodies. But any dosage below or above the optimal dose would result in low amounts of anti-lactococcal antibodies due to under dosing and immune tolerance respectively (Norton *et al*., 1994). In conclusion, BALB/c or C57BL/6 mice have been widely used for studies using *L. lactis* as vaccine vehicle depending on the type of pathogen targeted for protection (Pontes *et al*., 2003; Reveneau *et al*., 2002; Perez *et al*., 2005; Bermudez-Humaran *et al*., 2004).

Chapter 3: Materials and Methods

3.1 Cell culture

BHK-21 (baby hamster kidney ATCC number CCL-10) cells were grown and maintained in 75 cm^2 tissue culture flasks in RPMI 1640 (Gibco, Boston, MA, USA) supplemented with 10% fetal calf serum (FCS), 2% HEPES and 1.5% sodium bicarbonate.

When the cells were confluent, usually within 3-4 days, the cell monolayer was rinsed with 8 ml of PBS to remove the dead cells, and dislodged by the addition of 1ml of pre-heated (37^oC) 1 X trypsin/versene solution (Appendix 1.1.3) . 3ml of growth medium was added to dilute the trypsinized cells and split in a ratio of 1:4.

3.2 Preparation of Dengue 2 Virus Stock

BHK-21 monolayers were inoculated with 1ml of dengue virus type 2, New Guinea C (DEN2 NGC) strain which contains approximately 10^5 PFU. Virus adsorption was carried out by incubating the flasks at 37 ^oC for 1 hour with rocking at 15 min intervals. Following adsorption, 12 ml of complete growth medium (as described above) were added and the flasks were incubated at 37 ^oC for 10-14 days. The culture supernatant was then harvested and centrifuged at 10,000 g for 20 min to eliminate cellular debris. The resulting supernatant was aliquoted and stored at -80 ^oC, as the virus stock for this project.

3.3 Viral Quantitaion Using Plaque Assay

3.3.1 Determining of viable cell with hemocytometer

Trypan blue is one of several stains used for dye exclusion procedure for viable cell counting. This method is based on the principle that live cells do not take up certain dyes whereas dead cells do and appear blue under the microscope.

Equal volumes of the trypsinized cell suspension and 0.4% trypan blue solution (Sigma) were mixed together. This mixture was allowed to stand for 5 min at room temperature, then loaded into the chambers of the haemocytometer and observed under the microscope. Starting with one chamber of the haemocytometer, all the viable cells were counted in the 1 mm center square and the four 1 mm corner squares. The cells on top and those that touched the middle line of the perimeter of each square were counted while those touching the middle line at the bottom and right sides are not. The procedure was repeated for the other chamber. Each square of the haemocytometer with the cover slip in place represented a total volume of 0.1 mm^3 or 10^{-4} ml. Thus, the subsequent cell concentration per ml is determined as follows:

Viable cells per ml = the average count per square x *dilution factor of 2(dilution factor due to addition of Trypan blue) x* 10^4

3.3.2 Plaque assay

Two days prior plaque assay, BHK cells were seeded onto 24-well plates (Nunc, Roskilde, Denmark) at 2.5 x 10^5 cells per ml. Ten-fold serial dilutions of the virus (10^{-1} to 10^{-6}) were performed in RPMI 1640 culture medium. 100µl of undiluted and diluted virus suspensions were then added to each well and viral adsorption was carried out at 37^{o}C,

5% CO_2 for 1.5 hrs. Triplicates were done for each dilution. 1 ml of 1% (w/v) carboxymethyl cellulose (Appendix 1.2.1) in RPMI supplemented with 2% FCS (Appendix 1.1.1) was then added to each well and the cells were incubated at 37 ^{o}C, 5% CO_2 for 6 days. Visualization of plaques was achieved by fixing the cells with 20% formaldehyde solution followed by staining with approximately 200 µl of 0.5% crystal violet dissolved in 37% formaldehyde (Appendix 1.2.3) to each well. After thorough rinsing with water, the plates were dried and the plaques were scored.

3.4 Plaque reduction neutralization test (PRNT)

The presence of neutralizing antibodies in the mouse sera was determined by PRNT. Each of the mouse sera was heated at 56 ^{o}C for 30 min to inactivate complement. 2-fold serial dilutions of the sera (1:10 to 1: 320) were prepared in RPMI 1640 in 24-well plates. Each dilution was incubated with an equal volume containing 30PFU of dengue virus NGC strain. The virus-serum mixture was incubated at 37 ^{o}C for 1 hr with rocking every 15 min. Each mix (100µl) was transferred onto BHK monolayers grown in 24-well plates, and incubated at 37 ^{o}C for 1.5 hrs. The mix was decanted and subsequently processed according to the plaque assay described in section 3.3.2. The percentage of plaque reduction was calculated relative to the virus control without serum. The envelope-specific monoclonal antibody 3H5 (#8702, Chemicon International, USA) reactive against the NGC strain was used as the positive control at a concentration of 5 µg per well.

3.5 Bacterial strains and cultures

3.5.1 Bacterial strains

The *L. lactis* strains and plasmids used in this study are listed in the following table 3.1.

Strain	**Relevant features**	**Antibiotic resistance**	**Source/Reference**
Lactococcus lactis MG1363	Derivative of *L. lactis* subspecies cremoris	-	Pasteur Institute of Lille (Dr C. Grangette)
LLWE	*Lactococcus lactis* MG1363 transformed with pMG36E	Erythromycin	Lin W. (unpublished)
LLWE-EDIII	*Lactococcus lactis* MG1363 transformed with pMG36E-EDIII	Erythromycin	Lin W. (unpublished)
Plasmid	**Relevant features**	**Antibiotic resistance**	**Source/Reference**
pMGE-EDIII	pMG36E derivative with *SacI-PstI* E domain III fragment	Erthromycin	Lin W. (unpublished)

Table 3.1: *L. lactis* strains and plasmids

3.5.2 Media and growth conditions

All *L. lactis* strains were grown at 30°C overnight in fresh Difco™ M17 broth containing 0.5% glucose (GM17) without shaking, or on GM17 agar. 10 μg/ml of erythromycin was added to maintain antibiotic-resistant strains.

3.6 Immunization and persistence studies in mice

3.6.1 Immunization studies

3.6.1.1 Mouse strains

Two different strains of mice were used for both oral and nasal immunization studies, namely BALB/c and C57BL/6. They were purchased from Biological Resource Centre, Biopolis, Singapore. Groups of four to six 5-6 week old female mice were immunized.

3.6.1.2 Nasal immunization

Either recombinant *L. lactis* pMG36e/EDIII (LLWE-EDIII) or *L. lactis* pMG36E (LLWE) were cultured at 30^{o}C for 18 hours and harvested by centrifugation at 3,000 g for 10 mins at 4^{o}C. The cell pellets were washed three times with cold PBS, and then re-suspended in PBS to obtain 10^{8} CFU in 20µl (10^{10} CFU/ml) for each mice. BALB/c mice were anaesthesized using the anaesthetic cocktail (6% valium+ 10% atropine+ 20% ketamine) as described previously (Alonso *et al*, 2001) and C57BL/6 mice were anaesthesized using the same cocktail but with 3% valium instead of 6%. The sedated animals were inoculated with either LLWE-EDIII or LLWE suspensions using a micropipette and dropping 10 µl into each nostrils. This procedure was repeated at 14, 28, 70 and 84 days after the first administration (Fig 3.1A). This protocol was adopted from Mannam *et al.* (2004) with modifications for the third and forth boost.

3.6.1.3 Oral administration

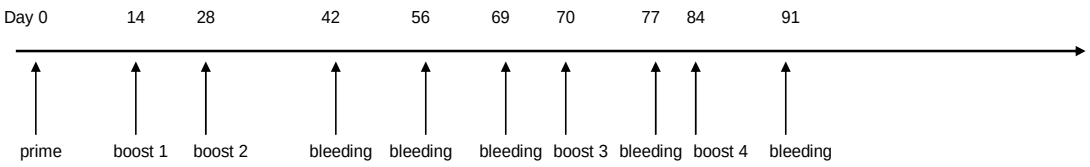
Non-sedated animals were inoculated with either recombinant LLWE-EDIII or LLWE harvested and re-suspended in PBS supplemented with 0.1% glucose at a concentration of $5x10^{10}$ CFU/ml. Water was restricted an hour before oral inoculation. For each mouse, 200µl of the bacterial suspensions were administred via intragastric gavage (Popper and Sons Inc, New York, USA). The bacteria suspensions were given on 3 consecutive days (0, 1, 2 days). A booster immunization was given at 14, 15 and 16 days, a second booster was given at 28, 29 and 30 days, a third booster was given at 70, 71 and 72 days and a forth booster was given at 84, 85 and 86 days (Fig 3.1B). This protocol was adopted from Lee *et al.* (2001) with modifications for the third and forth boost.

For the positive control, mice were injected intraperitoneally (ip) with 10^5 PFU of heat inactivated DEN 2 NGC. The inactivated virus was mixed with an equal volume of CFA (complete Freund's adjuvant) (Sigma-Aldrich, Inc. St Louis, Missouri, USA) for the priming dose, or with IFA (Incomplete Freund's adjuvant) for the booster dose, and 100µl of the mixture was injected.

3.6.1.4 Collection of sera

Blood was collected from sedated mice by retro-orbital puncture on day 42, 56, 69, 77 and 91 for both nasal and orally inoculated mice. Blood was allowed to clot overnight at 4 ^oC before centrifuging the specimens at 800 rpm for 10 min 4 ^oC. The serum was then transferred to a fresh collection tube and stored at -20^oC until use.

A



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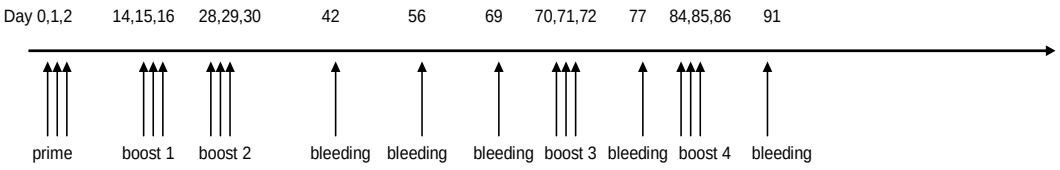


Figure 3.1: Nasal (A) and oral (B) immunization schedule and bleeding

3.6.1.5 ELISA

The titres of anti-EDIII and anti-lactococcal antibodies were measured by ELISA using purified envelope domain III protein (EDIII) and whole cell *Lactococcus lactis* as the coating antigen respectively. A culture of *Lactococcus lactis* was washed as stated in 3.6.1.1, resuspended in ice-cold PBS and sonicated five times for 45 sec each with intervals of 30 sec. The preparation was kept on ice during the sonication. Total protein concentration was estimated using NanoDrop® ND-1000 spectrophotometer (NanoDrop, Wilmington,DE, USA).

Purified EDIII or sonicated *L. lactis* were coated onto the 96-well plates (Corning costar, NY, USA) at 5µg/ml and 10µg/ml, respectively, in 0.1M $NaHCO_3$ buffer, pH 9.6 (50µl). The plate was incubated overnight at 4^{o}C. The plate was then washed three times with wash buffer solution (0.1% Tween 20 in PBS), and blocked with 2% BSA, 0.1% Tween 20 in PBS at 37 ^{o}C for 1 hr. After removal of the blocking solution, 50µl of the sera (1 in 50 dilutions for testing of EDIII and 1 in 500 dilutions for testing of *L. lactis*) were added. The plate was incubated at 37 ^{o}C for 1 hr. After three washings, the plate was treated with 50µl of anti-mouse IgG (H+L) HRP conjugate (Bio-rad, USA) (1:5000 dilutions) and incubated at 37 ^{o}C for 1 hr. The plate was washed and a yellow colour was generated after the addition of 50 µl of Sigma*Fast*™ O-phenylenediamine dihydrochloride substrate (stock solution; 1 tablet per 20ml of dH_2O, Sigma-Aldrich, Inc. St Louis, Missouri, USA), followed by incubation in the dark at room temperature for 1 hr. The reaction was stopped using 75 µl of 1M H_2SO_4 and measured using ELISA plate reader (Bio-rad model 680 microplate reader, USA) with the absorbance at OD_{490} were recorded using the microplate manager 5.2 software (Bio-rad, USA).

3.6.2. Persistence studies

3.6.2.1 Mouse strains

Mice were purchased from Biological Resource Centre, Biopolis, Singapore. Two strains of mice were used for both lung (nasal route) and intestine (oral route) colonization studies, namely BALB/c and C57BL/6. Four 5-6 week old female mice were used for each time point.

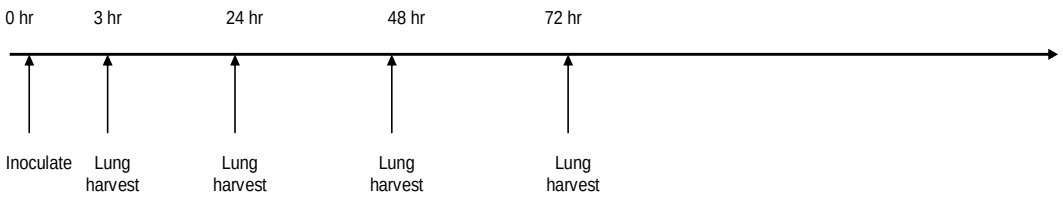
3.6.2.2 *L. lactis* persistence in the lungs

10^8CFU of bacterial suspension were intranasally inoculated into each mouse as described in section 3.6.1.2. At 3 hr, 24 hr, 48 hr and 72 hr post-inoculation, 4 mice per group were sacrificed, the lungs were harvested in 5ml sterile PBS and homogenized (Fig 3.2A). Appropriate dilutions of the suspension were then plated on GM17 agar containing 10 μg/ml erythromycin.

3.6.2.3 *L.lactis* persistence in the intestines

10^{10}CFU of recombinant *L. lactis* were orally administered to the animals as described in section 3.6.1.3. The fecal samples were collected at 3 hr, 24 hr, 48 hr and

A



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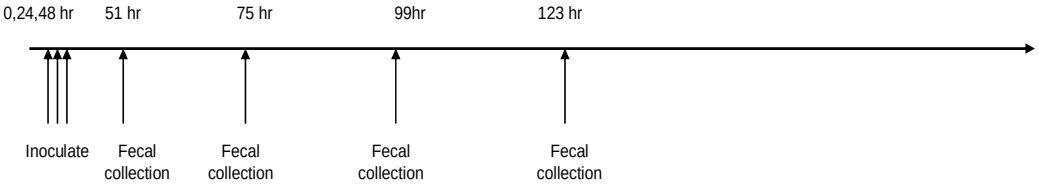


Figure 3.2: Persistence study schedule for nasal (A) and oral (B) inoculation

72 hr post-gavage, and pooled for each group (Fig 3.2B). These pellets were weighted and re-suspended in sterile PBS at a final concentration of 100mg/ml. After extensive homogenization, the suspension was allowed to settle for two hours. Appropriate dilutions of the suspensions were plated onto GM17 agar with 10 μg/ml of erythromycin.

3.7 Statistical analysis

Data was analyzed using Student's *t*-test for measurement of antibodies in the serum of the mice. Results were considered statistically significant when $P < 0.05$ and highly significant when $P < 0.01$.

Chapter 4: Results

4.1 Persistence studies of *L. lactis* in BALB/c and C57BL/6 mouse strains

In order to determine the ability of the EDIII-producing *L. lactis* strain (LLWE-EDIII) to persist in the lung and into the gastro-intestinal tract, persistence studies were carried out in both BALB/c and C57BL/6 mouse strains after nasal and oral single administration, respectively.

For the lung persistence studies, the mice received 10^8 CFU via the nasal route and, at different time points (3, 24, 48 and 72 hours), the lungs were collected, homogenized and plated as described in the Materials & Methods section. The results obtained showed that the bacteria were totally cleared from BALB/c mice 48 hours post-administration (Fig 4.1 A) whereas a significant amount (2 logs) of bacteria were still detected in C57BL/6 mice at the same time (Fig. 4.1B). These results indicate that the *L. lactis* LLWE-EDIII strain persists longer in the lungs of C57BL/6 than in BALB/c mice upon nasal administration.

The persistence ability of *L. lactis* in the gastro-intestinal tract was assessed by counting bacteria recovered in the feces of the animals. Feces homogenates were plated onto medium containing erythromycin in order to select for the EDIII-producing *L. lactis* bacteria (LLWE-EDIII) from the complex intestinal microbial flora. However, before oral administration of the LLWE-EDIII bacteria, a basal level (approximately 10^3 CFU/ml) of erythromycin-resistant bacteria was observed in both mouse strains (Fig 4.2A and Fig 4.2B).

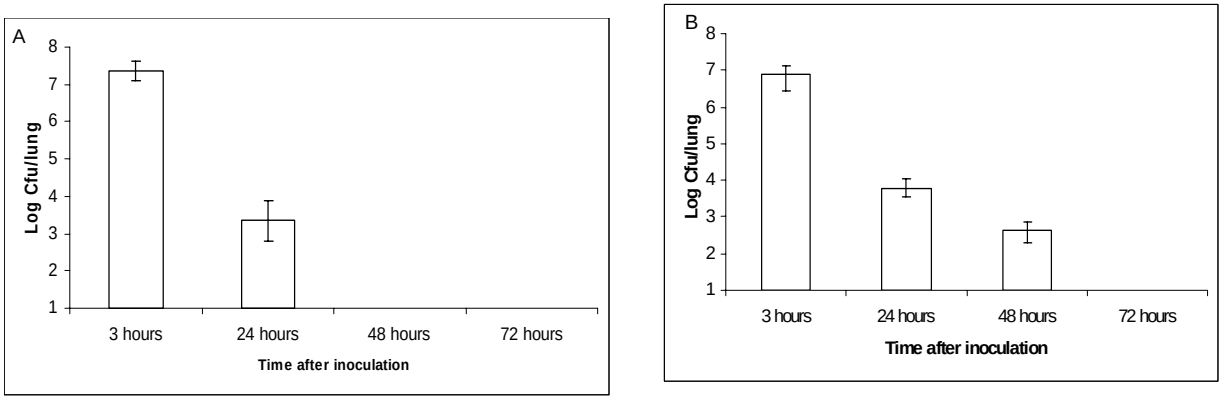


Figure 4.1: Lung persistence in BALB/c (A) and C57BL/6 (B) mice after nasal administration of *L. lactis* recombinant strain LLWE-EDIII.
10^8 CFU were nasally administered to the mice and at the indicated time points, the mice were sacrificed, the lungs were collected, homogenized and appropriate dilutions were plated on erythromycin containing GM17 agar. Four mice per group and per time point were individually assessed. The standard deviation is calculated from the CFU from each individual mouse.

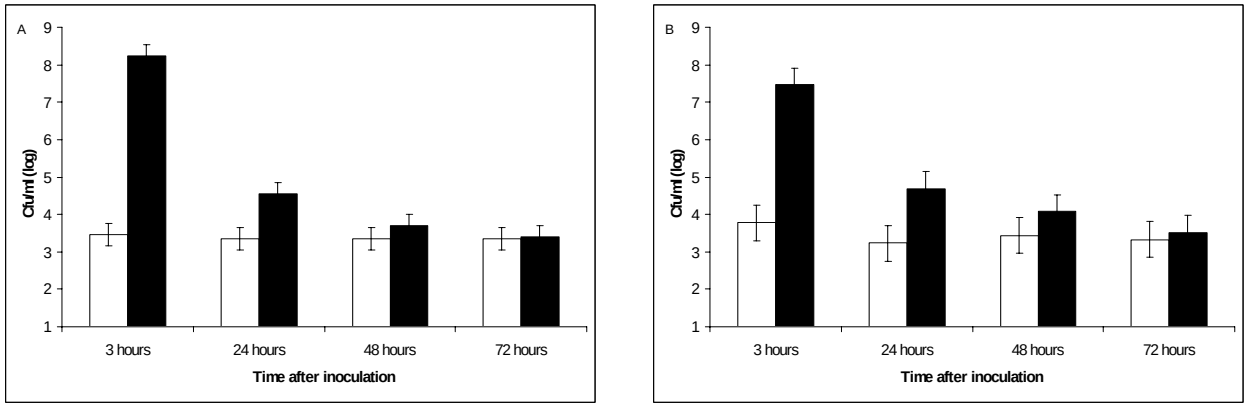


Figure 4.2: Intestine persistence in BALB/c (A) and C57BL/6 (B) mice after oral administration of *L. lactis* recombinant strain LLWE-EDIII.
Before administration of the *L. lactis* bacteria, the presence of erythromycin bacteria in the gastrointestinal tract of the animals was assessed (white bars). Then, 10^{10} CFU were given orally to the mice (black bars) and at the indicated time points, the pooled feces were collected, homogenized and appropriate dilutions were plated onto erythromycin containing GM17 agar plates. Groups of four mice were assessed for each time point. The standard deviation is from three different counts from the same pooled samples.

10^{10} CFU were orally administered to each mouse and at 3, 24, 48 and 72 hours, the feces were collected, homogenized and plated.

The results show that the bacteria are progressively cleared from the gastro-intestinal tract, coming back to the basal level of erythromycin-resistant bacteria within 72 hours (Fig 4.2A and Fig 4.2B). No significant difference was observed between BALB/c and C57BL/6 mice in the ability of *L. lactis* to persist in the gastro-intestinal tract.

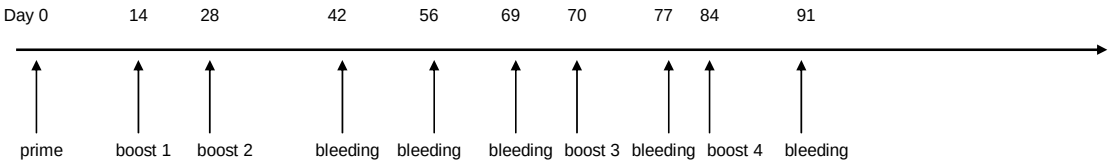
4.2 Sero-conversion of inoculated mice against *L. lactis*

The systemic anti-lactococcal total IgG response has been studied in three different groups of mice: naïve mice, mice which have received pMG36E backbone plasmid-transformed *L. lactis* strain (LLWE), and mice which have received EDIII-producing *L. lactis* strain (LLWE-EDIII). BALB/c and C57BL/6 mouse strains were studied after oral or nasal administration. The immunization schedules and bleeding time points are schemed in Figure 4.3.

Naive BALB/c mice showed a stable IgG against L. lactis from the ELISA reading of antibody at OD_{490nm} throughout the experiment (Fig 4.4A and Fig 4.5A), whereas naïve C57BL/6 mice were found to display a marked increase of the OD_{490nm} reading at day 77 and 91 (Fig. 4.4B and Fig. 4.5B).

BALB/c mice nasally inoculated with LLWE strain showed a significant increase of OD_{490nm} for bleeding at day 77 and 91 (Fig. 4.4A). For the BALB/c mice nasally inoculated with LLWE-EDIII strain, an overall constant OD_{490nm} reading was observed with values not significantly different from the values measured in naïve mice (Fig. 4.4A).

A



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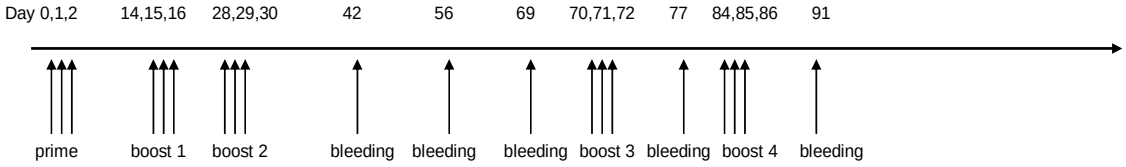

Figure 4.3: Immunization schedules and bleeding after nasal (A) or oral (B) administration of *L. lactis* strains (either LLWE or LLWE-EDIII).

The arrows indicate the days of manipulation of mice for either inoculation or bleeding. The naïve group of mice consist of 4 mice per group while the LLWE and LLWE-EDIII inoculated mice consist of 6 mice per group. On days of administration, the naïve mice were not inoculated but were bled on the same days as the inoculated groups.

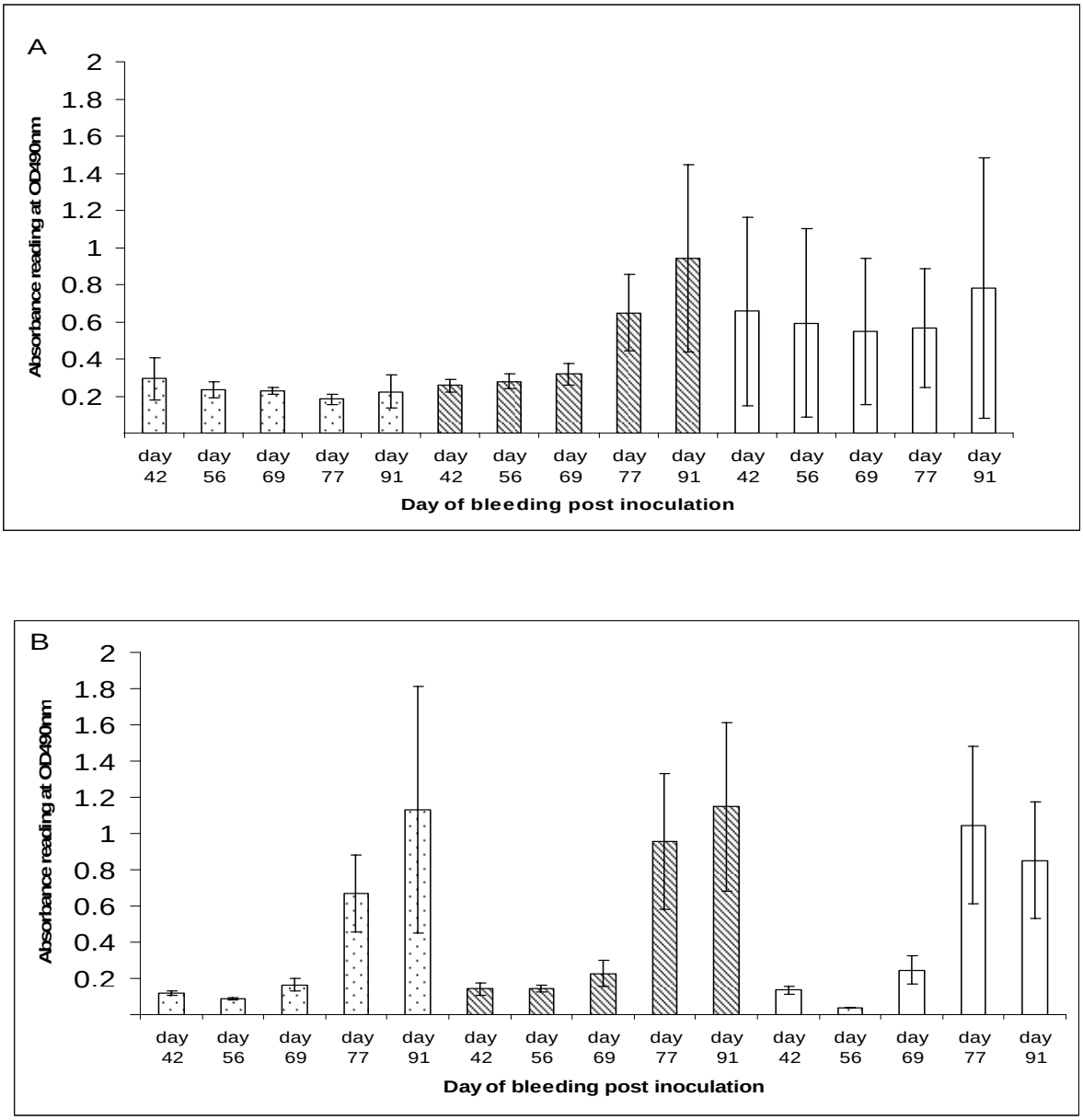

Figure 4.4 Detection of anti-*L. lactis* IgG antibodies in the serum of BALB/c (A) and C57BL/6 (B) mice after nasal administration of *L. lactis* strains.

The serum of six mice per group was assessed individually by ELISA. Results are expressed as the mean and standard deviation. Plates were coated with a total *L. lactis* lysate. Columns with dots represent naïve mice. Stripped columns represent LLWE inoculated-mice and white columns represent LLWE-EDIII inoculated-mice.

The nasally inoculated C57BL/6 mice showed instead a marked increase of the anti-lactococcal IgG response after the third and forth boosts (bleeding at day 77 and 91), and this trend was observed for all three groups (naïve, LLWE and LLWE-EDIII) (Fig 4.4B).

BALB/c mice orally inoculated with LLWE and LLWE-EDIII strains showed a marked increase of the anti-lactococcal IgG response after the third and fourth boosts (bleeding at days 77 and 91) (Fig 4.5A). For C57BL/6 orally inoculated with LLWE or LLWE-EDIII, the anti-lactococcal antibody response appears constant (if we exclude day 56 time point) throughout the experiment (Fig 4.5B).

In summary, BALB/c and C57BL/6 mice did not produce any significant anti-lactococcal antibody response before day 77. The third and fourth boosts clearly triggered the systemic production of anti-lactococcal antibodies in BALB/c mice (nasal and oral route of administration), whereas C57BL/6 did not show any significant differences compared to the naïve group.

In conclusion, our observations suggest that C57BL/6 mice appear more immune-tolerant to *L. lactis* bacteria compared to BALB/c mice.

4.3 Sero-conversion of inoculated mice against dengue NGC EDIII

We first compared the performance of an in-house EDIII-coated ELISA to the commercially available Dengue Indirect IgG ELISA Test kit

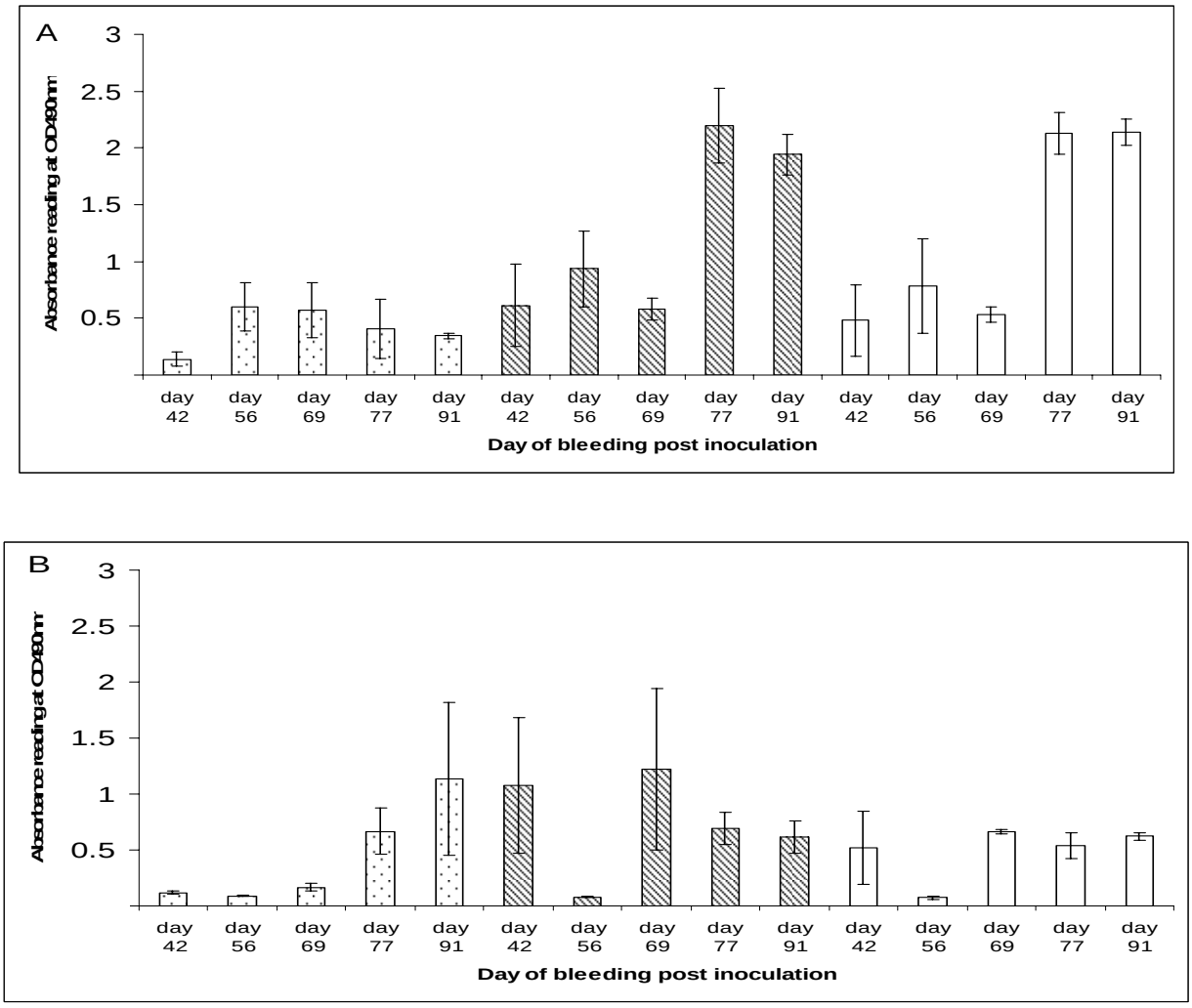

Figure 4.5 Detection of anti-*L. lactis* IgG antibodies in the serum of BALB/c (A) and C57BL/6 (B) mice after oral administration of *L. lactis* strains.

The serum of six mice per group was assessed individually by ELISA. Results are expressed as the mean and standard deviation. Plates were coated with a total *L. lactis* lysate. Columns with dots represent naïve mice. Stripped columns represent LLWE inoculated-mice and white columns represent LLWE-EDIII inoculated-mice.

(PanBio, Australia) where the four Dengue serotypes total viruses have been coated onto each well. The in-house EDIII-coated ELISA was found to be more sensitive and accurate for the detection of anti-EDIII antibodies (data not shown). All the readings were standardized using envelope-specific monoclonal antibody 3H5 (#8702, Chemicon international, USA) (OD_{490nm} reading of 1.019) as positive control. For both strains of mice, an additional group was intraperitoneally inoculated with heat-inactivated dengue serotype 2 (NGC strain) and was considered as the gold standard against which the production of anti-EDIII antibody production has been compared.

The serum of the naïve BALB/c mice gave an absorbance at OD_{490nm} comprised between 0.147 and 0.619 throughout the experiment (Fig 4.6A and Fig. 4.8A). Therefore, we considered that OD_{490nm} 0.619 represents the threshold value above which serum samples are positive. For the BALB/c mice nasally inoculated with LLWE strain, 5 mice at day 69 and 3 mice at day 77 showed an absorbance reading at OD_{490nm} above the threshold (Fig. 4.6B).

For the LLWE-EDIII inoculated-BALB/c mice, mice M1 and M3 were found with a positive absorbance reading at OD_{490nm} (above the threshold) at day 42, 56 and 69 (Fig. 4.6C). And were observed to be statistically significant for their sera readings at day 56 ($P < 0.05$). However, The OD_{490nm} values were significantly lower than that obtained for the NGC-injected mouse group (0.889). The naïve C57BL/6 mice gave an absorbance at OD_{490nm} below 0.48 throughout the experiment, with the exception of mice 4 at day 91 (Fig 4.7A).

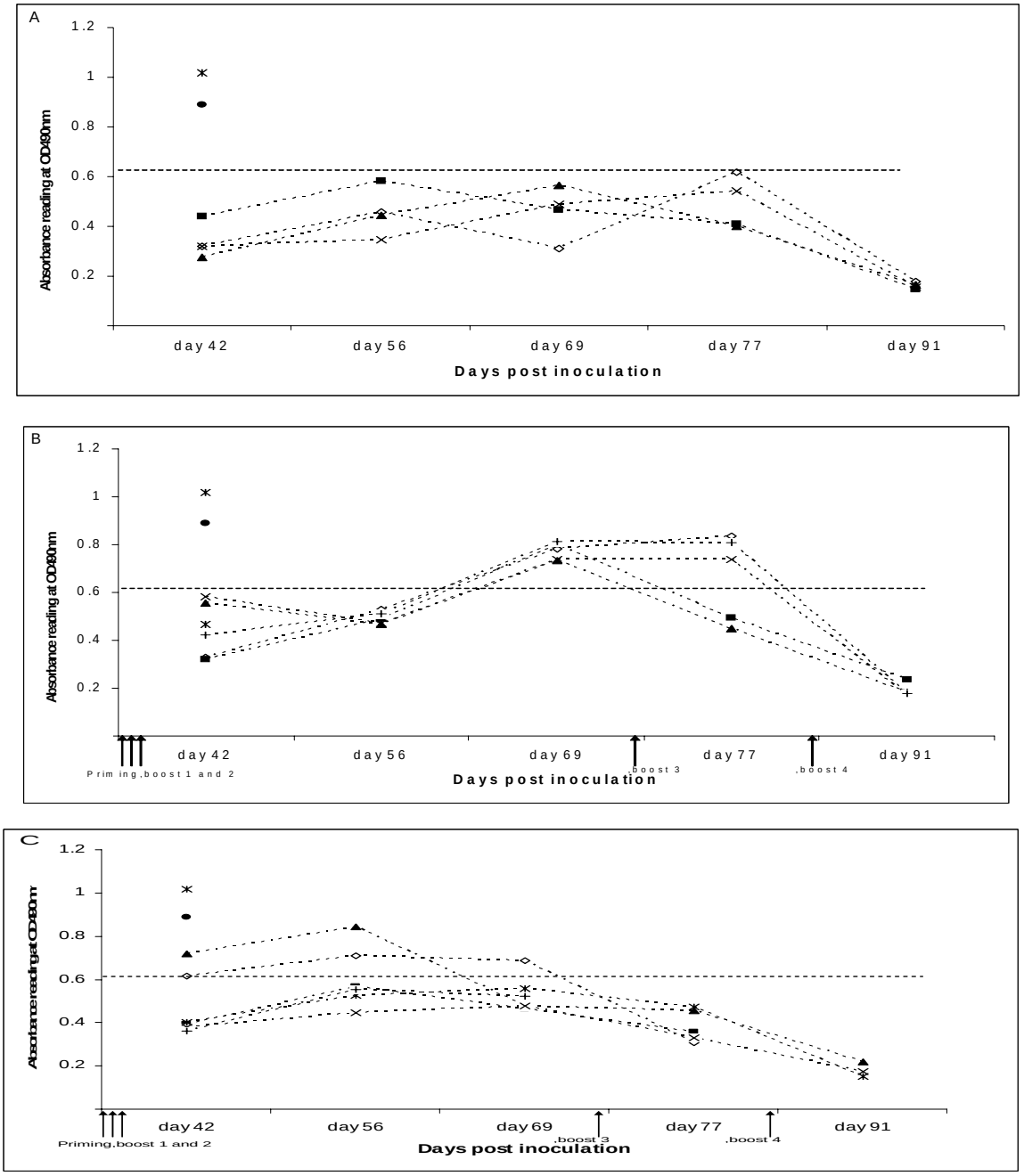

Figure 4.6: Detection of anti-EDIII IgG antibodies in the serum of BALB/c mice after nasal administration of *L. lactis* strains.

The serum of six mice per group was assessed individually by ELISA. Plates were coated with a purified EDIII. (A) naïve mice, (B) LLWE inoculated-mice, (C) LLWE-EDIII inoculated-mice. Solid arrows (↑) on the X axis denote priming and boostings. Horizontal dash line denotes cut-off value for serum positivity. The sera of the NGC dengue virus-injected mice were pooled before ELISA tested. Legend: * monoclonal antibodies (3H5)

⬣ mice intraperitoneally inoculated with heat-inactivated dengue (NGC); ◊ mouse 1; ■ mouse 2; ▲mouse 3; x mouse 4; □ mouse 5; + mouse 6.

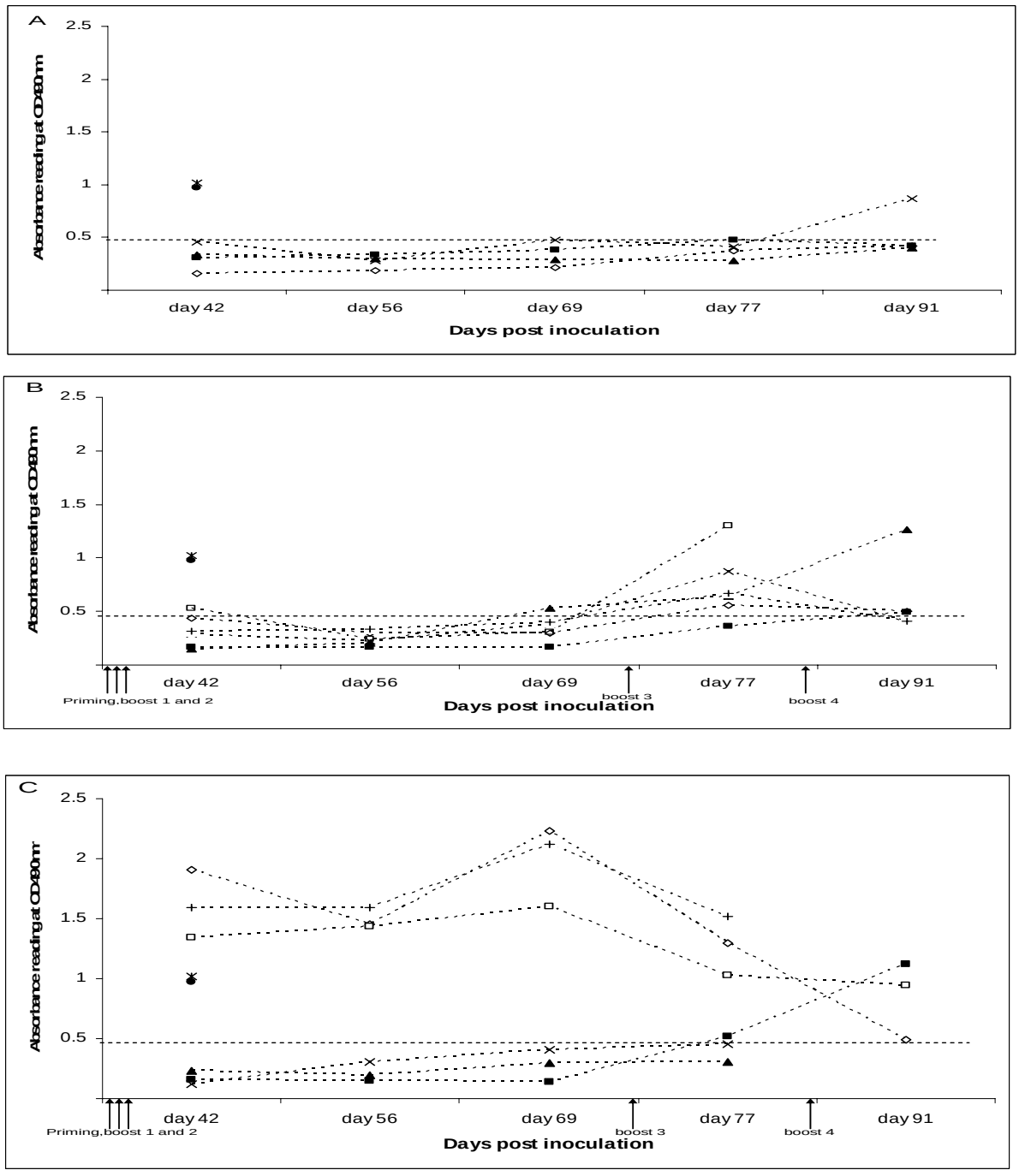

Figure 4.7: Detection of anti-EDIII IgG antibodies in the serum of C57BL/6 mice after nasal administration of *L. lactis* strains.

The serum of six mice per group was assessed individually by ELISA. Plates were coated with a purified EDIII. (A) naïve mice, (B) mice inoculated with LLWE strain and (C) mice inoculated with LLWE-EDIII strain. Solid arrows (↑) on the X axis denote priming and boostings. Horizontal dash line denotes cut-off value for serum positivity. Symbol legend: see Fig. 4.6

An OD_{490nm} of 0.48 was therefore considered as the threshold. For C57BL/6 mice nasally inoculated with LLWE strain, the absorbance reading remains below or similar to the threshold value up to day 69. However after the third boost (day 77), five mice were found transiently with OD_{490nm} readings clearly above the threshold, but wean to readings below the threshold on day 91, with the exception of mouse M3 (Fig 4.7B). Mouse M5 died after bleeding at day 77.

For the LLWE-EDIII nasally inoculated-C57BL/6 mice, 3 mice (M1, M4 and M5) out of 6 were found for all the bleeding time points with OD_{490nm} values above or similar to the value obtained for the NGC-injected group (0.889) (Fig. 4.7C). And were statistically significant ($P < 0.05$) for sera of days 42 and 56. The readings for sera of day 69 were highly significant ($P < 0.01$) for these mice. Mouse M2 was found with positive OD_{490nm} value after the fourth boost only (day 91) but was not statistically significant ($P > 0.05$). Mice M3 and M4 died after bleeding at day 77.

In conclusion, the C57BL/6 mice nasally inoculated with LLWE-EDIII clearly gave higher absorbance readings compared with BALB/c mice nasally inoculated with the same *L. lactis* recombinant strain. These results indicate that C57BL/6 mice produced a greater systemic anti-EDIII antibody response than BALB/c mice when nasally administered with the EDIII-producing *L. lactis* strain.

For the BALB/c orally inoculated with LLWE strain, the readings were found below the threshold with the exception of mouse M5 after day 69 (Fig 4.8B). For BALB/c orally inoculated with LLWE-EDIII, 5 mice (M1, M2, M3, M4 and M6) out of 6 displayed absorbance readings above the threshold on day 69 (Fig. 4.8C) and were statistically significant ($P < 0.05$).

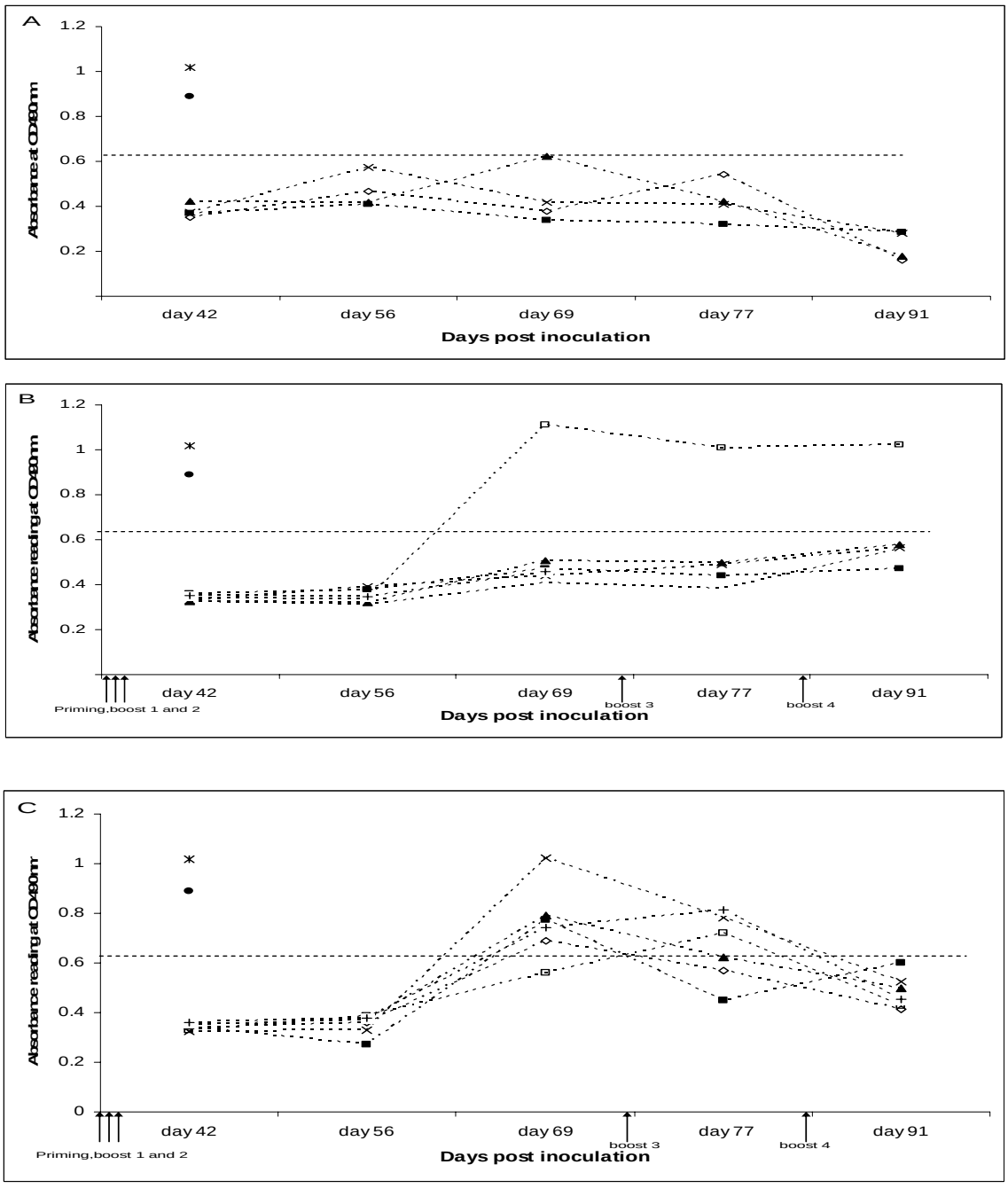

Figure 4.8: Detection of anti-EDIII IgG antibodies in the serum of BALB/c mice after oral administration of *L. lactis* strains.

The serum of six mice per group was assessed individually by ELISA. Plates were coated with a purified EDIII. (A) naïve mice, (B) mice inoculated with LLWE strain and (C) mice inoculated with LLWE-EDIII strain. Solid arrows (↑) on the X axis denote priming and boostings. Horizontal dash line denotes cut-off value for serum positivity.

Symbol legend: see Fig. 4.6.

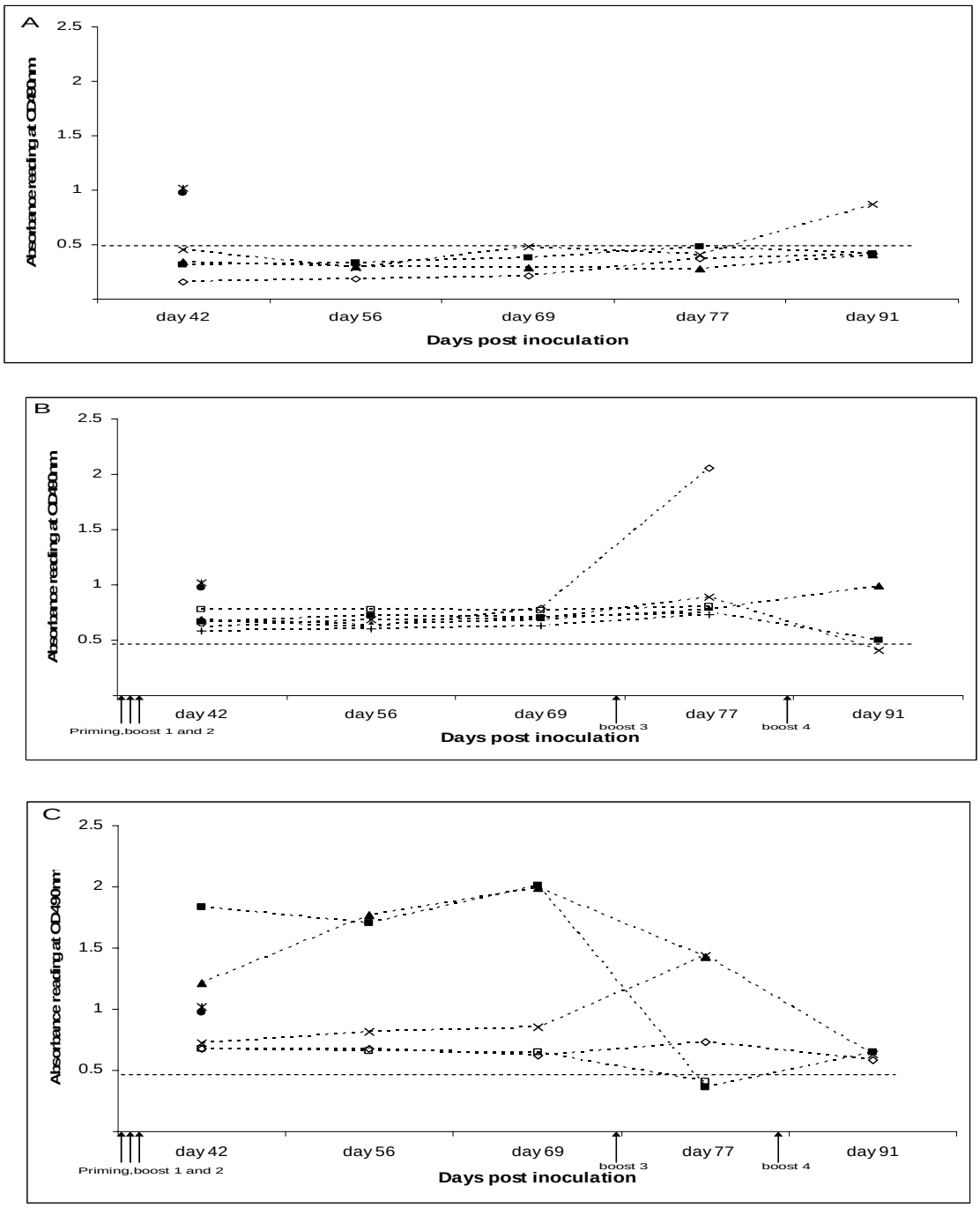

Figure 4.9: Detection of anti-EDIII IgG antibodies in the serum of C57BL/6 mice after oral administration of *L. lactis* strains.

The serum of six mice per group was assessed individually by ELISA. Plates were coated with a purified EDIII. (A) naïve mice, (B) mice inoculated with LLWE strain and (C) mice inoculated with LLWE-EDIII strain. Solid arrows (↑) on the X axis denote priming and boostings. Horizontal dash line denotes cut-off value for serum positivity.

Symbol legend: see Fig. 4.6.

However, only mouse M4 had an OD_{490nm} value above the value obtained for the NGC-injected group (0.889). This antibody response then decreased after the 3rd boost, and only three mice left (M4, M5 and M6) were found with OD_{490nm} values above the threshold at day 77 (Fig 4.8C). After the fourth boost, none of the mice showed positive OD_{490nm}.

The absorbance readings obtained for the C57BL/6 mice orally inoculated with LLWE strain, were not found significantly higher than the threshold ($P > 0.05$) (Fig 4.9B). However, the readings were stable throughout the experiment and did not fluctuate beyond the range of 0.6-0.9 except for mouse M1 on day 77. For C57BL/6 mice orally inoculated with LLWE-EDIII strain, all the six mice had reading values above the threshold, but only two of them (mice M2 and M3 at day 42, 56 and 69, and mice M3 and M4 at day 77) showed values above the value obtained for the NGC-injected group (Fig 4.9C). These readings were found highly significant ($P < 0.01$) when compared to the naïve and LLWE groups. Mouse M6 died after second boost and mice M3 and M5 died after bleeding at day 77.

In conclusion, the C57BL/6 mice orally inoculated with LLWE-EDIII gave higher readings when compared with BALB/c mice. The C57BL/6 group showed more mice than the BALB/c group having OD_{490nm} values higher than the value obtained for the NGC-injected group (3 C57BL/6 mice compared to 1 BALB/c mouse).

Altogether, our data indicate that C57BL/6 mice nasally inoculated with LLWE-EDIII strain produced the highest and the most sustained anti-EDIII antibody response, compared to BALB/c mice and compared to the oral route.

4.4 Detection of neutralizing antibodies in the recombinant *L. lactis*-inoculated mice

To further study the protective potential of the EDIII-producing *L. lactis* strain, the presence of neutralizing antibodies in the serum of the inoculated animals was determined by plaque reduction neutralizing test (PRNT). The envelope-specific monoclonal antibody 3H5 (#8702, Chemicon international, USA) was used as positive control, since it has been shown to neutralize dengue viral challenge and since neutralizing epitopes were found to be located within domain III (EDIII) (Crill *et al.*, 2001; Beasley *et al.,* 2002; Hermmida *et al*., 2006). The serum from the NGC-injected group was also used as a positive control at a sera dilution of 1:10. The sera of naïve and LLWE-inoculated mice were pooled and used at a dilution of 1:10. For the LLWE-EDIII inoculated-mice, the individual sera for which high anti-EDIII IgG titers were obtained were selected and assayed in PRNT. As such, the serum from BALB/c mouse M3 of orally inoculated with LLWE-EDIII was tested (bleeding at day 69, see Fig.4.8C). And the serum from C57BL/6 mice M2 and M3 orally inoculated with LLWE-EDIII were also tested (bleeding at day 69, see Fig 4.9C).

The results showed that the pooled sera from the naïve and LLWE inoculated-mice have no neutralizing activity against the dengue virus (NGC strain) (Fig 4.10A and B). Instead, the serum from BALB/c M3 mouse was found to efficiently neutralize the dengue virus (Fig. 4.10A). The percentage of inhibition obtained for the sera diluted 1/10 indicated that the neutralizing potential of the M3 serum might be lower than the serum from the NGC-injected group (43.4% and 62.5% inhibition, respectively). However, similar neutralizing efficiencies were found at dilution 1/20

(about 25% inhibition). This observation suggests that the neutralizing ability of the serum from the orally inoculated BALB/c M3 mouse is comparable to the neutralizing ability of the serum from the NGC-immunized mice.

The serum from the orally inoculated C57BL/6 M2 mouse showed a lower ability to neutralize dengue (NGC) compared to the serum from the NGC-immunized mice (Fig. 4.10B). However, at dilution 1/40, both sera gave similar percentage of inhibition (around 10%). The serum from M3 mouse was found more efficient to neutralize the virus than the serum from the NGC-immunized group, as it gave consistent higher percentages of inhibition at all dilutions tested (Fig. 4.10B).

In conclusion, the sera from orally LLWE-EDIII inoculated-mice (BALB/c and C57BL/6) were able to neutralize the NGC dengue virus in an *in vitro* plaque reduction neutralizing test (PRNT), with an efficacy comparable to or even higher than the one obtained with the serum from mice immunized with the whole NGC virus.

The serum from mice nasally inoculated with LLWE-EDIII strain is being currently tested in the laboratory.

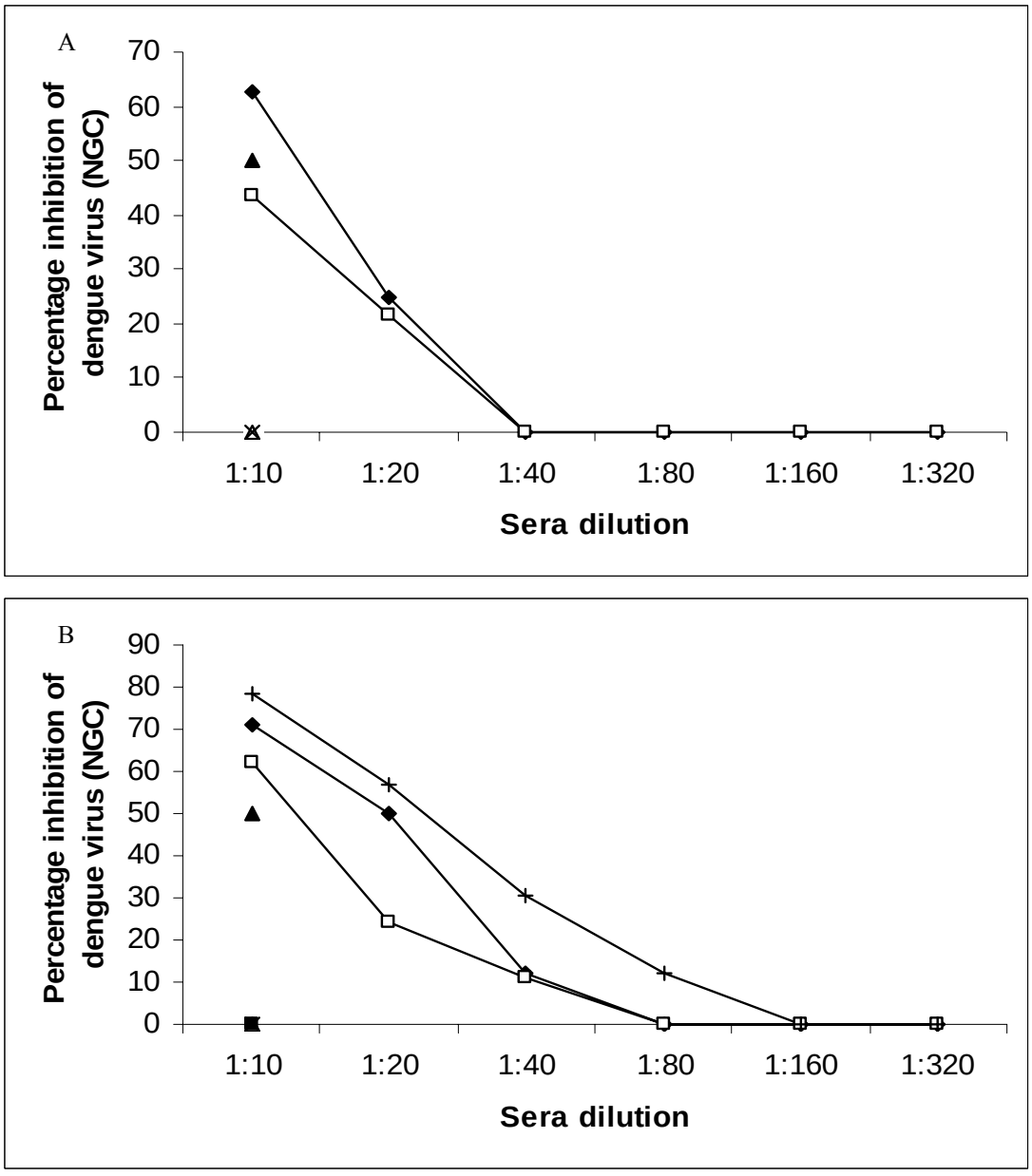

Figure 4.10: PRNT of orally inoculated BALB/c (A) and C57BL/6 (B) with LLWE-EDIII

Legend: ▲monoclonal antibodies (3H5); ◆ mice intraperitoneally inoculated with heat-inactivated dengue (NGC); ■ pooled naïve mice; x pooled mice inoculated with LLWE mice; individual mice inoculated with LLWE-EDIII , (panel A)□ mouse 4 and for (panel B) □ mouse 2; + mouse 3

Chapter 5: Discussion

Mucosal vaccines, which are administered by oral or intranasal route, are more convenient than the usual parenteral vaccines due to their ease of administration and low cost. Both are priorities for developing countries plagued by infectious diseases when considering vaccination for public health policy. Moreover, mucosal vaccines are able to elicit serum-IgG and mucosal-IgA antibodies to neutralize toxins and viruses and induce cytotoxic T lymphocytes (CTL) activities (Levine *et al*., 1998).

In this context, we have embarked on the study of the use of *Lactococcus lactis* as a possible vaccine vector targeting dengue virus. This is a further study from previous work by Lin, W. (2006) who constructed a recombinant *L. lactis* strain producing in its cytoplasm the E domain III (EDIII) antigen from DEN2 virus, Singapore strain. *L. lactis* is a noninvasive, nonpathogenic, gram-positive bacterium which has a long history of widespread use in the food industry for the production of fermented milk products, thus it has a generally-regarded as safe (GRAS) status (Adam *et al*., 1995). Its GRAS status coupled to its inability to colonize the digestive and the respiratory tracts of both humans and mice, except gnotobiotic mice, make *L. lactis* a safe and attractive vaccine delivery vehicle for human use (Gruzza *et al*., 1994; Klijin *et al*., 1995; Vesa *et al*., 2000).

This study aims to study the immunization efficacy of the EDIII-producing *L. lactis* strain (LLWE-EDIII), via measuring the systemic anti-EDIII antibody response generated in two different mouse strains, BALB/c and C57BL/6, after nasal or oral administration.

Previous studies have established a correlation between persistence efficiency of lactic acid bacteria in the host and their protective potential (Grangette *et al*., 2002;

Oliveira *et al*., 2006). The persistence of the LLWE-EDIII bacteria in the respiratory and gastro-intestinal tracts was determined in both BALB/c and C57BL/6 mice after nasal and oral administration, respectively. The recombinant bacteria administered via the nasal or oral route were progressively cleared from the respiratory or gastro-intestinal tract of the animals within 48 or 72 hours. Bacteria were found to persist longer in the respiratory tract of C57BL/6 mice compared to the BALB/c mice, as a significant amount of bacteria (approx. 2.5 log) were still recovered from the lungs of C57BL/6 mice after 48 hours whereas the lungs of BALB/c mice were totally sterile at the same time point. No difference in the persistence of LLWE-EDIII bacteria was observed between both mouse strains after oral inoculation. Our observations are in agreement with previous studies which reported varying abilities to recover inoculated *L. lactis* in different mouse strains and via different routes of inoculation (Norton *et la*., 1994; Klijin *et al*., 1995; Drouault *et al*., 1999; Grangette *et al*., 2002; Oliveira *et al*., 2006). In these studies, the persistence of *L. lactis* bacteria ranged from none to three days post-inoculation. This study was carried out to study whether the cytoplasmic production of the EDIII antigen in *L. lactis* affects the persistence of the bacteria in the respiratory tract of the animals. A correlation between persistence of *L. lactis* and antibody raised against it has indeed been demonstrated (Norton *et al*., 1984).

Moreover, we have observed that naïve BALB/c and C57BL/6 mice displayed a basal level (10^3 CFU/ml) of erythromycin resistant bacteria. This basal level of inherent erythromycin-resistant bacteria has also been observed by Grangette *et al*. (2002) although it only appeared 4 days after administration of recombinant *L. lactis* strain producing tetanus toxin fragment C.

LAB have been described to induce tolerance which is one of the advantage to use them as vaccine delivery vehicle, as repeated administrations will not lead to a faster clearance of the recombinant bacteria (Wells *et al*., 1995; see survey of literature section 2.5.2). The systemic anti-lactococcal antibody response was determined in BALB/c and C57BL/6 naïve mice and in mice nasally or orally inoculated with LLWE or LLWE-EDIII strains.

The naïve C57BL/6 mice displayed a marked increase of the OD_{490nm} reading at day 77 and 91. Our observation is in agreement with a previous study describing the increase of enteric anti-*L. lactis* IgA antibodies in naïve C57BL/6 mice as they grew older (Norton *et al.*, 1994). The authors postulated that environmentally encountered cross-reacting bacteria might be responsible for the positive detection of anti-lactococcal antibodies. We observed instead a basal and constant OD_{490nm} reading for the naïve BALB/c mice, suggesting that BALB/c mice are more tolerant to environmental bacteria than C57BL/6 mice.

The C57BL/6 mice nasally inoculated with LLWE or LLWE-EDIII bacteria showed a comparable profile and similar OD_{490nm} values to that observed for the naïve C57BL/6 mice. The C57BL/6 mice orally inoculated produced an overall constant anti-lactococcal antibody response throughout the experiment, with OD_{490nm} values similar to the values obtained for naïve C57BL/6 mice at day77 and 91. These results suggest that the repeated nasal or oral administrations of *L. lactis* bacteria do not trigger the production of anti-lactococcal antibodies in the serum of C57BL/6 mice.

Instead, the BALB/c mice orally inoculated with LLWE or LLWE-EDIII showed a marked increase of the anti-lactococcal antibody response after the 3^{rd} and 4^{th} boosts. A

previous study has also described that the systemic anti-lactococcal IgG antibody response was enhanced in BALB/c mice after oral inoculation of *L. lactis* (Norton *et al*., 1984). Similarly, BALB/c mice nasally inoculated with LLWE strain showed a marked increase of the anti-lactococcal antibody response after the 3rd and 4th boosts.

Altogether, these data indicate that C57BL/6 mice are less prone than BALB/c mice to develop a systemic anti-lactococcal antibody response, and therefore appear more tolerant to repeated nasal or oral administrations of *L. lactis* bacteria than the BALB/c mice.

The specific anti-EDIII antibody response was determined in the serum of BALB/c and C57BL/6 mice nasally or orally inoculated with the recombinant *L. lactis* strain LLWE-EDIII. An in-house ELISA assay was developed in which purified His-tagged EDIII was used as coating antigen. The pooled sera of C57BL/6 or BALB/c mice intraperitoneally injected with heat-inactivated dengue virus (NGC strain) provided a gold standard reference against which the OD_{490nm} readings were compared.

Overall, our results showed clearly that i) C57BL/6 mice inoculated with LLWE-EDIII bacteria produced a higher systemic anti-EDIII IgG response than the BALB/c counterparts, and ii) the nasal route triggered a stronger and more sustained systemic response than the oral route in those C57BL/6 mice. Three out of six C57BL/6 mice nasally inoculated with LLWE-EDIII bacteria were found indeed with antibody titers significantly higher than the titer obtained for the NGC-injected group. Such titers were observed already after the second boost (bleeding at day 42) and were sustained up to at least day 77 for the three mice. Instead, only two C57BL/6 mice orally inoculated with LLWE-EDIII bacteria showed antibody titers above the titer obtained for the NGC-

injected group, and such a response decreased markedly after the third boost (bleeding at day 77), coming back to the threshold value at day 91. The observation that the nasal administration of recombinant *L. lactis* triggered a better systemic antibody response than the oral route is in opposition to previous reports in which the oral administration of recombinant *L. lactis* bacteria was found better than the nasal administration (Pei *et al*., 2005; Xin *et al*., 2003). However, these studies were all carried out in BALB/c mice and true enough, if we compare the anti-EDIII antibody response triggered in BALB/c mice nasally or orally inoculated with LLWE-EDIII bacteria, it appears that indeed the oral route clearly triggered higher titers than the nasal route (Fig 4.6 and Fig 4.8).

In the nasally inoculated C57BL/6 mice, only three mice out of six were found to produce significant anti-EDIII IgG antibodies in their serum. Similarly, Kim *et al.* (2005) described that only two out of five C57BL/6 mice orally inoculated with a recombinant *L. lactis* MG1363 strain expressing an *H. pylori* vaccine candidate on the pMG36 plasmid, generated *H. pylori* antigen-specific antibodies. Perez and co-workers (2005) also reported that only two out of five mice (BALB/c) inoculated with rotavirus antigen secreting-*L. lactis* elicited a specific-antibody response. Another study using an HPV antigen-producing *L. lactis* strain reported that only 35% of the mice (C57BL/6) were protected from tumour formation (Bermudez-Humaran *et al*., 2005). It appears therefore that the nasal or oral administration of recombinant *L. lactis* strains generally triggers a significant specific (protective) immune response in only 50% or less of the immunized animals. It is not clear what technical and/or physiological parameter(s) might be critical during mucosal administration. The lack of full understanding of mucosal immunity might contribute to the inability to ensure that the majority of the mice respond to

inoculation. However, the expression of heterologous antigens with cytokines in *L. lactis* has been shown to enhance the immunity against such antigens (Bermudez-Humaran *et al*., 2004; Grangette *et al*., 2002; Steidler *et al*., 1998, 2000) The use of *L. lactis* expressing IL-10 has also been approved by Dutch authorities for phase I clinical trials for treatment of inflammatory bowel diseases (Steidler *et al*., 2003).

Overall, we have observed a decrease in the anti-EDIII antibody titer after the third boost for most of the mice from both strains and regardless of the route of inoculation. This phenomenon might be due to the induction of immune tolerance to EDIII antigen after the third boost. Antigen tolerance has also been observed in mice inoculated with recombinant *L. lactis* bacteria expressing the urease subunit B of *H. pylori* (Lee *et al*., 2001), or producing the MSP-1_{19} antigen from *Plasmodium yoelii* (Zhang *et al*., 2005). The induction of antigen tolerance could depend on several factors including the dose, frequency and interval of administration of the recombinant bacteria (Weiner, 1994). Therefore, the third and forth boosts might be non-beneficial or even detrimental to the production of a strong and sustained specific antibody response.

The importance of neutralizing antibodies to protect from dengue has been demonstrated by the protection of mice passively transferred with monoclonal antibodies (Kaufman *et al*., 1987; 1989). Moreover, due to a lack of a reliable animal model for dengue, neutralizing antibody titers and plaque reduction neutralizing test (PRNT) are widely used as surrogate markers of protective immunity (Putnak *et al.*, 1991; Delenda *et al*., 1994). The sera of the mice for which a significant anti-EDIII antibody titer was measured, were assayed in a PRNT.

Our results showed that the serum from mice orally inoculated with LLWE-EDIII strain was able to neutralize NGC virus at least as efficiently as the serum from mice immunized with the whole NGC virus. The serum from mice immunized with NGC virus does contain antibodies against the whole virus, whereas the serum from LLWE-EDIII inoculated-mice does contain EDIII-antibodies only. Moreover, the sera from the orally inoculated-mice tested in PRNT showed anti-EDIII titers significantly higher than the titer obtained for the NGC-immunized mice. This observation indicates that when EDIII is the only antigen used for vaccination, higher anti-EDIII antibody titers might be necessary in order to achieve a neutralizing activity comparable to the one obtained with a whole virus-immunization. Our data therefore suggest that EDIII antigen does contain important neutralizing epitopes, however some additional neutralizing epitopes are present in the virus as well. Alternatively, another hypothesis can be proposed: The EDIII antigen produced in the LLWE-EDIII strain has been cloned from the Dengue 2 SING/99 strain, whereas Dengue 2 NGC strain has been used to carry out the PRNT. A recent study has shown that the antibodies raised against SING/99 strain are able to neutralize NGC strain despite amino acid variation of 3% between the two homotypic strains (Lim *et al*., 2006). Within the EDIII region, the two strains differ in 3 amino acids (Lim *et al*., 2006). Thus, the ability of anti-EDIII (SING/99) antibodies to neutralize NGC virus might be lower than anti-EDIII(NGC) antibodies, which might require a higher antibody titer to achieve similar protection levels.

Chapter 6: Conclusion and future directions

This study was carried out in order to determine the suitability of *L. lactis* for dengue antigens vaccine delivery. Recombinant *L. lactis* bacteria producing intracellularly EDIII antigen from DEN2 Singapore strain, were administered to BALB/c or C57BL/6 mice via the oral or the nasal route. The systemic specific anti-EDIII IgG responses were compared. Our data indicate that the nasal administration of EDIII-producing *L. lactis* bacteria triggers a strong and sustained antibody response against EDIII antigen in the serum of C57BL/6 mice. Moreover, we have shown that the sera from orally inoculated mice for which high anti-EDIII titers were measured, are able to efficiently neutralize Dengue NGC strain in an *in vitro* neutralization assay. The sera from the nasally inoculated mice are being currently tested in the laboratory.

Despite the lack of a proper animal model, there is still a need to prove the protective ability of our recombinant *L. lactis* producing EDIII antigen against viral challenge *in vivo*. The various current mice models (refer to survey of literature 2.6.1) can be taken as reference for our future studies with the various means of dengue virus infection and proof of protection in various strains of mice could also be taken into consideration.

It would also be of interest to study the T cell response in the immunized animals by monitoring the magnitude of cell proliferation and production of cytokines such as IFN-γ (a Th1 cytokine) and IL-4 (a Th2 cytokine) upon activation *in vitro* by purified EDIII antigen of the splenocytes isolated from immunized mice. The importance of T cell response for dengue infection has been observed by Khanam *et*

al. (2006) and An *et al*. (2005) where higher Th1 response was found to be involved in the virus clearance. Moreover, T cells obtained from PBMC of dengue infected patients were able to recognize E proteins (Rothman *et al*., 1996). The ability of recombinant *L. lactis* in eliciting a T cell response was observed by Pei *et al.* (2005) and Ramasamy *et al*. (2006). The involvement of both Th1 and Th2 $CD4^+$ cell activity was also observed in C57BL/6 inoculated with recombinant *L. lactis* expressing tetanus toxin fragment C (Norton *et al*., 1997).

Due to the distinct four serotypes of dengue, a tetravalent vaccine is required. The construction of recombinant *L. lactis* strains expressing each EDIII antigen of each serotype may be an interesting approach. However, the administration of a tetravalent vaccine at a single site of inoculation might result in viral interference or immuno-dominance against a single serotype. The inoculation at different sites (oral, nasal…) which drains to physiologically distinct lymph nodes might be of particular interest. Targeting of distinct lymph nodes has shown promise in Sendai virus and cancer experiments in mice (Cole *et al*., 1997; Makki *et al*., 2002).

In order to lower the anti-carrier response so as to increase the number of immunizations, *L. lactis* bacteria can be acid pre-treated to remove surface components, leaving a non-living particle that is termed Gram-positive enhancer matrix (GEM) (Steen *et al*., 2003; Bosma *et al*., 2006). GEM has been shown to trigger a lower anti-carrier response compared to live *L. lactis*, but still efficiently elicit antibodies against the heterologous antigen they produce (Ramasamy *et al*., 2006). Recombinant *L. lactis* bacteria secreting a *H. pylori* vaccine candidate has been shown to trigger a higher systemic antigen-specific antibody response than

recombinant *L. lactis* bacteria which produced the vaccine antigen intracellularly (Nouaille *et al*., 2003). Cell anchored HPV antigen in recombinant *L. lactis* was also shown to elicit a higher immune response than *L. lactis* intracellularly expressing the same antigen (Bermudez-Humaran *et al*., 2005). Thus, the construction of recombinant *L. lactis* strains secreting or surface-expressing EDIII antigen might be undertaken in order to further improve the specific immune response in C57BL6 mice upon nasal administration.

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Appendix 1: Reagents for BHK cells culture

1.1 Growth and Maintenance Media

1.1.1 Fetal Calf Serum (FCS)

FCS (Gibco BRL, USA) was inactivated by heating at 56 ^{o}C for 30 min, aliquoted and stored at -20^{0}C.

1.1.2 10x Phosphate Buffered Saline (PBS) (pH7.4)

(per litre)

NaCl	80.0g
KCl	2.0g
KH_2PO_4	2.0g
$Na_2.HPO_4$	11.5g

To prepare the 10x PBS stock solution, the specified constituents were added to 900ml of nanopure water and pH adjusted to 7.4. The stock solution was top up to 1 L and then autoclaved at 121 ^{o}C for 15 min. The stock solution was then stored at room temperature.
To prepare the working (1x) PBS, 10x PBS stock solution was diluted 1:10 with nanopure water. The solution was then autoclaved at 121^{o}C for 15min and subsequently stored at 4 ^{o}C.

1.1.3 Trypsin/Versene Solution

The 10X trypsin/versene solution (JRH biosciences, Kansas, USA) was diluted to 1 X trypsin/versene solution using 1 x PBS solution as diluent.

1.1.4 RPMI medium 1640

RPMI medium 1640 with L-glutamine	1 packet
HEPES buffer solution (1M)	20ml
$NaHCO_3$	15ml
FCS	10% final concentration for growth; 2% final concentration for maintenance in plaque assays.

1 packet of RPMI medium 1640 powder was dissolved in 500ml of nanopure water. Two ml of $NaHCO_3$ (Sigma-Aldrich Inc. was then added and the pH adjusted to 7.2. The final volume was then increased to 900ml and sterilization carried out by filtration through a 0.2µm membrane.
The media was then stored in aliquots at 4 ^oC. FCS was added as required prior to use.

To make double strength RPMI medium for use in plaque assay, the same constituents were dissolved in a final volume of 480ml of nanopure water instead of 900ml. FCS was added to a final concentration of 2%.

1.2 Reagents for Plaque Assay

1.2.1 2% Carboxymethylcellulose (CMC)

To make 100ml of 2% CMC, 2.0g of carboxymethylcellulose (Aquacide II, Calbiochem) was resuspended in 100ml of nanopure water. The suspension was then autoclaved for 15 minutes at 121^0C and then kept at 4^0C.

1.2.2 Medium used for plaque assay

Two hundred ml of plaque assay medium were made by combining 100ml of 2% CMC with 299ml of double strength RPMI

1.2.3 Solution for staining plaque assay plates

Crystal violet (Sigma,USA)	5g (1% final)
37% formaldehyde (Merck)	270ml (20% final)
PBS (Appendix 1.1.2)	230ml