Feasibility Study Examining the Association between Gut Microbiota and Immune Response to Seasonal Influenza Vaccination in Healthy Adults.

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Abstract

Research into the effect of the gut microbiota on host immune response is continuing to shed new light on the underappreciated role of the microbiota in human health. Recent research using mice has shown that the microbiota is critical to the host immune response to influenza infection. Whilst there is great variation in the human gut microbiota, classifications called stool community types can be used to classify individuals based on the abundance of major bacterial taxa.

The primary objective of this study was to investigate the feasibility of using the study protocol for a large randomised controlled trial.

Healthy adult participants (n=125) aged 18 to 64 were recruited from the general population and vaccinated with the seasonal trivalent influenza vaccine. Participants were followed up over a period of six months, during which time, both stool and blood samples were collected. Blood samples were collected at Day Zero, Three, Seven, 28 and 180 to measure immune response. The immune response to vaccination was measured by HAI antibody titres at Day Zero and Day 28. Stool samples were collected at Day Zero and Day 28 to assign participants to one of the four stool community types and assess stability over time. Stool samples were assigned to stool community types using the proportions of major taxa present. The association between stool community type and either post vaccination HAI titre, seroconversion rates or seroprotection rates was also assessed.

The results obtained in this study supported the feasibility of a large randomised controlled trial using the study protocol. The study demonstrated a high participant retention rate (97.6%; 95% CI = 93.1% to 99.5%), as well as high participant adherence to the study protocol and good success obtaining the required blood and stool samples.
All participants were able to be grouped into one of the four stool community types; SCT-A (n=64), SCT-B (n=4), SCT-C (n=23) and SCT-D (n=32). A large variation in baseline and post vaccination HAI titres to the H1N1 and H3N2 strains of the vaccine was found in this study.

For the stool community types there was modest variation in the post vaccination HAI titre, seroconversion and seroprotection rates, although the differences were not significant in this small sample.

The results of this study indicate the suitability of this study design for a future large randomised controlled trial. Researchers should expect high levels of participant retention and protocol adherence. Resources should be focused on the stool sampling labelling and blood sampling on Day Zero.

The proportions of participants with each of the stool community types were identified, but this feasibility study did not have sufficient power to investigate the association between stool community type and immune response. Future research with adequately powered samples should be undertaken to investigate the associations further. Those studies should take into account the feasibility findings of this study in order to improve study protocol design.
Acknowledgements

We all know that scientific research is rarely a solo endeavour and while it is slightly clichéd to say it this way, there are many people without whom this thesis would not have been possible, and I must express my gratitude.

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Table of Contents

Abstract.................................................................................................................................................. i
Acknowledgements................................................................................................................................... iii
Table of Contents .................................................................................................................................... v
List of Tables ........................................................................................................................................... ix
List of Figures .......................................................................................................................................... xi
List of Abbreviations ............................................................................................................................... xiii
Chapter 1: Introduction ........................................................................................................................... 1
  1.0 Introduction to Thesis Topic............................................................................................................. 1
  1.1 Definition of the Gut Microbiota ...................................................................................................... 1
    1.1.1 The Gut Microbiota ................................................................................................................... 1
    1.1.2 Enterotypes ............................................................................................................................... 3
  1.2 Potential Influence Factors on Microbiota Composition and Stability ............................................. 4
  1.3 Immune System .............................................................................................................................. 6
    1.3.1 Innate Immune System ............................................................................................................ 7
    1.3.2 Adaptive Immune System ......................................................................................................... 7
  1.4 Interaction between the Gut Microbiota and the Immune System ................................................. 9
    1.4.1 Interactions between the Gut Microbiota and the Innate Immune System ................................ 9
    1.4.2 The Effect of the Gut Microbiota on the Adaptive Immune System ....................................... 10
      1.4.2.1 T Cells ............................................................................................................................. 10
      1.4.2.2 B Cells ............................................................................................................................ 11
  1.5 Vaccination as a Proxy for Immune Response................................................................................. 11
  1.6 Vaccination ....................................................................................................................................... 12
    1.6.1 Influenza ..................................................................................................................................... 12
      1.6.1.1 Influenza Virus .................................................................................................................. 12
      1.6.1.2 Influenza Vaccine ............................................................................................................. 13
1.6.1.3 The Effect of the Gut Microbiota on the Immune Response to Influenza ........................................... 13

1.7 Summary ..................................................................................................................................................... 14

Chapter 2: Methods ........................................................................................................................................ 17

2.1 Study Setting ................................................................................................................................................. 17

2.2 Vaccine ......................................................................................................................................................... 17

2.2.1 Measure of Antibody Response .......................................................................................................... 18

2.2.1.1 Seroconversion and Seroprotection ................................................................................................. 19

2.3 Objectives ..................................................................................................................................................... 19

2.4 Participant Timeline ...................................................................................................................................... 20

2.5 Sample Size .................................................................................................................................................. 23

2.6 Recruitment .................................................................................................................................................. 23

2.7 Eligibility Criteria .......................................................................................................................................... 23

2.8 Data Collection Methods and Data Management ....................................................................................... 24

2.9 Statistical Methods ..................................................................................................................................... 26

2.9.1 Completion of the Lifestyle Questionnaire .......................................................................................... 26

2.9.2 Blood Sample Collection ....................................................................................................................... 27

2.9.3 Potential Exclusion Criteria .................................................................................................................. 27

2.10 Study Procedures ....................................................................................................................................... 27

2.10.1 Stool Sample Collection and Analysis ............................................................................................... 27

2.10.2 Blood Sample Collection and Analysis .............................................................................................. 28

Chapter 3: Results .............................................................................................................................................. 29

3.1 Participant Flow ............................................................................................................................................. 29

3.2 Participant Characteristics .......................................................................................................................... 31

3.2.1 Characteristics for all Participants ...................................................................................................... 31

3.2.2 Characteristics by Stool Community Type ............................................................................................ 32

3.3 Feasibility ..................................................................................................................................................... 35

3.3.1 Feasibility Study Completion ................................................................................................................ 35

3.3.2 Lifestyle Questionnaire Completion ................................................................................................... 35

3.3.3 Stool Sample Collection ....................................................................................................................... 35
3.3.4 Blood Sample Collection

3.3.5 Potential Exclusion Criteria

3.4 Stool Community Types at Day Zero vs Day 28

3.5 Response to Influenza Vaccination

3.5.1 All Participants

3.5.2 Stool Community Type A

3.5.3 Stool Community Type B

3.5.4 Stool Community Type C

3.5.5 Stool Community Type D

3.6 Association between Stool Community Type and Response to Influenza Vaccination

3.6.1 HAI Titre

3.6.2 Seroconversion

3.6.3 Seroprotection

Chapter 4: Discussion

4.1 Key Findings

4.2 Feasibility

4.2.1 Proportion of Participants that Completed the Study

4.2.2 Completion of the Lifestyle Questionnaire

4.2.3 Stool Samples

4.2.4 Blood Sample Collection

4.2.5 Possible Exclusion Criteria

4.3 Stool Community Types

4.3.1 Stability of Stool Community Types

4.4 Response to Influenza Vaccination

4.5 Association between Stool Community Type and Response to Influenza Vaccination

4.6 Future Research Path

4.7 Conclusion

References

Appendix I: Participant Information Sheet
Appendix II: Lifestyle Questionnaire........................................................................................................83
Appendix III: Haemagglutination Inhibition Assay Protocol..........................................................111
List of Tables

Table 1. Schedule of Procedures ............................................................... 22
Table 2. Demographic characteristics of participants, displayed overall as well as broken down by stool community type ............................................................... 33
Table 3. Proportions and 95% confidence intervals related to the feasibility for a possible larger randomised controlled trial ............................................................... 37
Table 4. Stool Community Type assignments at Day Zero vs Day 28 ............................................................... 38
Table 5. Antibody response generated by participants to Influenza vaccination, displayed overall as well as broken down by stool community type ............................................................... 43
Table 6. P values generated by univariate and multivariate association analysis between stool community type and antibody titre, seroconversion and seroprotection ............................................................... 47
List of Figures

Figure 1. Flow of participants ................................................................. 30

Figure 2: Displayed in Tukey boxplots are the relative abundance of genera in the samples (Day Zero and Day 28) broken down by stool community type ................................................................. 39

Figure 3. Comparison of pre-vaccination and post vaccination log transformed HAI titres to the H1N1 strain (A), H3N2 strain (B), and B strain (C) ............................................................................. 45
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIVC</td>
<td>Australian Influenza Vaccine Committee</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Analysis of Covariance</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>ANZCTR</td>
<td>Australian New Zealand Clinical Trials Registry</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>BMR</td>
<td>Basal Metabolic Rate</td>
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<tr>
<td>CI</td>
<td>Confidence Interval</td>
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<tr>
<td>CRF</td>
<td>Case report forms</td>
</tr>
<tr>
<td>GF</td>
<td>Germ Free</td>
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<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GMT</td>
<td>Geometric mean titre</td>
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<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HAI</td>
<td>Haemagglutination-inhibition assay</td>
</tr>
<tr>
<td>IBS</td>
<td>Irritable bowel syndrome</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive Care Unit</td>
</tr>
<tr>
<td>ID</td>
<td>Identification</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>ILI</td>
<td>Influenza like illness</td>
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I.M. Intramuscular
LPS Lipopolysaccharide
M2 Matrix 2
MIMR Malaghan Institute of Medical Research
MRINZ Medical Research Institute of New Zealand
NA Neuraminidase
PAMP Pathogen associated molecular pattern
PRR Pathogen recognition receptors
PSA Polysaccharide A
RCT Randomised controlled trial
REDCap Research Electronic Data Capture
rRNA Ribosomal RNA
SARI Severe acute respiratory infection
S.C. Subcutaneous
SCFA Short chain fatty acids
SCT Stool community type
SNP Single Nucleotide Polymorphism
TCR T cell receptor
TLR Toll-like receptor
Treg Regulatory T cells
URTI Upper respiratory tract infection
Chapter 1: Introduction

1.0 Introduction to Thesis Topic
The underlying hypothesis of this study was that certain interactions between the host immune system and gut microbiota may be influential on the protective immune response against influenza infection. This hypothesis is supported by recent research which shows that antibiotic use alters gut microbiota composition and negatively affects host immune response\(^1-4\). To date there has been no clinical research in humans investigating the association between enterotype and immune response to influenza. This study aimed to explore the feasibility of undertaking a large randomised controlled trial (RCT) involving a dietary intervention designed to change enterotype in order to maximise response to the influenza vaccine. It also investigated the association between the gut microbiota, classified into enterotypes, and the protective immune response to influenza in adult humans, using the annual trivalent influenza vaccine (i.m), a standardised immune challenge, as a proxy for influenza infection.

1.1 Definition of the Gut Microbiota

1.1.1 The Gut Microbiota
While the exact number is under debate it is widely accepted that there are more bacterial cells present on the body than there are human cells. The bacteria that are present inside and outside the body are termed the “microbiota” and the collective genomes of these bacteria are termed the “microbiome”\(^5\). These bacteria reside in many sites across the body including the oral cavity, skin, airways, vagina and gut.

The gut, specifically the distal ileum and colon, contains the greatest density of bacterial cells\(^6\), approximately $10^{11}$-$10^{12}$ bacterial cells per ml of luminal content\(^7\).
The first major bacterial colonisation of humans occurs during birth. The source of colonisation differs depending on the route of delivery. The first exposure for naturally born babies is the mother’s vaginal flora while babies born through caesarean section are exposed to epidermal flora first. The next exposure for vaginally born infants is skin and oral flora. This exposes the infants to bacterial species that are similar to the first exposure for babies born via caesarean section. The infant gut microbiota fluctuates in density and composition over the first 18-36 months of life, but slowly begins to transition towards a profile that resembles a generalised adult microbiota community. The generalised adult profile is represented by a high level of Bacteroides and Firmicutes, moderate levels of Verrucomicrobia, and very low levels of Proteobacteria and aerobic Gram-negative bacteria.

Under normal conditions the gut of the human host provides a warm environment rich in nutrients. These are optimal growth conditions for many bacteria allowing them to proliferate rapidly and form a stable bacterial ecosystem. The bacteria in the distal ileum and colon digest complex carbohydrates that have passed through the small intestine, the complex carbohydrates are indigestible by the human host and are a source of nutrition that would be lost in the absence of the microbiota. Short chain fatty acids (SCFAs) like acetate, butyrate and propionate are metabolites produced by the gut microbiota when they breakdown the complex carbohydrates. These SCFAs can then be absorbed by the intestine and act as an important energy source for the human host. The also potentially assist in the inhibition of pathogens.

Not only does the microbiota complement normal digestive functions they also provide protection from pathogens through competitive exclusion. Bacteria that are present from shortly after birth outcompete pathogens for nutrients and space. This can prevent pathogenic bacteria from forming substantial colonies and thereby removing their opportunity to cause disease in humans.

The bacteria that make up the gut microbiota are often referred to as “commensals”. However, this is not strictly true as most gut bacteria exhibit a mutualistic (mutual benefit) relationship with the human host instead of commensal (where one organism benefits without affecting the other). The term commensal came from a lack of understanding about the
importance of gut microbiota in normal human health. The interactions above demonstrate the mutualistic relationship between the gut microbiota and human host. The bacteria benefit from optimal growth conditions and nutrition provided by the diet of the human host. The human host benefits from increased nutritional intake and protection from potential pathogens.

Another aspect of the mutualistic relationship is the role the gut microbiota plays in the maturation and normal function of the human immune system. This interaction was the focus of this research and will be expanded on below.

1.1.2 Enterotypes
One of the difficulties faced when working with the human gut microbiota is the diversity of gut microbiota between and within individuals\(^\text{19}\). An enterotype is a categorical label that can be applied to a gut microbiota sample based on the abundance of different bacterial genera\(^\text{20}\). Enterotypes are commonly known as stool community types (SCTs).

Arumugam \textit{et al} first proposed that individuals could be assigned to one of three SCTs based on the abundance of \textit{Bacteroides}, \textit{Prevotella} and \textit{Ruminococcus}\(^\text{20}\). Using a 16S rRNA data set of 300 individuals collected as part of the Human Microbiome Project (HMP), Ding and Schloss identified four distinct SCTs present in the gut\(^\text{21}\) using Dirichlet multinomial mixture (DMM) models. The SCTs were categorised as A, B, C and D (hereafter referred to as SCT-A, SCT-B, SCT-C and SCT-D). SCT- A lacked \textit{Prevotella} and Ruminococcaceae but had the highest levels of \textit{Bacteroides}. SCT-B was dominated by populations associated with the Firmicutes and had the least \textit{Bacteroides}. SCT-C had greater amounts of \textit{Alistipes}, \textit{Faecalibacterium} and \textit{Ruminococcaceae} but a lack of \textit{Prevotella} and a lower relative abundance of \textit{Bacteroides}. SCT-D had higher levels of \textit{Prevotella} than SCT-A and SCT-C but fewer \textit{Bacteroides}. It is these SCTs that were of interest in this study.
1.2 Potential Influence Factors on Microbiota Composition and Stability

One of the major environmental aspects that can affect a person’s gut microbiota is diet. Microbes from food have been found to colonise the gut which suggests that not only is food a source of environmental selection for the gut microbiota but it is also a source of new strains\(^\text{22}\).

Research has demonstrated evidence that diet can affect the gut microbiota. A study of obese participants undergoing diet therapy found that when participants lost a significant amount of weight they had an increase in Bacteriodetes and decrease in Firmicutes in their gut microbiota\(^\text{23}\). Additionally, significant differences in gut microbiota composition have been observed between children from Burkina Faso in Africa and Florence, Italy\(^\text{24}\). As well as the higher levels of Bacteroidetes and lower levels of Firmicutes, children from Burkina Faso were found to have a unique abundance of bacteria from the *Prevotella* genus and the *Xylanibacter* genus, which are known to contain genes for cellulose and xylan hydrolysis. These bacteria were absent from the Italian children and correlated with the plant matter rich diet of the children from Burkina Faso\(^\text{24}\).

Wu *et al* observed that a change in microbial composition could be detected within 24 hours of a change to a high-fat/low-fibre or high-fibre/low-fat diet\(^\text{25}\). A significant difference between microbiota samples has also been observed from previously vegetarian individuals following the consumption of an animal based diet\(^\text{22}\). The changes occurred one day after the dietary intake reached the gut microbiota in the distal intestine and the microbiota returned to the original state two days after the animal based diet was ceased.

In terms of dietary influence on the gut microbiota, probiotics and prebiotics are of particular interest. Probiotics are live microbes while prebiotics are non-digestible food ingredients that have been shown to stimulate the growth of selected beneficial bacteria\(^\text{10}\). Decreased levels of *Bifidobacterium* have been demonstrated in the gut microbiota of elderly individuals\(^\text{26}\) and low *Bifidobacterium* levels have been associated with irritable bowel syndrome (IBS), coeliac disease and obesity\(^\text{27,28}\). Current research has shown that probiotic supplementation in elderly individuals can increase levels of Bifidobacteria and decrease levels of deleterious bacteria\(^\text{29}\).
Supplementation with prebiotics has also been found to increase *Bifidobacterium* levels in elderly participants\(^4\).

Of particular relevance to this study, a 2014 Cochrane review of probiotics and upper respiratory tract infections (URTIs) found that probiotics were more effective than placebo at reducing the number of acute URTI episodes and their duration in humans\(^30\). However, the researchers did note a low quality of evidence and a need for more trials with better protocols.

The use of probiotics has also been shown to decrease the proportional change of Firmicutes and Proteobacteria that occurs during antibiotic treatment for *Helicobacter pylori* infection\(^31\).

Antibiotic use is another factor with potential impact on the composition and stability of the gut microbiota. While antibiotics are beneficial in fighting infection they may have the unintended side effect of altering the normal composition of the microbiota leading to opportunities for pathogens to exploit and negatively impact the hosts health\(^32\).

A study looking at the effects of antibiotics on the gut microbiota of elderly patients found a 10-fold decrease in the relative abundance of *Bacteroides-Prevotella* group. Complete elimination of some bacterial communities was also observed in a few of the participants taking antibiotics\(^32\).

Administration of ciprofloxacin was shown to result in a compositional change in the microbiota of healthy, young participants\(^33\). The compositional change observed continued after the antibiotic treatment ceased. The data obtained also suggested that there was an increase in strains resistant to ciprofloxacin.

While it has been shown that the composition of the gut microbiota may change due to dietary interventions, these changes may not lead to a change in SCT. For example, two of the SCTs identified by Arumugam *et al*\(^20\) (*Bacteroides* and *Prevotella*) were later confirmed by Wu *et al*\(^25\) and shown to be associated with the individual’s long term diet but not short term dietary changes. The *Bacteroides* SCT was associated with a “Western-type” diet which is high in proteins and fats and the *Prevotella* SCT was associated with plant fibre intake.
Collectively these data suggest that a short term change in diet alone may not be sufficient to induce a change in stool community type\textsuperscript{24,25}. Pre and probiotics in combination with either a short term or long term dietary intervention may be more successful in altering SCT in an adult population, although more research is needed before robust conclusions can be drawn.

While dietary intake is the focus of this thesis it should be noted that the host’s genetic make-up exerts some degree of influence on gut microbiota composition\textsuperscript{34–36}. This genetic influence may act as a confounding factor when analysing results and should be kept in mind when designing study protocols.

### 1.3 Immune System

The human immune system is made up of two distinct yet interconnected components: the innate and adaptive immune systems.

The main features of the innate immune system are the physical barriers of the body, myeloid cells, cytokines and the complement cascade\textsuperscript{37–39}. The innate immune system allows organisms to deal with pathogens in a general sense, it is not specific to certain pathogens and does not confer lasting immunity\textsuperscript{40}.

The adaptive immune system consists mainly of T and B lymphocytes. These lymphocytes differentiate into many different sub classes depending on the signals provided. The response generated by the adaptive immune response is pathogen specific and long lasting\textsuperscript{41}.

While this study focused on using the production of antibodies by B cells as a measure of immune response it is important to acknowledge that the effects of microbiota on the immune system extend further than simply B cell activity. Below is a brief overview of the normal function of the immune system and the observed effects of gut bacteria on these functions.
1.3.1 Innate Immune System
Epithelial layers, mucous, cilia clearance and low pH are just some of the components that make up the anatomical and physiological barriers within the human body\textsuperscript{39}. These barriers act as the first line of defence against infection.

The innate immune system detects potential pathogens through a set of germline encoded receptors that are sometimes referred to as pattern recognition receptors (PRRs)\textsuperscript{16,42}. There are many different receptor families including Toll-like receptors (TLRs), NOD – like receptors and RIG-1-like receptors\textsuperscript{16}. These germline receptors detect pathogens through conserved molecular patterns which are sometimes referred to as pathogen associated molecular patterns (PAMPs)\textsuperscript{42,43}. They include lipopolysaccharide (LPS), peptidoglycan, viral double stranded RNA and bacterial flagellar proteins\textsuperscript{37}. These conserved targets are usually crucial for the proper function of the microbe and as such are seldom altered by mutation\textsuperscript{38,39}. This allows for the recognition of a large number of foreign microbes from a small set of germline encoded receptors. After a receptor is activated there are many potential downstream responses based on the signal provided. These responses can range from inducing neutrophil differentiation and recruitment, to activating the complement cascade, to the activation of natural killer cells\textsuperscript{44}.

1.3.2 Adaptive Immune System
The adaptive immune system consists primarily of T and B cells\textsuperscript{14,41,45,46}. T cells develop in the thymus and after going through positive and negative selection they migrate to the lymph nodes\textsuperscript{41,44}. In the lymph nodes they are presented with antigens by antigen presenting cells (APC’s) such as macrophages and dendritic cells\textsuperscript{41}. Dendritic cells are a subset of immune cells which are crucial to the maturation of T and B cells\textsuperscript{52}. Dendritic cells specialise in the uptake and processing of antigens which are presented to naïve T Cells. Full T cell activation requires three separate signals: antigen recognition through the T cell receptor (TCR), costimulatory signals through CD28 and signalling from cytokines\textsuperscript{46,47}. Once these signals are received the T cell differentiates into one of many T cell sub classes such as T helper cells, cytotoxic T cells, and regulatory T cells (Treg)\textsuperscript{37}.
T helper cells come in multiple forms. The main forms are Th1, Th2 and Th17. Th1 cells release cytokines which activate mononuclear phagocytes, cytotoxic T cells and natural killer cells\(^\text{41}\). Th2 cells secrete cytokines that provide help to B cell antibody production\(^\text{41}\). Th17 cells are involved in increasing the response of neutrophils and promoting inflammation\(^\text{41}\).

The primary role of cytotoxic T cells is the induction of apoptosis (programmed cell death) of cells infected by intracellular microbes\(^\text{37,48}\). Cytotoxic T cells achieve apoptosis of the target cell through the secretion of granzymes and perforin as well as the activation of the Fas receptor\(^\text{41}\).

Treg cells are important in regulating the immune response and preventing autoimmunity. They accomplish this by supressing self-reactive T and B cell responses\(^\text{49}\). Treg cells are also crucial to host tolerance of the gut microbiota\(^\text{50}\).

B cells are formed in the bone marrow and are responsible for generation of antibodies\(^\text{41,44}\). They are able to recognise and bind antigens without the need for the antigen to be presented by an APC\(^\text{51}\). B cells can be activated by antigen in a T cell dependent manner or a T cell independent manner\(^\text{41}\). T cell independent activation leads to B cell maturation into antibody producing plasma cells\(^\text{51}\). T cell dependent activation results in proliferation and differentiation into either plasma cells or initiation of a germinal centre reaction\(^\text{51}\). Germinal centres are an important part of the B cell response and result in B cells that produce high affinity antibodies and memory B cells\(^\text{45,51}\).

Antibodies produced from plasma cells bind strongly to their corresponding antigen and depending on the antibody target are able to neutralise toxins, prevent receptor binding, activate the complement cascade or target the bacteria for phagocytosis\(^\text{44}\).

During the initial immune response memory T and B cells are produced which are long lived and are able to be activated quickly during a subsequent exposure of the same antigen\(^\text{41}\).
1.4 Interaction between the Gut Microbiota and the Immune System

The gut microbiota interacts with both components of the immune system to support appropriate immune responses, and helps to strengthen the response to pathogens. Research on Germ free (GF) animals has given great insight into the role the microbiota has in normal development. GF rodents are typically reared in a plastic film isolator using germ free techniques\textsuperscript{53}. The term germ free is used when the animal is free from all demonstrable forms of life including bacteria, viruses, fungi, parasites etc. GF animals can be studied in this state or can be colonised by known microbial life to determine specific interactions\textsuperscript{54}. GF animals that have been colonised by known microbial species are termed gnotobiotic\textsuperscript{55}. Another type of lab animal used for microbiota research are termed specific pathogen free. These animals are colonised by symbiotic microbes, but free from specific pathogens that may produce infections, either clinical or sub-clinical, which may cause experimental bias\textsuperscript{55}.

GF rodents display marked phenotypic changes compared with normally colonised rodents including an altered intestinal cell morphology, and reduced gastrointestinal motility\textsuperscript{55}. As well as these GI tract changes there are also many defects observed in the GF rodent immune system. Smaller Peyer’s patches and mesenteric lymph nodes have been observed as well as far fewer isolated lymphoid follicles when compared to non-GF rodents. Lower antibody levels and a higher susceptibility to allergy have also been observed in GF mice\textsuperscript{55}. Collectively these observations suggest the microbiota has an effect on both mucosal and systemic immunity.

1.4.1 Interactions between the Gut Microbiota and the Innate Immune System

In the case of the gut, the major component of the innate immune system is the physical barrier of the intestinal epithelial cells and leukocytes located in the lamina propria\textsuperscript{16,56}. The barrier is formed by the intestinal epithelial cells and is complemented by a layer of mucous made up of glycoproteins which are secreted by goblet cells in the epithelium\textsuperscript{14}. In the colon this mucus layer is separated into two layers, an outer layer and a more viscous inner layer\textsuperscript{57}. The inner layer acts as a barrier to bacterial penetration of the epithelium, while the structure of the outer layer allows bacterial adhesion and colony formation. The gut microbiota that
infiltrate the inner layer and intestinal epithelium are phagocytosed by macrophages in the lamina propria and broken down for presentation to cells of the adaptive immune system.

The innate immune system is not simply a passive presence when it comes to the gut microbiota. Instead of just simply dealing with bacteria that infiltrate the epithelium it actively influences the composition of the microbiota present in the mucus layer\textsuperscript{16}. This is evidenced by the activity of the NOD2 receptor. NOD2 is highly expressed on the Paneth cells in the small intestine and is activated by microbial peptidoglycan\textsuperscript{16}. When activated, NOD2 triggers many cellular responses including cytokine secretion, epithelial regeneration, induction of autophagy, and production of microbial peptides. These processes combine to affect the microbial composition of the intestine\textsuperscript{58}.

Macrophages are a part of the innate immune response but are also important APCs for the adaptive immune response. When stimulated via TLR5, a PRR which recognises bacterial flagellin, they produce IL-6\textsuperscript{59}. Macrophage produced IL-6 has been shown to support antibody production by B cells\textsuperscript{2}. This illustrates that interactions between the innate immune system and the microbiota can have downstream consequences for the adaptive immune system.

1.4.2 The Effect of the Gut Microbiota on the Adaptive Immune System

1.4.2.1 T Cells

Bacterial colonisation of the intestinal mucosa contributes to Th1, Th2 and Th17 differentiation as well as the development of Tregs\textsuperscript{60}. GF mice are normally skewed towards a Th2 immune response but this skewing can be rebalanced following colonisation of GF mice with \textit{Bacteroides fragilis}\textsuperscript{50,61}. It has also been found that polysaccharide A (PSA) from \textit{B. fragilis} induces CD4+ T cell development\textsuperscript{62}.

Th17 cells are absent in the small intestine of GF mice but can be restored after colonisation of normal mouse microbiota\textsuperscript{50}. Th17 cells are a predominant source of IL-17 which is involved with maintaining the intestinal mucus layer and elimination of pathogens in the tissues. In addition
Foxp3+ Treg differentiation and IL-10 production in the gut have been shown to be stimulated by the gut microbiota\(^{61,63}\).

Tfh cells are a subset of helper T cells that have recently caught the attention of researchers\(^{64}\). They are essential for germinal centre formation, the development of high-affinity antibodies and memory B cells\(^{65}\). One of the main regulators of Tfh differentiation is IL-6\(^{65}\) which has been shown to be upregulated by TLR5 activation\(^2\). While the effect of bacteria on Tfh and B cells remains unclear the TLR5 – IL-6 pathway provides potential insight into this relationship.

1.4.2.2 B Cells
Bacteria that penetrate the inner mucosal layer and the epithelial layer are quickly phagocytosed by dendritic cells\(^{57}\). These dendritic cells migrate to the Peyer’s patches and interact with T and B cells, resulting in induction of B cells to produce IgA specific for the intestinal bacteria. These B cells migrate to the lamina propria and secrete IgA. IgA moves through the epithelial layer into the inner mucus layer and binds to bacteria preventing them from crossing the epithelial layer\(^{57}\).

Through animal models it has been demonstrated that the gut microbiota have a crucial role in B cell response to influenza vaccination through the TLR5 signalling pathway\(^2\). It has also been shown that antibiotic treated mice exhibit a decreased antibody response to trivalent inactivated influenza vaccine and generate fewer memory B cells\(^2\). However, more research needs to be conducted to establish if this effect is reproducible in humans.

1.5 Vaccination as a Proxy for Immune Response
One of the best ways to test the human immune response is through vaccination. It is delivered in a standard dose, the safety profile for vaccines are well known and the vaccination benefits the participant once study participation has concluded. For this study the influenza vaccine was selected due to previous studies using mice\(^1,2\).
When using the influenza vaccine as a proxy for immune response there is the added benefit of a widely accepted assay used to assess the response to the vaccine. This assay is known as a haemagglutination-inhibition (HAI) assay. The HAI assay detects the levels of HA antibody in the blood sample and is a correlate of protection from infection\textsuperscript{66}. A post vaccination HAI titre of \( \geq 40 \) is determined to be protective against infection and is termed as seroprotection\textsuperscript{67}. Other measures of vaccine effectiveness are seroconversion and change in geometric mean titre (GMT). Seroconversion is defined as a \( \geq 4 \) fold increase in HAI titre from baseline or a post vaccination HAI titre of 4 times the lower detection limit if the baseline HAI titre is below the lower detection limit\textsuperscript{66}.

1.6 Vaccination

Vaccination is the process of priming the immune system, so it can rapidly and strongly respond to a specific pathogen when it is encountered again in the future. The primary types of vaccine are live attenuated and inactivated. Live attenuated vaccines contain viruses or bacteria that are still alive but are unable to cause disease in a human host\textsuperscript{68}. Inactivated vaccines do not contain live viruses or bacteria. Instead the organism has been destroyed while its antigenic components are left intact\textsuperscript{69}.

When a vaccine is administered the immune system reacts to the antigenic content in the same way it would to an actual infection. The innate and adaptive immune systems are activated leading to local inflammation of the administration site as well as naive B cell maturation which leads to production of antibodies specific to the antigens contained in the vaccine.

1.6.1 Influenza

1.6.1.1 Influenza Virus

The influenza A, B and C viruses come from the Othomyxoviridae family and are three of the five genera in this family\textsuperscript{70}. The influenza viruses are composed of 7-8 segmented single stranded RNA sequences surrounded by a lipid membrane\textsuperscript{70–72}. Influenza A is of avian origin and
transmits to other species now and then, while Influenza B almost exclusively infects humans. Fever, cough, myalgia and weakness are the most common symptoms associated with Influenza infection.

Influenza A has the proteins HA, NA and M2 in its surface membrane while Influenza B has HA, NA, NB and BM2. 16 HA subtypes and 9 NA subtypes have been found.

The standard nomenclature for influenza strains is virus type; species that it was isolated from (if not human); geographic location where it was first isolated; isolate number; isolate year; HA and NA subtype (Influenza A viruses only).

1.6.1.2 Influenza Vaccine
Both live attenuated and inactivated vaccines are used to vaccinate against influenza. The inactivated vaccine is more commonly used and more is known about its mechanism of protection. Influenza vaccines are most commonly administered via injection while some newer vaccines are able to be administered via nasal spray. Most common seasonal influenza vaccines are trivalent. The term trivalent means the vaccine contains three strains representing influenza A H1N1, influenza A H3N2 and influenza B. The strains contained in the vaccine change season to season and are chosen based on predictions of the major strains that will be circulating later in the coming influenza season. The chosen strains are combined with a laboratory strain adapted to grow in eggs. This process creates reference strains which are grown in fertilised eggs before being harvested. The influenza vaccine prompts the body to produce antibodies to the HA and NA membrane proteins.

1.6.1.3 The Effect of the Gut Microbiota on the Immune Response to Influenza
Previous studies using germ free and antibiotic treated mouse models have looked at the role of gut microbiota in normal immune response to influenza vaccination. Ichinohe et al. found that neomycin sensitive bacteria were crucial to normal immune response in the lungs. Mice treated with neomycin had reduced CD4 T cell, CD8 T cell and B cell responses to influenza.
infection. Both the T cell and B cell responses were able to be rescued by a single dose of lipopolysaccharide (LPS), a TLR agonist, either locally (nasal) or distally (rectal) mimicking bacterial colonisation.

Oh et al found that mice deficient in TLR5, a PRR that recognises bacterial flagellin, had reduced antibody titres and lower frequencies of antibody secreting plasma cells post vaccination with the trivalent influenza vaccine. They also demonstrated that a lack of TLR5 signalling resulted in impaired function of memory B cells. The effect of TLR5 signalling demonstrated by Oh et al was shown to be specific to non-adjuvanted subunit vaccines and could not be replicated using adjuvanted or live attenuated vaccines.

The data from Oh et al also suggests a greater importance on multiple groups of bacteria rather than specific bacteria strains on regulation of host immune response to the trivalent influenza vaccine.

1.7 Summary
The hypothesis for this study was that certain host-microbiota interactions have a positive effect on the immune response of the host to influenza; and that an individual’s SCT can be predictive of their immune response to influenza. If this were the case, then future double-blind, randomised, placebo-controlled RCTs might be designed whereby possible dietary interventions aimed at switching SCTs associated with a weak immune response to a SCT associated with a strong immune response could be undertaken using vaccinations such as a seasonal influenza vaccine as a proxy for infection. The interaction between gut microbiota and immune response has mainly been studied through the use of mouse models. These RCTs would be the first of their kind undertaken in humans and would give further insight into the translation of mouse model data to humans.

Due to the lack of RCT’s of this nature, there are a number of feasibility issues that may impede the success of a large RCT. This study aimed to provide answers to these feasibility issues in order to enable the successful completion of a large RCT. The feasibility issues could be loosely
categorised into; likely proportion of participants that can complete a 6-month study, participant adherence to RCT requirements, testing and sampling issues, and possible exclusion criteria.

This study therefore, focused on two main issues. Firstly, if a large RCT were to be developed with the intention of trialling dietary interventions, with the aim of changing gut enterotypes in participants, and thereby influencing their immune response, what are the feasibility issues that should be considered when allocating resources and calculating the numbers of participants who would need to be enrolled into a RCT? Secondly, is there an association between SCT and responsiveness to the seasonal influenza vaccine that suggests individuals with one SCT have a better immune response than individuals with another SCT?
Chapter 2: Methods

2.1 Study Setting
This study was conducted at the Malaghan Institute of Medical Research (MIMR) and the Medical Research Institute of New Zealand (MRINZ), both in Wellington, New Zealand. The majority of the study visits were conducted at MIMR and a small number of consent visits were conducted at MRINZ. The study was approved by the Central Health and Disability Ethics Committee, reference number 15/CEN/207. The study was registered on the Australian New Zealand Clinical Trial Registry (ANZCTR) with the trial id ACTRN12615001365550.

2.2 Vaccine
Participants were vaccinated with the Medsafe approved 2016 Influvac® (Mylan, Illinois, USA) seasonal influenza vaccine. Influvac® is a clear colourless suspension for injection. It is an egg-grown, inactivated influenza virus vaccine based on isolated surface antigens of A and B strains of myxovirus influenza. The type and amount of viral antigens in Influvac® conformed to the requirements of the Australian Influenza Vaccine Committee (AIVC) and the New Zealand Ministry of Health for the winter of 2016. The strains chosen for vaccine manufacture are endorsed by the AVIC as being antigenically equivalent to the reference virus strains.

Each 0.5mL of the Influvac® vaccine contains antigens representative of the following type:

- A/California/7/2009 (H1N1)pdm09-like strain (A/California/7/2009, X-181) 15 µg haemagglutinin/dose
- A/Hong Kong/4801/2014 (H3N2)-like strain (A/New Caledonia/71/2014, X257A) 15 µg haemagglutinin/dose
- B/Brisbane/60/2008-like strain (B/Brisbane/60/2008, wild type) 15 µg haemagglutinin/dose
Inactive compounds in the Influvac® vaccine include: potassium chloride, monobasic potassium phosphate, disodium hydrogen phosphate anhydrous, sodium chloride, calcium chloride, magnesium chloride and water for injections.

Participants were vaccinated by a trained nurse or doctor. Accountability of the vaccine was managed as follows: the time and body location of vaccination was recorded on the Day Zero study worksheet. The vaccinator and a second member of the study staff signed the study worksheet to confirm the vaccine was given. The sticker from the label on the syringe was retained and stuck to the study worksheet. The empty vaccine syringes were disposed in a sharps bin.

With the exception of one, all vaccinations were administered intramuscularly. The remaining vaccination was administered subcutaneously due to participant use of warfarin.

As the vaccine only required one dose, there was no concern regarding treatment adherence or the need to discontinue the intervention.

2.2.1 Measure of Antibody Response
Haemagglutination inhibition (HAI) assays were used to assess participant antibody response to the influenza vaccination. The HAI assay measures antibody titre based on the ability of the serum sample to prevent agglutination of chicken blood exposed to a matching influenza virus strain. The HAI titre is based on serial dilutions e.g. 1:10, 1:20, 1:40 and so on. The assay used in this study had a minimum limit of detection of 1:10 and a maximum limit of detection of 1:2560. For participants with HAI titres below the limit of detection (<10) their result was deemed to be half of the lower limit of detection (5). The standard protocol for an HAI assay can be found in Appendix III.
2.2.1.1 Seroconversion and Seroprotection
Seroconversion and seroprotection are traditional measures of response to influenza vaccination. Seroconversion is measured as a ≥4 fold increase in HAI titre if pre vaccination HAI titre was ≥1:10; or a post vaccination HAI titre of 1: 40 if pre vaccination HAI titre was <1:10.

Seroprotection is measured as a post vaccination HAI titre of ≥1:4067.

Seroconversion and seroprotection rates were calculated for each strain individually. These numbers were used to calculate the number of participants who seroconverted to, and were seroprotected against, all strains in the vaccine.

2.3 Objectives
There were a number of objectives in order to establish the feasibility of a large RCT which were:

- Establishing the expected withdrawal rate for a large RCT.
- Measuring the likely completion rate of the lifestyle questionnaire in a large RCT by estimating the proportion of participants who completed a minimum of 90% of the questions in the Lifestyle Questionnaire.
- Establishing the proportion of participants who provided stool samples at Day Zero and Day 28, and the proportion of samples that were adequately labelled at Day Zero and Day 28.
- Establishing the likelihood of obtaining viable blood samples at all visits for the proposed RCT by estimating the proportion of blood samples physically obtained versus planned, and the proportion of consequently analysable samples obtained at the visits on Day Zero, Day Three, Day Seven, Day 28 and Day 180.
- Identification of the likely exclusion criteria for participants in a large RCT, with particular focus on:
- The proportion of participants who took systemic antibiotics and/or systemic corticosteroids within 30 days of Day Zero, and between Day Zero and the Day 180 follow up visit.

- The proportion of participants who had an influenza vaccine in the previous two years.

- The proportion of participants who were pregnant at Day Zero, or became pregnant during the study.

- Establishing that a New Zealand population could be mapped to SCT-A, SCT-B, SCT-C and SCT-D, and understanding the stability of within-participant SCT over time. In order to achieve this objective, the following proportions were determined:

  - The proportion of participants who can be mapped to any of the four pre-specified SCTs at Day Zero and at Day 28.

  - The proportion of participants who mapped to each of the SCTs at Day Zero and at Day 28.

- Establish whether there is an association between SCT and post vaccination HAI titre/seroconversionseroprotection to EACH and ALL strains of the influenza vaccine at Day 28.

2.4 Participant Timeline
Participants were enrolled in the study at least seven days before Day Zero. In the seven days leading up to Day Zero the participants completed a lifestyle questionnaire (electronically or on paper), seven-day food diary and collected a stool sample once at least three days of the food diary had been completed. Participants had a blood sample collected on Day Zero and were vaccinated once the blood sample had been collected. The lifestyle questionnaire, stool sample and food diary were given to study staff pre-blood sample collection. The participants then had follow up appointments on Day Three and Day Seven. A blood sample was collected at each
visit. At the visit on Day Seven the participants were given a second food diary and stool sample collection kit. In the seven days leading up to their follow up visit on Day 28, the participants filled in the food diary. The participants also collected a stool sample once at least three days of the food diary had been completed. On Day 28 participants gave their stool sample and food diary to study staff before a blood sample was collected. The final follow up visit occurred 180 days after Day Zero and involved the collection of a final blood sample. Concomitant medication data was collected at each visit from consent to Day 28. Adverse event data was collected from Day Zero to Day 28.
### Table 1. Schedule of Procedures

<table>
<thead>
<tr>
<th>Day -14 to Day -7</th>
<th>Day -7 to Day Zero</th>
<th>Day Zero</th>
<th>Day Three</th>
<th>Day Seven</th>
<th>Day 21 to Day 28</th>
<th>Day 28</th>
<th>Day 180</th>
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<td><strong>Informed Consent</strong></td>
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<td><strong>Stool Sample</strong></td>
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<tr>
<td><strong>Vaccination</strong></td>
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<td></td>
<td>X</td>
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<tr>
<td><strong>Blood Sample</strong></td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><strong>Concomitant medications</strong></td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
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<tr>
<td><strong>Adverse Events</strong></td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

\[\text{a}\] 1 x 6ml Serum tube, 7 x 10 ml and 1 x 6ml Heparin tubes, and 1 x 3ml CBC tube.  
\[\text{b}\] 1 x 10mL and 1 x 3mL Heparin tubes.  
\[\text{c}\] 5 x 10 mL Heparin tubes.  
\[\text{d}\] 1 x 6ml Serum tube.  
\[\text{e}\] 1 x 6mL Serum tube, 1 x 6ml EDTA tube, and 2 x 10mL Heparin tubes.
2.5 Sample Size
To ensure that estimates of proportions had 95% confidence intervals of around +/- 10% a minimum of 100 participants were required to complete the study in its entirety. It was anticipated that a 20% withdrawal and drop-out rate was possible, therefore a sample size of 125 participants was chosen.

2.6 Recruitment
Participants were recruited using email and poster advertising. A database of previous study participants from MRINZ were emailed asking if they were interested in taking part in the study and the MIMR sent similar email correspondence to their database. The School of Biological Sciences and Facilities Management at Victoria University, Wellington also sent out emails to their staff and post-graduate students. Posters were displayed at the Kelburn Campus of Victoria University, Wellington, with information about the study and who to contact. Potential participants were initially contacted via email and received a brief overview of the study. If they expressed interest in being part of the study, they were then emailed a copy of the participant information sheet to read. The potential participants who were still interested after reading the information sheet were emailed a study schedule which included the available Day Zero (vaccination) dates as well as the follow up dates for each different Day Zero. Once the potential participant selected a suitable Day Zero date they were booked in for a first visit at least one week before their pre-selected Day Zero date.

2.7 Eligibility Criteria
Participants were eligible for the study if they were aged between 18 and 64 years, able to give informed consent, and able to comply with all trial requirements.

Exclusion criteria were:

- A known severe reaction or allergy to any components of the influenza vaccine.
- Any contra-indications to vaccination per recommendations of vaccine manufacturer.

- A history of Guillain-Barre Syndrome within six weeks of receiving a previous influenza vaccine.

- An impaired immune system that may confound immune response testing i.e. any condition that impairs participant immune response through either the condition or through the treatment of the condition.

- Already received the 2016 seasonal influenza vaccine.

- Any clinical condition which the investigator deemed relevant for exclusion from the study.

There were no prohibited treatments or medications while participants were participating in the study. Due to the exploratory nature of this study, participants were not excluded based on antibiotic or corticosteroid use. Participant use of antibiotics and corticosteroids was recorded in order to determine the effect on the feasibility of a future RCT if their use were to be an exclusion criteria.

2.8 Data Collection Methods and Data Management

Data was collected using paper-based case report forms (CRFs) designed by the study team. Study data were collected and managed using REDCap (Research Electronic Data Capture) electronic data capture tools hosted at MRINZ79. REDCap is a secure, web-based application designed to support data capture for research studies, providing; an intuitive interface for validated data entry, audit trails for tracking data manipulation and export procedures, automated export procedures for seamless data downloads to common statistical packages; and procedures for importing data from external sources.

Data was entered into REDCap using double data reconciliation as a quality control. All investigators collecting data were instructed on how to fill out the CRFs before commencement
of the study. Data entered into REDCap from the CRFs included consent details, demographic information, concomitant medication, adverse events, and details of each study visit. The Day Zero full blood count report was also entered into REDCap.

The data that was not entered into REDCap was food diary data, lifestyle questionnaire data, results from the HAI assay and results from the stool sample analysis.

Dietary data was collected using a paper-based food diary (provided by Sally Poppitt, Director of the Human Nutrition Unit, University of Auckland, New Zealand). It required the participant to record the amount of food consumed and the time the food was consumed in writing. Dietitians were available at Day Zero and Day 28 visits to review diaries with participants and assist with quality control of nutritional data. The diaries, once collected, were sent to the Human Nutrition Unit at the University of Auckland, New Zealand and analysed using FoodWorks 7 (Xyris Software, Australia) software.

The lifestyle questionnaire was collected using either an online survey (created using www.wufoo.com) or a paper-based copy. The online survey was the preferred option and the paper-based copy acted as a backup if the participant was unable to use the online survey. If the participant completed the paper-based copy, then the data was entered into the online survey system by an investigator and reviewed for entry errors by a second investigator. The lifestyle questionnaire was based on the questionnaire administered by the American Gut arm of the Human Microbiome Project and adapted for use in a New Zealand population.

The antibody level results and stool sample analysis results were sent to the investigators in Excel spreadsheet format by the MIMR and stored in the ‘Study’ folder on the MRINZ server.

Participants received reminder texts and emails to prompt them that they had a visit coming up or that they needed to record food diary data.

All data collected up until the point of withdrawal/loss to follow up was used in final analysis.

When participants were enrolled into the study they were given an identification number (ID number) that consisted of the letter “M” followed by three digits from “001” to “125”. If a participant missed their vaccination visit they were given a new number to indicate that they
were completing a second food diary and collecting a second stool sample. Those numbers started from “126” and participants kept the secondary number for the rest of the study.

Participants were given their study materials in a blue folder which was labelled with their study ID number. The folder allowed the participants to store their stool sample discreetly as well as store all study related materials including the food diary, lifestyle questionnaire and instructions.

2.9 Statistical Methods
Proportions were calculated with a 95% confidence interval (CI).

Exact binomial confidence intervals for dichotomous variables were calculated using the Clopper-Pearson method.

Logistic regression was used to explore the association between SCT and post vaccination HAI titre/seroconversion/seroprotection using ANOVA for univariate analysis and ANCOVA for the multivariate analysis, which included possible confounding variables of body mass index (BMI), age and vaccination within the last two years. Normality assumptions for absolute values of HAI titres were not met, so HAI values were logarithm transformed.

The back-transformation by exponentiation of a difference in logarithms is interpreted as a ratio of geometric means.

SAS version 9.4 was used.

2.9.1 Completion of the Lifestyle Questionnaire
Lifestyle questionnaire completion was defined as an answer provided for ≥90% of the questions contained in the questionnaire. Any sub-questions that did not require an answer were discounted when calculating completion percentage.
2.9.2 Blood Sample Collection
The number of tubes of blood that were obtained from each participant were recorded and compared to the number of tubes of blood that were planned to be collected. These numbers were used to calculate the proportion of participants for whom all blood samples were successfully collected.

2.9.3 Potential Exclusion Criteria
The lifestyle questionnaire recorded pregnancy status at Day Zero along with influenza vaccination history. Participants were asked at their first visit if they had used antibiotics or steroids in the 30 days prior to the study visit. At each subsequent visit they were asked if they had used antibiotics or steroids since the previous study visit.

2.10 Study Procedures
2.10.1 Stool Sample Collection and Analysis
Stool samples were collected by participants using the OMNIgene GUT collection kit (DNA Genotek, Ontario, Canada). Samples were collected by participants in the three-day period prior to both Day Zero and Day 28. At the visits investigators checked the labels of the stool samples to ensure that both date and time were present and legible. If the date and/or time were not present/legible then the investigator adjusted the label with input from the participant. Participant provision of stool samples was recorded at each visit along with whether the labelling required investigator intervention.

The stool samples were stored at room temperature by the participant after collection. DNA extraction from the stool samples was performed at MIMR (Nucleospin Soil kit, Macherey-Nagel (Düren, Germany)), and extracted DNA was sent to New Zealand Genomics Limited for 16S rRNA sample library preparation and sequencing. SCTs were assigned using the following method: First bacteria were assigned to the taxonomic groups: Bacteroides genus, Prevotella genus, Alistipes genus, Ruminococcaceae family and Faecalibacterium genus. Then samples
were assigned to the different SCTs based on the abundance of the 5 taxa. The steps are detailed below:

1. Samples with *Bacteroides* proportions in the upper 90% (10\textsuperscript{th} to 100\textsuperscript{th} percentile) were assigned to SCT-D.

2. Samples with *Prevotella* proportions in the upper 60% (40\textsuperscript{th} to 100\textsuperscript{th} percentile) were assigned to SCT-C.

3. Samples with *Bacteroides* proportions in the upper 40% (60\textsuperscript{th} to 100\textsuperscript{th} percentile) were assigned to SCT-A.

4. Samples with *Prevotella* proportions in the upper 10% (90\textsuperscript{th} to 100\textsuperscript{th} percentile) were assigned to SCT-D.

5. All remaining unclassified samples were assigned to SCT-B.

The sequence of steps was determinate of the final assignment rather than each individual step.

### 2.10.2 Blood Sample Collection and Analysis

Blood samples were collected using a butterfly needle and vacutainer system (Beckton, Dickinson and Company, New Jersey, USA). Phlebotomy was performed by trained study staff. Serum samples were processed and stored at MIMR, and HAI assays were performed for each influenza strain by the National Centre for Biosecurity & Infectious Disease at the Institute of Environmental Science and Research Limited, Porirua, New Zealand.
Chapter 3: Results

3.1 Participant Flow

As displayed in Figure 1, 133 potential participants received the participant information sheet after registering interest about the study. Of those 133 potential participants, four declined to participate and two did not meet the inclusion criteria. The remaining 127 potential participants consented to the study. Two participants withdrew consent shortly after due to parking availability concerns. This left 125 participants in the study.

Of the 125 participants who progressed to Day Zero, 123 received the vaccination. With regards to the remaining two participants, one was too sick to attend their Day Zero and elected to withdraw from the study rather than reschedule. For the second participant, study staff were unable to obtain a baseline blood sample for HAI testing prior to their vaccination resulting in their withdrawal from the study. Of the 123 participants who received the intervention, only one was unable to attend the final visit on Day 180. All participants who consented to the study were included in the final analysis as part of the feasibility analysis. Only the 123 vaccinated participants were included in the association analysis.
Figure 1. Flow of participants

Enrolment

Sent study information (n=133)

Consent to Day Zero

Excluded (n=6)
- Not meeting inclusion criteria (n=2)
- Declined to participate (n=4)

Consented (n=127)

Withdraw consent (n=2)

Withdrawn (n=2)
- Unable to get baseline bloods (n=1)
- Ill on vaccination day (n=1)

Vaccination

Received vaccination (n=123)
Analysed up to Day 0 (n=125)

Day One to 28

Lost to follow-up (n=0)
Analysed up to Day 28 (n=123)

Day 29 to 180

Lost to follow-up (n=1)
- Out of country during visit window (n=1)
Analysed up to Day 180 (n=122)
3.2 Participant Characteristics

Participant characteristics are detailed in Table 2.

3.2.1 Characteristics for all Participants

Of the 125 participants that consented to the study, 77 (62.6%) were female and the mean (SD) age was 34.5 years (12.4). The mean (SD) BMI was 24.8 kg/m² (4.4).

Of the 123 participants who received the vaccination, 100 participants (81.3%) identified as New Zealand European or European while seven (7.7%) identified as Maori. Additionally, one participant (0.8%) identified as a Pacific Islander and seven (5.7%) identified as Asian.

When looking at environmental factors potentially affecting gut microbiota composition, 26 of the participants (21.7%) were delivered by caesarean section and 77 (69.4%) were primarily breast fed as infants.

Lifestyle questionnaire data indicated 10 (8.3%) of the participants identified as vegetarian and 22 (17.9%) were currently smoking. Two participants (1.6%) reported daily alcohol consumption while seven participants (5.7%) reported never drinking alcohol. Collection of medication use data revealed 12 participants (9.6%) had used antibiotics in the 30 days prior to their vaccination date.

When looking at potential factors affecting immune response, 101 participants (82.0%) had received an influenza vaccine previously of which 76 participants (61.8%) had received an influenza vaccine in the last two years. Additionally, 10 participants (8.0%) used corticosteroids in the 30 days prior to their vaccination date.
3.2.2 Characteristics by Stool Community Type

In SCT-A (n=64), the mean (SD) age was 33.7 years (12.7), the mean (SD) BMI was 24.2 kg/m² (4.2) and 42 participants (65.6%) were female. The number (percentage) of participants, who received an influenza vaccination in the previous two years was 42 (65.6%). Antibiotics were used by seven participants (10.9%) assigned to SCT-A in the 30 days prior to their Day Zero.

In SCT-B (n=4), the mean (SD) age was 38.3 years (9.6), the mean (SD) BMI was 31.7 kg/m² (8.5) and two participants (50.0%) were female. The number (percentage) of participants, who received an influenza vaccination in the previous two years was two (50.0%). Antibiotics were used by none of the participants assigned to SCT-B in the 30 days prior to their Day Zero.

In SCT-C (n=23), the mean (SD) age was 35.1 years (11.7), the mean (SD) BMI was 23.5 kg/m² (3.3) and 15 participants (65.2%) were female. The number (percentage) of participants, who received an influenza vaccination in the previous two years was 13 (56.5%). Antibiotics were used by three participants (13.0%) assigned to SCT-C in the 30 days prior to their Day Zero.

In SCT-D (n=32), the mean (SD) age was 35.3 years (13.3), the mean (SD) BMI was 25.9 kg/m² (3.9) and 18 participants (56.3%) were female. The number (percentage) of participants, who received an influenza vaccination in the previous two years was 19 (59.4%). Antibiotics were used by two participants (6.3%) assigned to SCT-D in the 30 days prior to their Day Zero.
Table 2. Demographic characteristics of participants, displayed overall as well as broken down by stool community type

<table>
<thead>
<tr>
<th>Stool community type (SCT) at Day Zero (n=123)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All (n=123)</td>
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<tr>
<td>----------</td>
</tr>
<tr>
<td>**Mean (SD)</td>
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<tr>
<td><strong>Age</strong></td>
</tr>
<tr>
<td><strong>BMI</strong> (Kg/m²)</td>
</tr>
<tr>
<td><strong>Proportion Female</strong></td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
</tr>
<tr>
<td>- NZ European / European</td>
</tr>
<tr>
<td>- Maori</td>
</tr>
<tr>
<td>- Pacific Islander</td>
</tr>
<tr>
<td>- Asian</td>
</tr>
<tr>
<td>- Other</td>
</tr>
<tr>
<td><strong>Obesity (BMI&gt;30 Kg/m²)</strong></td>
</tr>
<tr>
<td><strong>Caesarean birth</strong></td>
</tr>
<tr>
<td><strong>Vegetarian</strong></td>
</tr>
<tr>
<td><strong>Diet as an infant</strong></td>
</tr>
<tr>
<td>- Primarily Breast Milk</td>
</tr>
<tr>
<td>- Primarily Formula</td>
</tr>
<tr>
<td>- Mix of Breast Milk and Formula</td>
</tr>
<tr>
<td><strong>Alcohol consumption</strong></td>
</tr>
<tr>
<td>- Daily</td>
</tr>
<tr>
<td>- Regularly (3-5 times/week)</td>
</tr>
<tr>
<td>- Occasionally (1-2 times/week)</td>
</tr>
<tr>
<td>- Rarely</td>
</tr>
<tr>
<td>- Never</td>
</tr>
</tbody>
</table>
### Table 2 (cont). Demographic characteristics of participants, displayed overall as well as broken down by stool community type

<table>
<thead>
<tr>
<th>Stool community type (SCT) at Day Zero (n=123)</th>
<th>All (n=123)</th>
<th>SCT-A (n=64)</th>
<th>SCT-B (n=4)</th>
<th>SCT-C (n=23)</th>
<th>SCT-D (n=32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/123 (%)</td>
<td>N/64 (%)</td>
<td>N/4 (%)</td>
<td>N/23 (%)</td>
<td>N/32 (%)</td>
<td></td>
</tr>
<tr>
<td>Currently smoking</td>
<td>22 (17.9)</td>
<td>15 (23.4)</td>
<td>1 (25)</td>
<td>2 (8.7)</td>
<td>4 (12.5)</td>
</tr>
<tr>
<td>Diagnosis of IBS</td>
<td>10 (8.2)</td>
<td>9 (14.1)</td>
<td>0 (0)</td>
<td>0/22 (0)</td>
<td>1 (3.1)</td>
</tr>
<tr>
<td>Previous Influenza vaccination</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Ever</td>
<td>101 (82.1)</td>
<td>52 (81.3)</td>
<td>4 (100)</td>
<td>20 (87.0)</td>
<td>25 (78.1)</td>
</tr>
<tr>
<td>- Last two years</td>
<td>76 (61.8)</td>
<td>42 (65.6)</td>
<td>2 (50.0)</td>
<td>13 (56.5)</td>
<td>19 (59.4)</td>
</tr>
<tr>
<td>CMV positive</td>
<td>58 (47.2)</td>
<td>32 (55.2)</td>
<td>2 (50)</td>
<td>11 (47.8)</td>
<td>13 (40.6)</td>
</tr>
<tr>
<td>N/125^ (%)</td>
<td>N/64 (%)</td>
<td>N/4 (%)</td>
<td>N/23 (%)</td>
<td>N/32 (%)</td>
<td></td>
</tr>
<tr>
<td>Antibiotics used between Day -30 and Day Zero</td>
<td>12 (9.6)</td>
<td>7 (10.9)</td>
<td>0 (0)</td>
<td>3 (13.0)</td>
<td>2 (6.3)</td>
</tr>
<tr>
<td>Corticosteroids used between Day -30 and Day Zero</td>
<td>10 (8.0)</td>
<td>5 (7.8)</td>
<td>0 (0)</td>
<td>3 (13.0)</td>
<td>2 (6.3)</td>
</tr>
</tbody>
</table>

Abbreviations: SD, standard deviation; BMI, body mass index; IBS, irritable bowel syndrome; CMV, cytomegalovirus; SCT, stool community type.

* Sample size differs from the number provided at the top of the table due to participants selecting “not sure” as an answer or not providing information.

^ Change in sample size as data from withdrawn participants included in this analysis.

a Self-reported height and weight were recorded in the lifestyle questionnaire and used to calculate BMI.
3.3 Feasibility
Data collected around feasibility issues are displayed in Table 3.

3.3.1 Feasibility Study Completion
Of the 125 participants who consented to participate in the study, 123 attended the Day 28 follow up visit. This represents a Day 28 completion rate of 98.4% (95% CI 93.2% to 99.5%).

Furthermore, 122 participants attended the final follow up visit at Day 180. This represents an overall study completion rate of 97.6% (95% CI 93.1% to 99.5%).

The overall study completion rate of 97.6% corresponds to a withdrawal rate of 2.4%.

3.3.2 Lifestyle Questionnaire Completion
All 125 participants that submitted the lifestyle questionnaire successfully completed >90% of the questions.

3.3.3 Stool Sample Collection
All participants provided a stool sample when required. One of the participants who was withdrawn still supplied a stool sample at Day Zero which accounts for the discrepancy in totals between Day Zero and Day 28.

Of the 124 stool samples received at Day Zero, 29 (23.4%; 95% CI 16.3% to 31.8%) were labelled incorrectly and required investigator intervention.

Of the 123 stool samples received at Day 28, 23 (18.7%; 95% CI 12.2% to 26.7%) were labelled incorrectly and required investigator intervention.
3.3.4 Blood Sample Collection
The number (percentage) of participants for whom, all planned blood samples were collected was 118 (95.2%), 123 (100%), 121 (99.2%), 123 (100%), 119 (97.5%) for Day Zero, Three, Seven, 28 and 180 respectively.

The number (percentage) of participants who had analysable samples was 118 (95.2), 109 (88.6%), 121 (99.2%), 123 (100%) and 120 (98.4%) for Day Zero, Three, Seven, 28 and 180 respectively.

3.3.5 Potential Exclusion Criteria
Collection of medication use data revealed 12 participants (9.6%; 95% CI 5.1% to 16.2%) used antibiotics in the 28 days prior to Day Zero and 32 participants (26.2%; 95% CI 18.7% to 35.0%) used antibiotics between Day Zero and Day 180.

Additionally, 10 participants (8.0%; 95% CI 3.9% to 14.2%) used steroids in the 28 days prior to Day Zero and 15 participants (12.3%; 95% CI 7.1% to 19.5%) used steroids between Day Zero and Day 180.

In total, 76 participants (61.8%; 95% CI 52.5% to 70.2%) had received an Influenza vaccination in the last two years.

No participants were pregnant at recruitment, or became pregnant over the course of the study.
### Table 3. Proportions and 95% confidence intervals related to the feasibility for a possible larger randomised controlled trial

<table>
<thead>
<tr>
<th>Study Completed to</th>
<th>N/N (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Day 28</td>
<td>123/125 (98.4)</td>
<td>93.2 to 99.5</td>
</tr>
<tr>
<td>- Day 180</td>
<td>122/125 (97.6)</td>
<td>93.1 to 99.5</td>
</tr>
<tr>
<td>Completed &gt;90% of lifestyle questionnaire</td>
<td>125/125 (100)</td>
<td></td>
</tr>
<tr>
<td>Provided a stool sample at</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Day Zero</td>
<td>124/124 (100)</td>
<td></td>
</tr>
<tr>
<td>- Day 28</td>
<td>123/123 (100)</td>
<td></td>
</tr>
<tr>
<td>Provided a stool sample labelled incorrectly at</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Day Zero</td>
<td>29/124 (23.4)</td>
<td>16.3 to 31.8</td>
</tr>
<tr>
<td>- Day 28</td>
<td>23/123 (18.7)</td>
<td>12.2 to 26.7</td>
</tr>
<tr>
<td>All blood samples obtained at</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Day Zero</td>
<td>118/124 (95.2)</td>
<td>89.8 to 98.2</td>
</tr>
<tr>
<td>- Day Three</td>
<td>123/123 (100)</td>
<td></td>
</tr>
<tr>
<td>- Day Seven</td>
<td>121/122 (99.2)</td>
<td>95.5 to 100</td>
</tr>
<tr>
<td>- Day 28</td>
<td>123/123 (100)</td>
<td></td>
</tr>
<tr>
<td>- Day 180</td>
<td>119/122 (97.5)</td>
<td>92.9 to 99.5</td>
</tr>
<tr>
<td>Blood samples contained sufficient material at</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Day Zero</td>
<td>118/124 (95.2)</td>
<td>89.8 to 98.2</td>
</tr>
<tr>
<td>- Day Three</td>
<td>109/123 (88.6)</td>
<td>81.6 to 93.6</td>
</tr>
<tr>
<td>- Day Seven</td>
<td>121/122 (99.2)</td>
<td>95.5 to 99.8</td>
</tr>
<tr>
<td>- Day 28</td>
<td>123/123 (100)</td>
<td></td>
</tr>
<tr>
<td>- Day 180</td>
<td>120/122 (98.4)</td>
<td>94.2 to 99.8</td>
</tr>
<tr>
<td>Systemic antibiotics used</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- within 30 days of Day Zero</td>
<td>12/125 (9.6)</td>
<td>5.1 to 16.2</td>
</tr>
<tr>
<td>- between Day Zero and Day 180</td>
<td>32/122 (26.2)</td>
<td>18.7 to 35.0</td>
</tr>
<tr>
<td>Systemic corticosteroids used</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- within 30 days of Day Zero</td>
<td>10/125 (8.0)</td>
<td>3.9 to 14.2</td>
</tr>
<tr>
<td>- between Day Zero and Day 180</td>
<td>15/122 (12.3)</td>
<td>7.1 to 19.5</td>
</tr>
<tr>
<td>Influenza vaccination during last two years</td>
<td>77/125 (61.6)</td>
<td>52.5 to 70.2</td>
</tr>
<tr>
<td>Pregnant at start of study or became pregnant during study</td>
<td>0/122 (0)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval;
3.4 Stool Community Types at Day Zero vs Day 28

SCT assignments for Day Zero and Day 28 are displayed in Table 4. The relative abundance of genera in each SCT are displayed in Figure 2.

All Day Zero and Day 28 stool samples were able to be mapped to a SCT.

The numbers (percentage) of participants in each SCT at Day Zero were; SCT-A, 64 (52.0%); SCT-B, four (3.3%); SCT-C, 23 (18.7%); SCT-D, 32 (26.0%). At Day 28 there were 59 participants (48.0%) in SCT-A, six participants (4.9%) in SCT-B, 26 participants (21.1%) in SCT-C and 32 participants (26.0%) in SCT-D.

Eighty-four of the participants (68.3%; 95% CI 59.3 to 76.4) had the same SCT at Day 28 as they did at Day Zero. The remaining 39 (31.7%) participants changed to another of the SCTs. The pattern of change from Day Zero to Day 28 is detailed in Table 2.

### Table 4. Stool Community Type assignments at Day Zero vs Day 28

<table>
<thead>
<tr>
<th>Stool Community Type Day Zero N/123 (%)</th>
<th>SCT-A N/59 (48)</th>
<th>SCT-B N/26 (4.9)</th>
<th>SCT-C N/26 (21.1)</th>
<th>SCT-D N/32 (26.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCT-A 64 (52)</td>
<td>51</td>
<td>2</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>SCT-B 4 (3.3)</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>SCT-C 23 (18.7)</td>
<td>4</td>
<td>0</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>SCT-D 32 (26)</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>21</td>
</tr>
</tbody>
</table>

Abbreviation: SCT, stool community type.
Figure 2: Displayed in Tukey boxplots are the relative abundance of genera in the samples (Day Zero and Day 28) broken down by stool community type. The boxes represent the interquartile range (IQR) while the whiskers represent the lowest and highest data points still within 1.5 IQR of the upper and lower quartile respectively. The dots represent outliers and the central line is the median (A, B, C and D correspond to SCT-A, SCT-B, SCT-C and SCT-D respectively).
3.5 Response to Influenza Vaccination
Participant response to influenza vaccine is displayed in Table 5 and Figure 3.

3.5.1 All Participants
The baseline mean (SD) HAI titres were 116.5 (231.3), 114.7 (307.6) and 7.8 (6.5) for the H1N1, H3N2 and B strains respectively.

Post vaccination mean (SD) HAI titres were 252.0 (291.8), 188.8 (339.5) and 26.3 (62.4) for the H1N1, H3N2 and B strains respectively.

This represents a ratio of geometric means (SD) of 8.7 (17.7) for the H1N1 strain, 7.3 (17.8) for the H3N2 strain and 3.8 (7.7) for the B strain.

Analysis of HAI titres revealed 43 participants (35%) seroconverted to the H1N1 strain, 45 participants (36.6%) seroconverted to the H3N2 strain, 20 participants (16.3%) seroconverted to the B strain and 8 participants (6.5%) seroconverted to all three strains of the vaccine.

Additionally, 107 participants (87.0%) were seroprotected against the H1N1 strain, 98 participants (79.7%) were seroprotected against the H3N2 strain, 27 participants (22.0%) were seroprotected against the B strain and 19 participants (15.5%) were seroprotected against all three strains.

3.5.2 Stool Community Type A
For SCT-A (n=64), the baseline mean (SD) HAI titres were 134.3 (270.7) for the H1N1 strain, 164.0 (408.5) for the H3N2 strain and 7.6 (6.4) for the B strain.

Post vaccination mean (SD) HAI titres were 249.2 (293.3) for the H1N1 strain, 201.8 (305.0) for the H3N2 strain and 31.0 (81.7) for the B strain.

This represents a ratio of geometric means (SD) of 8.2 (18.9) for the H1N1 strain, 6.9 (18.2) for the H3N2 strain and 4.4 (9.1) for the B strain.
Analysis of HAI titres revealed 22 participants (34.4%) seroconverted to the H1N1 strain, 20 participants (31.3%) seroconverted to the H3N2 strain, 11 participants (17.2%) seroconverted to the B strain and 4 participants (6.3%) seroconverted to all three strains.

Additionally, 54 participants (84.4%) were seroprotected against the H1N1 strain, 53 participants (82.8%) were seroprotected against the H3N2 strain, 15 participants (23.4%) were seroprotected against the B strain and 11 participants (17.2%) were seroprotected against all three strains.

3.5.3 Stool Community Type B
For SCT-B (n=4), the baseline mean (SD) HAI titres were 41.3 (30.7) for the H1N1 strain, 92.5 (152.6) for the H3N2 strain and 6.3 (2.5) for the B strain.

Post vaccination mean (SD) HAI titres were 82.5 (61.3) for the H1N1 strain, 211.3 (293.4) for the H3N2 strain and 18.8 (15.5) for the B strain.

This represents a ratio of geometric means (SD) of 2.0 (0.01) for the H1N1 strain; 3.75 (3.1) for the H3N2 strain and 3.5 (3.3) for the B strain.

No participants seroconverted to the H1N1 strain, two participants (50.0%) seroconverted to the H3N2 strain, one participant (25.0%) seroconverted to the B strain and no participants seroconverted to all three strains.

Additionally, three participants (75.0%) were seroprotected against the H1N1 strain, three participants (75.0%) were seroprotected against the H3N2 strain, one participant (25.0%) was seroprotected against the B strain and no participants were seroprotected to all three strains.

3.5.4 Stool Community Type C
For SCT-C (n=23), the mean (SD) baseline HAI titres were 145.9 (267.6) for the H1N1 strain, 77.2 (145.4) for the H3N2 strain and 9.1 (8.1) for the B strain.
Post vaccination mean (SD) HAI titres were 381.7 (399.1) for the H1N1 strain, 267.2 (572.6) for the H3N2 strain and 27.4 (33.6) for the B strain.

This represents a ratio of geometric means (SD) of 11.0 (17.7) for the H1N1 strain; 10.0 (26.2) for the H3N2 strain and 4.1 (6.5) for the B strain.

Analysis of HAI titres revealed 11 participants (47.8%) seroconverted to the H1N1 strain, 10 participants (43.5%) seroconverted to the H3N2 strain, 5 participants (21.7%) seroconverted to the B strain and 4 participants (17.4%) seroconverted to all three strains.

Additionally, 22 participants (95.7%) were seroprotected against the H1N1 strain, 17 participants (73.9%) were seroprotected against the H3N2 strain, six participants (26.1%) were seroprotected against the B strain and four participants (17.4%) were seroprotected against all three strains.

### 3.5.5 Stool Community Type D

For SCT-D (n=32), the mean (SD) baseline HAI titres were 69.1 (80.4) for the H1N1 strain, 45.9 (70.9) for the H3N2 strain and 7.5 (6.7) for the B strain.

Post vaccination mean (SD) HAI titres were 185.6 (161.7) for the H1N1 strain, 103.8 (105.6) for the H3N2 strain and 7.5 (6.7) for the B strain.

This represents a ratio of geometric means (SD) of 8.8 (16.4) for the H1N1 strain; 6.5 (9.0) for the H3N2 strain and 2.6 (5.4) for the B strain.

Analysis of HAI titres revealed 10 participants (31.3%) seroconverted to the H1N1 strain; 13 participants (40.6%) seroconverted to the H3N2 strain, three participants (9.4%) seroconverted to the B strain and two participants (6.3%) seroconverted to all three strains.

Additionally, 28 participants (87.5%) were seroprotected against the H1N1 strain, 25 participants (78.1%) were seroprotected against the H3N2 strain, five participants (15.6%) were seroprotected against the B strain and four participants (12.5%) were seroprotected against all three strains.
Table 5. Antibody response generated by participants to Influenza vaccination, displayed overall as well as broken down by stool community type

<table>
<thead>
<tr>
<th>Stool community type (SCT) at Day Zero (n=123)</th>
<th>All (n=123)</th>
<th>SCT-A (n=64)</th>
<th>SCT-B (n=4)</th>
<th>SCT-C (n=23)</th>
<th>SCT-D (n=32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-vaccination HAI titre</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- H1N1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>116.5 (231.3)</td>
<td>134.3 (270.7)</td>
<td>41.3 (30.7)</td>
<td>145.9 (267.6)</td>
<td>69.1 (80.4)</td>
</tr>
<tr>
<td>- H3N2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>114.7 (307.6)</td>
<td>164.0 (408.5)</td>
<td>92.5 (152.6)</td>
<td>77.2 (145.4)</td>
<td>45.9 (70.9)</td>
</tr>
<tr>
<td>- B&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.8 (6.4)</td>
<td>7.6 (5.7)</td>
<td>6.3 (2.5)</td>
<td>9.1 (8.1)</td>
<td>7.5 (6.7)</td>
</tr>
<tr>
<td>Post vaccination HAI titre</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- H1N1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>252.0 (291.8)</td>
<td>249.2 (293.3)</td>
<td>82.5 (61.3)</td>
<td>381.7 (399.1)</td>
<td>185.6 (161.7)</td>
</tr>
<tr>
<td>- H3N2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>188.8 (339.5)</td>
<td>201.8 (305.0)</td>
<td>211.3 (293.4)</td>
<td>267.2 (572.6)</td>
<td>103.8 (105.6)</td>
</tr>
<tr>
<td>- B&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26.3 (62.4)</td>
<td>31.0 (81.7)</td>
<td>18.8 (15.5)</td>
<td>27.4 (33.6)</td>
<td>17.0 (28.4)</td>
</tr>
<tr>
<td>Pre-vaccination logarithm titres</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- H1N1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.75 (1.43)</td>
<td>3.79 (1.51)</td>
<td>3.34 (1.20)</td>
<td>3.96 (1.52)</td>
<td>3.58 (1.25)</td>
</tr>
<tr>
<td>- H3N2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.35 (1.55)</td>
<td>3.62 (1.63)</td>
<td>3.17 (1.99)</td>
<td>3.15 (1.52)</td>
<td>2.95 (1.29)</td>
</tr>
<tr>
<td>- B&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.89 (0.49)</td>
<td>1.88 (0.47)</td>
<td>1.78 (0.35)</td>
<td>2.00 (0.58)</td>
<td>1.85 (0.49)</td>
</tr>
<tr>
<td>Post vaccination logarithm titres</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- H1N1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.91 (1.23)</td>
<td>4.84 (1.30)</td>
<td>4.04 (1.20)</td>
<td>5.44 (1.08)</td>
<td>4.77 (1.10)</td>
</tr>
<tr>
<td>- H3N2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.37 (1.34)</td>
<td>4.52 (1.31)</td>
<td>4.21 (2.07)</td>
<td>4.29 (1.65)</td>
<td>4.14 (1.04)</td>
</tr>
<tr>
<td>- B&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.61 (0.96)</td>
<td>2.64 (1.04)</td>
<td>2.65 (0.89)</td>
<td>2.91 (0.84)</td>
<td>2.32 (0.85)</td>
</tr>
<tr>
<td>Ratio of geometric means</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- H1N1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.7 (17.7)</td>
<td>8.2 (18.9)</td>
<td>2.0 (0.01)</td>
<td>11.0 (17.7)</td>
<td>8.8 (16.4)</td>
</tr>
<tr>
<td>- H3N2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.3 (17.8)</td>
<td>6.9 (18.2)</td>
<td>3.75 (3.1)</td>
<td>10.0 (26.2)</td>
<td>6.5 (9.0)</td>
</tr>
<tr>
<td>- B&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.8 (7.7)</td>
<td>4.4 (9.1)</td>
<td>3.5 (3.3)</td>
<td>4.1 (6.5)</td>
<td>2.6 (5.4)</td>
</tr>
<tr>
<td>Seroconversion&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- H1N1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43 (35.0)</td>
<td>22 (34.4)</td>
<td>0 (0)</td>
<td>11 (47.8)</td>
<td>10 (31.3)</td>
</tr>
<tr>
<td>- H3N2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45 (36.6)</td>
<td>20 (31.3)</td>
<td>2 (50)</td>
<td>10 (43.5)</td>
<td>13 (40.6)</td>
</tr>
<tr>
<td>- B&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20 (16.3)</td>
<td>11 (17.2)</td>
<td>1 (25)</td>
<td>5 (21.7)</td>
<td>3 (9.4)</td>
</tr>
<tr>
<td>- All strains</td>
<td>8 (6.5)</td>
<td>4 (6.3)</td>
<td>0 (0)</td>
<td>2 (8.7)</td>
<td>2 (6.3)</td>
</tr>
</tbody>
</table>
Table 5 (cont). Antibody response generated by participants to Influenza vaccination, displayed overall as well as broken down by stool community type

<table>
<thead>
<tr>
<th>Seroprotection*</th>
<th>All (n=123)</th>
<th>SCT-A (n=64)</th>
<th>SCT-B (n=4)</th>
<th>SCT-C (n=23)</th>
<th>SCT-D (n=32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/123 (%)</td>
<td>N/64 (%)</td>
<td>N/4 (%)</td>
<td>N/23 (%)</td>
<td>N/32 (%)</td>
<td></td>
</tr>
<tr>
<td>H1N1a</td>
<td>107 (87.0)</td>
<td>54 (84.4)</td>
<td>3 (75.0)</td>
<td>22 (95.7)</td>
<td>28 (87.5)</td>
</tr>
<tr>
<td>H3N2b</td>
<td>98 (79.7)</td>
<td>53 (82.8)</td>
<td>3 (75)</td>
<td>17 (73.9)</td>
<td>25 (78.1)</td>
</tr>
<tr>
<td>Bc</td>
<td>27 (22.0)</td>
<td>15 (23.4)</td>
<td>1 (25)</td>
<td>6 (26.1)</td>
<td>5 (15.6)</td>
</tr>
<tr>
<td>All strains</td>
<td>19 (15.5)</td>
<td>11 (17.2)</td>
<td>0 (0)</td>
<td>4 (17.4)</td>
<td>4 (12.5)</td>
</tr>
</tbody>
</table>

Abbreviations: SCT, stool community type; SD, standard deviation; HAI, haemagglutination inhibition.

* Influenza A (H1N1) A/California/7/2009, X-181
b Influenza A (H3N2) A/New Caledonia/71/2014, X257A
c Influenza B (B/Brisbane/60/2008, wild type)
d Seroconversion: a ≥4-fold increase in HAI titre if pre-vaccination HAI titre was ≥1:10; or a post vaccination HAI titre of 1:40 if pre-vaccination HAI titre was <1:10.
e Seroprotection: a post vaccination HAI titre of ≥1:40.
Figure 3. Comparison of pre-vaccination and post vaccination log transformed HAI titres to the H1N1 strain (A), H3N2 strain (B), and B strain (C). Pre and post vaccination log transformed HAI titre is shown for all participants and broken down by stool community type. Error bars represent the standard error.
3.6 Association between Stool Community Type and Response to Influenza Vaccination

Results of both the univariate analysis and multivariate analysis are displayed in Table 6.

Each variable was analysed separately, and then a multivariate analysis was undertaken incorporating the pre-specified variables of age, BMI, and vaccination in the last 2 years (yes or no) in the model.

A p-value of <0.05 was used to assess significance for all association analyses.

3.6.1 HAI Titre
Both univariate and multivariate p-values were >0.05. There was no significant association between SCT and HAI titre post vaccination for each of the strains: H1N1, H3N2 and B.

3.6.2 Seroconversion
Both univariate and multivariate p-values were >0.05. There was no significant association between SCT and seroconversion post vaccination for each of the strains (H1N1, H3N2, B) and all strains together.

3.6.3 Seroprotection
Both univariate and multivariate p-values were >0.05. There was no significant association between SCT and seroprotection post vaccination for each of the strains (H1N1, H3N2, B) and all strains together.
Table 6. *P* values generated by univariate and multivariate association analysis between stool community type and antibody titre, seroconversion\(^a\) and seroprotection\(^b\)

<table>
<thead>
<tr>
<th></th>
<th>Univariate p value</th>
<th>Multivariate p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HAI titre</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- H1N1(^c)</td>
<td>0.075</td>
<td>0.099</td>
</tr>
<tr>
<td>- H3N2(^d)</td>
<td>0.60</td>
<td>0.98</td>
</tr>
<tr>
<td>- B(^e)</td>
<td>0.17</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Seroconversion(^a)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- H1N1(^c)</td>
<td>0.15</td>
<td>0.08</td>
</tr>
<tr>
<td>- H3N2(^d)</td>
<td>0.62</td>
<td>0.79</td>
</tr>
<tr>
<td>- B(^e)</td>
<td>0.57</td>
<td>0.42</td>
</tr>
<tr>
<td>- All strains</td>
<td>0.87</td>
<td>0.64</td>
</tr>
<tr>
<td><strong>Seroprotection(^b)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- H1N1(^c)</td>
<td>0.43</td>
<td>0.40</td>
</tr>
<tr>
<td>- H3N2(^d)</td>
<td>0.81</td>
<td>0.71</td>
</tr>
<tr>
<td>- B(^e)</td>
<td>0.77</td>
<td>0.59</td>
</tr>
<tr>
<td>- All strains</td>
<td>0.62</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Abbreviation: HAI, haemagglutination inhibition

\(^a\) Seroconversion: a \(\geq\)4-fold increase in HAI titre if pre vaccination HAI titre was \(\geq\)1:10; or a post vaccination HAI titre of 1:40 if pre vaccination HAI titre was <1:10.

\(^b\) Seroprotection: a post vaccination HAI titre of \(\geq\)1:40.

\(^c\) Influenza A (H1N1) A/California/7/2009, X-181

\(^d\) Influenza A (H3N2) A/New Caledonia/71/2014, X257A

\(^e\) Influenza B (B/Brisbane/60/2008, wild type)
Chapter 4: Discussion

4.1 Key Findings

The results of this study showed that it was feasible to run a large RCT using a similar study protocol. The protocol underpinning the study led to a low withdrawal rate, good success with obtaining all necessary samples and high protocol adherence from the participants, all of which are cornerstones of a successful study. All participants were able to be assigned to the stool community types (SCTs) identified by Ding and Schloss\(^{21}\), and approximately two thirds had the same SCT at Day 28 as they had at Day Zero. It is important to note that only four participants were found to be in SCT-B on Day Zero. The sample size for SCT-B should be taken into account when interpreting the demographic, stability, and association data obtained for this SCT.

There was large variation in both the baseline and post vaccination HAI titres between participants for the H1N1 and H3N2 strains in the vaccine. Less variation was observed in both the baseline and post vaccination HAI titres to the B strain.

Contrary to the hypothesis, this study found no association between SCT and immune response to influenza vaccine. Both univariate and multivariate analysis were unable to demonstrate a significant association \((p < 0.05)\). However, two of the \(p\) values trended towards significance, suggesting this study was not adequately powered to detect an association.

4.2 Feasibility

4.2.1 Proportion of Participants that Completed the Study

The dropout rate for the study was surprisingly low when considering the length of the study and the nature of the samples that were required. Initially, it was anticipated that
a withdrawal rate of 20% would be likely and the sample size of the study was adjusted accordingly. However, only three participants did not complete the study. Of these three participants only one received the vaccination and this participant attended all visits except the final Day 180 visit. These numbers give a completion rate of 97.6% (95% CI 93.1% to 99.5%), representing a withdrawal rate of 2.4%. This low withdrawal rate may have been in part due to the ability for participants to pick their own study schedule from a range of possible options. A number of other retention methods were also employed including text message reminders and splitting the participant reimbursement between Day 28 and the Day 180 visit.

When calculating the required sample size for a large RCT, the inverse of the lower bound of the 95% CI should be used in order to remain conservative. In this case the expected withdrawal rate in a large RCT may be 6.9%.

### 4.2.2 Completion of the Lifestyle Questionnaire

Completion of the lifestyle questionnaire was required before Day Zero as it informed the demographic data for the study and acted as a second source of screening information for the study exclusion criteria. Because of this, it was important to determine the likely proportion of participants that would provide data for >90% of the questions in the lifestyle questionnaire in a large RCT. In this study all participants provided data for >90% of the questions in the lifestyle questionnaire despite the length of the questionnaire (Appendix II).

### 4.2.3 Stool Samples

For this study protocol to be successful in a large RCT it was crucial that participants were willing and able to collect stool samples in the three days prior to their Day Zero visit.
All participants who attended their Day Zero and Day 28 visits provided the required stool samples. Of the 124 stool samples provided at Day Zero, 23.4% required investigator intervention as they were not labelled correctly with the date and/or time of collection. Of the stool samples provided at Day 28, 18.7% required investigator intervention.

These findings suggest that researchers can be confident all participants will be able to provide required stool samples at multiple time points. However, resources should be focused on improving participant labelling for Day Zero stool samples in particular, as the upper limit of the 95% CI (31.8%) suggests that almost one third of samples could have insufficient labelling.

### 4.2.4 Blood Sample Collection

An important feasibility issue for this study to address was the ability of study investigators to obtain all planned blood samples using standard phlebotomy equipment. This was considered a potential issue as Day Zero required approximately 80ml of blood to be collected in 10 tubes. The following visits presented less concern as they each required a lower number of tubes than Day Zero. There was concern that the high number of tubes required for Day Zero would lead to a higher chance of the participants vein collapsing, preventing collection of adequate samples. Collection of a blood sample to determine baseline HAI titre was crucial to study participation and a low success rate would be a large barrier to study completion.

The results show that it is feasible for investigators to use standard phlebotomy equipment when collecting the blood samples from participants. The percentage of successful blood sample collections was consistently greater than 95%. The lowest proportion of blood samples collected during the study was on Day Zero. There was a 95.2% success rate for blood sample collection at Day Zero with a 95% confidence interval of 89.8% to 98.2%. This suggests that in a large study a minimum success rate of 89.8% could be expected. This corresponds to a conservative blood sample collection
failure rate of 9.2%. Resources in a large RCT should be focused on blood collection at Day Zero to improve the success rate of baseline blood sample collection.

Success of blood collection improved over the course of the study as shown in *Table 3* but decreased at the Day 180 visit. Both timing of the blood collection and temperature of the room were kept consistent (within reasonable expectations) over the course of the study, so are likely to have had minimal impact on the decreased success of the blood collection at Day 180. There are two potential factors which may have caused this trend in the success rate. The first is increased participant familiarity with the blood collection procedure and learning which arm and veins are the easiest to obtain blood from which they can pass on phlebotomists. The gap in time between the Day 28 visit and the Day 180 visit likely resulted in a loss of this knowledge leading to the decrease in successful blood collection. Additionally there were less phlebotomists available for the Day 180 visits which meant that a second phlebotomist was not available after the initial blood collection attempts were unsuccessful.

### 4.2.5 Possible Exclusion Criteria

This study needed to inform the proportion of potential participants likely to be excluded based on theorised exclusion criteria. The theorised exclusion criteria were antibiotic use, steroid use, vaccination history and pregnancy.

Antibiotic use can have a major effect on the gut microbiota\(^{33}\) and should ideally be controlled for in a large RCT looking to change SCT. 9.6% of participants used antibiotics in the 30 days prior to their vaccination date and 26.2% of participants used antibiotics between Day Zero and Day 180.

Corticosteroids can dampen the immune response\(^{61}\) and would ideally not be used throughout the duration of a large RCT. 8.0% of participants used corticosteroids in the 30 days prior to their vaccination date and 12.3% used corticosteroids between Day Zero and Day 180.
Another potential exclusion criteria affecting immune response was a previous influenza vaccination within the last two years. By excluding potential participants who have been vaccinated in the last two years there is an increased chance of having a sample which does not already have high antibody titres to the current influenza vaccine strains in use. In this study, 76 participants (61.8%) had received an influenza vaccine in the last two years. The large proportion of participants who had received a vaccination in the last two years makes this an unfavourable exclusion criteria in terms of recruitment as it results in the elimination of a large proportion of the potential participant pool. People willing to participate in research involving the influenza vaccine are more likely to have received an influenza vaccination so previous exposure numbers will be high.

Pregnancy is another potential exclusion criteria to be considered for an interventional study due to the potential risk to the health of the foetus. In this study no participants were pregnant at consent or became pregnant during the study. This suggests that pregnancy as an exclusion criteria will result in very few exclusions from the potential participant pool.

If all the proposed exclusion criteria were applied to this study sample, then 74% of the participants that took part in this study would have been ineligible. This would result in a large increase in the number of potential participants that would need to be screened before the sample size was met.

Additional analysis of the data collected for this thesis may be undertaken to determine the effect of these proposed exclusion criteria on SCT stability and immune response. However, the sample size in this study may be insufficient to obtain meaningful results.

### 4.3 Stool Community Types

Arunugam et al.\(^2^0\) were the first to suggest using SCTs as a method of categorising the human gut microbiota. Using a multidimensional cluster analysis and principal component analysis they identified three distinct SCTs dominated by *Bacteroides*,
*Prevotella* and *Ruminococcus* respectively. Ding and Schloss\textsuperscript{21} expanded on the SCT concept by using a Dirichlet multinomial mixture (DMM) model. The DMM model identified four SCTs. SCT-A, SCT-C and SCT-D were dominated by *Bacteroides*, Ruminococcaceae and *Prevotella* respectively. These SCTs resembled those identified by Arumugam et al\textsuperscript{20}. The fourth SCT (SCT-B) was dominated by Firmicute affiliated populations.

The SCTs investigated in this study were those determined by Ding and Schloss\textsuperscript{21}. SCT assignments were performed through a best fit model using proportions of major taxonomic groups instead of the Dirichlet multinomial mixture (DMM) model, this was done to ensure that the SCTs assigned were representative of the SCTs identified by Ding and Schloss\textsuperscript{21}. If the same DMM model had been used it would have likely found different SCTs due to differences in region, sequencing technology etc.

Using the parameters detailed in the methods section, it was possible to assign all participants to one of the four SCTs. SCT-A was the most prevalent with 52\% of the participants assigned to it at Day Zero. Next was SCT-D with 26\% and SCT-C with 18.7\%. SCT-B was the least represented with only four participants (3.3\%) assigned at Day Zero. Interestingly these percentages align with the percentages found by Ding and Schloss\textsuperscript{21} (SCT-A, 37.0\%; SCT-B, 2.5\%; SCT-C, 13.4\%; SCT-D, 47.1\%). There was a difference in the percentages of SCT-D and SCT-A, which likely reflects differences in New Zealand microbiota as compared to the HMP Consortium sample\textsuperscript{80} used by Ding and Schloss\textsuperscript{21}. This study is the first time that the New Zealand adult gut microbiota has been assessed via enterotype and the data obtained suggests that the majority of the New Zealand population may have a high proportion of *Bacteroides* and low proportion of *Prevotella* in their gut microbiota.

Since this study focused on the SCTs identified by Ding and Schloss\textsuperscript{21} the samples were assigned SCTs based on the proportions of major taxa which resulted in SCTs representative of those they identified. It may have been more advantageous for this study to determine the SCTs using the DMM model as grouping participants into the
SCTs based on major taxonomic proportions may have led to groups not representational of the New Zealand gut microbiota.

4.3.1 Stability of Stool Community Types
In order to be able to assess the ability for an intervention to change an individual’s SCT, it was important to measure the likely stability of the SCTs over a 28-day period. The percentage of participants that had the same SCT at Day 28 as they had at Day Zero was 68.3%. This translates to 31.7% of participants having a different SCT on Day Zero and Day 28.

SCT-A was the most stable community type over the 28-day period with 79.7% of participants classified as SCT-A on Day Zero remaining SCT-A on Day 28. SCT-C was the least stable with only 43.5% of SCT-C participants staying the same on Day 0 and Day 28. This differs from the findings by Ding and Schloss\textsuperscript{21}, who found that SCT-D was the most stable while SCT-B was the least. The increased stability of SCT-B was likely due to the small number of samples that were assigned to SCT-B at Day Zero (n=4).

Ding and Schloss\textsuperscript{21} also found overall higher levels of within sample stability than was found in this study. This may have been due to the greater time between sample collection in Ding and Schloss’ study\textsuperscript{21}. The greater time difference may have allowed short term fluctuations in gut microbiota to revert to the original state. Alternatively these findings could lend strength to the argument that instead of discrete enterotypes, the gut microbiota is best modelled by continuous variation\textsuperscript{82}. Knight \textit{et al}\textsuperscript{82} argue that while discrete enterotypes are an appealing concept, the current evidence suggests that there is no barrier to switching between enterotypes and that the major taxa of the gut microbiota are in constant fluctuation.

Using the data collected in this study it is impossible to state whether or not the participants who had the same SCT on Day Zero and Day 28 switched to another SCT between sample collections. Further research, ideally focussing on daily SCT stability
over an extended period, is needed to determine the best model for the gut microbiota. A continuous variation model would pose issues for the proposed RCT as there would be no fixed SCT for the gut microbiota to change from or to.

The food diary data collected in this study may provide insight into any dietary factors that account for the SCTs at Day Zero and Day 28. If an association is found, then a diet plan may improve the stability of SCTs in a large RCT. However, this would run counter to current research that suggests SCT is associated with long term diet rather than short term\textsuperscript{25}.

### 4.4 Response to Influenza Vaccination

The response to the influenza vaccine was assessed using the HAI assay which measures the amount of antibody produced specific to the HA antigen. The study sample were recruited from the general population and not selected based on baseline HAI titre or vaccination history. Due to this, the pre-vaccination HAI titres show a large amount of variation between participants for the H1N1 strain (5 to 1280) and the H3N2 strain (5 to 2560). The H3N2 strain in particular had a range that extended from below the limit of detection to the upper limit of the assay. The pre-vaccination titres for the B strain suggested that the study sample could be classified as seronegative toward the B strain with a mean HAI titre well below the <1:40 criteria for seronegativity. The Influvac\textsuperscript{®} vaccine that was used in this study contained two previously used strains (H1N1 and H3N2) and one new strain (B). The study sample was unlikely to have encountered the B strain previously through infection and/or vaccination as it was a novel strain in the 2016 influenza vaccine. This led to a high number of participants (n=120) in the sample that were seronegative to the B strain, compared to only 44 for the H1N1 strain and 67 for the H3N2 strain.

There are three measures used by regulatory authorities to assess vaccine efficacy\textsuperscript{83,84} These measures are seroconversion, seroprotection and increase in geometric mean
titre. To approve a seasonal inactivated influenza vaccine, the FDA requires that HAI seroconversion should be ≥40%; and HAI seroprotection should be ≥70%.

Seroconversion is measured as a ≥4-fold increase in HAI titre if pre-vaccination HAI titre was ≥1:10; or a post vaccination HAI titre of 1:40 if pre-vaccination HAI titre was <1:10. Seroconversion rates for the study sample were low with only 35.0%, 36.6% and 16.3% seroconverting to the H1N1, H3N2 and B strains respectively.

Seroconversion rates for the study sample were low with only 35.0%, 36.6% and 16.3% seroconverting to the H1N1, H3N2 and B strains respectively.

Seroprotection is measured as a post vaccination HAI titre of ≥1:40. Seroprotection rates were high for the H1N1 and H3N2 strain but this may have been due to participants already achieving the seroprotection criteria at baseline. The B strain of the vaccine provided a more unbiased look at seroprotection due to the seronegativity of the study sample. 22.0% of the participants achieved seroprotection to the B strain of the vaccine.

Collectively these measures suggest that the participants tested in this study had a lower overall response than should be expected when using an approved influenza vaccine. As was suggested previously this may have been due to the fact that the participants in this study were not selected for seronegativity to the vaccine strains or vaccination history. Seidman et al. found a correlation between high baseline antibody titres (≥40) and low seroconversion numbers. Participants with high baseline HAI titres are likely to have reached the threshold for seroprotection but may be unable to produce the fourfold increase in antibody titre required to achieve seroconversion. Alternatively, the low overall response may have been due to this vaccine having low immunogenicity.

Recruitment of adults with low baseline antibody titres is likely to be difficult as almost all adults will have been exposed to the standard strains at some point in their life. A better option may be to exclude participants based on previous vaccination history, or an abnormally high baseline antibody titre. Removal of participants that received an influenza vaccine in the last two years caused overall seroconversion numbers to increase to 68.1%, 63.8% and 31.9% for the H1N1, H3N2 and B strains respectively. It
should be noted that this resulted in the exclusion of 76 participants (61.8%) from the calculation which only left 47 participants.

Use of the influenza vaccine leads to a few difficulties when assessing immune response. Firstly, the vaccine contains three strains which makes analysis more complicated and can cloud results. Secondly, influenza vaccination is common, and the influenza virus is a commonly encountered pathogen by the general population. This leads to a large variation in the baseline antibody levels of potential participants. Using a less commonly administered vaccine with a single strain would likely alleviate these issues. However, more research needs to be done to select a suitable vaccine.

4.5 Association between Stool Community Type and Response to Influenza Vaccination

Association between SCT and response to influenza vaccination was assessed using three different measures of antibody response. These were post vaccination HAI titre, the number of participants who achieved seroconversion and the number of participants who achieved seroprotection. No significant association was found between SCT and any of the measures assessed. This finding is contrary to the initial hypothesis that at least one of the SCT’s would be associated with a stronger or weaker immune response.

While not statistically significant, multivariate analysis of SCT vs seroconversion to the H1N1 Strain (p value = 0.08) and univariate analysis of SCT vs H1N1 HAI titre (p value = 0.075) trended towards significance. This suggests that a larger study, adequately powered, may identify an association that was not detected in this feasibility study.

While not borne out in this thesis, further research in the area of human gut microbiota and immune response is justified when one considers the animal studies that have shown the effect gut microbiota has on host immune response to influenza\(^1\).\(^2\).
There are a number of factors that could contribute to no association being found in this study despite the evidence obtained from animal models. GF mice used in animal models are raised in very specific conditions with a specific diet. This removes a lot of variability in terms of immune history and environmental factors which cannot be easily controlled for in humans.

The low number of participants who were assigned to SCT-B affected the ability to model associations for this SCT. Due to the seeming rarity of SCT-B it may be worth pre-screening participants SCTs for future studies to ensure equal groups for analysis. However, due to the instability of the SCTs observed in this study over a short period this may be futile.

Results of a currently ongoing US clinical trial (clinicaltrials.gov identifier: NCT02154061) looking at the effect of antibiotic use on immune response to the influenza vaccine may shed more light on the role the microbiota plays in the human immune response to influenza vaccination.

**4.6 Future Research Path**

Follow up studies with adequate power should be performed to validate and expand on the results obtained in this study. These studies can be improved using the results from the exploration of feasibility issues to help inform the study design.

As the concept of SCTs is relatively young, there are a number of potential future research paths that expand on the knowledge provided by this study.

There is evidence that antibiotic treated mice generate fewer memory B cells to the trivalent influenza vaccine, potentially impacting their response to a subsequent infection. A potential path for future research may be to test the association between SCT and response to a previously encountered antigen. This could be achieved by bringing back the original cohort involved in this study and re-exposing them to the
same vaccine. The Day 180 blood samples collected could also be analysed to determine if any significant differences exist in the level of circulating antibodies after the initial response has waned.

Another potential path for future research is further investigation of the 16S rRNA data collected in this study, used to determine the SCTs. While there was no significant association found when using the SCTs identified by Ding and Schloss\textsuperscript{21}, there may be an association when looking at different SCTs. There may also be merit in focusing on the association between immune response and individual bacterial species or taxa rather than SCTs.

Additionally, this study only used antibody titre as a measure of immune response. It could be beneficial in future research to use flow cytometry in order to assess any difference in immune cell subset levels post vaccination (e.g. antibody secreting plasma cells or memory B cells).

Finally, the stability of SCTs and sensitivity to lifestyle and dietary influence should be assessed in greater detail. In this study 30% of the participants had a different SCT at Day Zero and Day 28. Future research could focus on whether or not there was a nutritional or lifestyle impact on the SCTs. The lifestyle questionnaire collected data about many lifestyle factors including; smoking history, long term diet, hygiene and medical history. The short term dietary data collected by the food diary combined with the lifestyle questionnaire data could be mined to determine associations between lifestyle and SCT as well as reasons for the changes in SCT that were observed.
4.7 Conclusion

The findings from this study show that a large RCT using this study protocol is feasible. In a large RCT study staff can be confident that there will be high participant adherence to study procedures, but resources should be focused on improving the Day Zero blood collection and participant labelling of Day Zero stool samples. While there is likely to be high retention of participants once they have started the study, there will also likely be a large proportion of the potential participant pool excluded based on the theorised exclusion criteria. While the study protocol was feasible, the sample size of 123 participants was insufficient to detect an association between SCT and immune response to the influenza vaccine.

Despite this, the field of microbiota research is rapidly evolving and further research into this area is indicated to offer insights into human gut microbiota in health and disease.
References:


Appendix I: Participant Information Sheet
You are invited to take part in a feasibility study looking at the relationship between the bacteria that live in your gut and your immune response to the influenza (‘flu’) vaccination.

Whether or not you take part is your choice. If you don’t want to take part, you don’t have to give a reason. If you do want to take part now, but change your mind later, you can pull out of the study at any time.

You will not be eligible to take part in this study if

- You are younger than 18 or older than 64.
- You are allergic to any component of the flu vaccine
  - Eggs
  - Chicken
  - Formaldehyde
  - Cetrimonium bromide
  - Polysorbate 80
  - Gentamicin
- You have any condition that impairs your immune response through either the condition itself, or through the treatment of the condition
• You have a history of Guillain-Barre Syndrome within 6 weeks of receiving a previous flu vaccine
• Have had a previous vaccination for the current flu season.
• Have any other clinical condition which the investigator deems relevant for exclusion from the study

This Participant Information Sheet will help you decide if you would like to take part. It sets out why we are doing the study, what your participation would involve, what the benefits and risks to you might be, and what would happen after the study ends. We will go through this information with you, and answer any questions you may have. We expect this will take about 10 minutes.

You may also want to talk about the study with other people, such as family, whānau, friends, or healthcare providers. Feel free to do this.

The study has been approved by the Central Health and Disability Ethics Committee, reference number 15/CEN/207

If you agree to take part in this study, you will be asked to sign the Consent Form on the last page of this document. You will be given a copy of both the Participant Information Sheet and the Consent Form to keep.

This document is 10 pages long, including the Consent Form. Please make sure you have read all the pages. If you require an interpreter, this will be arranged.

**Why are we doing this study?**

Everyone has bacteria living in their gut, but the types and corresponding amounts of these bacteria can vary from person to person. Recent research has shown that individuals can be grouped into four different categories (known as ‘Stool Community Types’) based on what bacteria are present in their gut. Even though the bacterial population in your gut is the most stable, it can be influenced by a number of different lifestyle factors including your diet, antibiotic use, stress and where you live.

Research conducted on mice has shown a link between the bacteria present in the gut and the immune response to the flu vaccine. Mice with no gut bacteria, or diminished gut bacterial populations, had a lower response to the seasonal flu vaccine.

We are interested in examining if an individual’s gut bacterial community type has an effect on their immune systems response the seasonal flu vaccine. We are conducting this study with the idea that it will lead to a larger study, that will ultimately determine if it is possible for someone
to change their ‘stool community group’ from one type to another with the goal of improving their immune response.

In order for us to determine your body’s immune response, you will receive the seasonal flu vaccine, to measure how well your immune system works in response to it. We will monitor your body’s response to the vaccine by analysing small blood samples taken from you over the 6 months of the study.

With this study we want to find out the following:

- Can we categorise a general New Zealand population into gut bacterial community types
- Can we see a difference in immune response to the flu vaccine based on these gut bacterial community types
- Will the information we gather help us to develop a well-designed larger study

**What will my participation involve?**

If you agree to take part in this study we will need to see you for six visits spread out over six months.

Here is an outline of the timing of the visits and what will happen during each visit.

**Visit 1 - 14 to 7 days prior to vaccination**

After reading the information sheet and signing the informed consent form, the study investigator will collect a brief medical history to ensure you are eligible for the study. Next you will be given a lifestyle questionnaire to complete either online or on paper between Visit 1 and Visit 2 which contains questions related to past and present lifestyle choices, medical conditions and other situations that may affect the bacteria in your gut. You will also be given a collection kit to collect a sample of your bowel motions (stool sample), a 7-day food diary and a visit schedule. You will get instructions on how to fill out the food diary and how to collect a stool sample using the collection kit.

You need to do three things before we see you at visit 2:

1. Complete the lifestyle questionnaire. Using either the paper copy provided or the online version of the form.
   a. If you utilise the online option the information you enter into the system will be encrypted and stored overseas on secure servers
2. Complete the 7-day food diary so that the last day is the same as your vaccination (Visit 2) day
3. And you need to collect a stool sample no more than 3 days before Visit 2 (Day Zero).

Bring the questionnaire, the food diary and the stool sample with you to Visit 2, including the blank lifestyle questionnaire, even if you filled it out online.

Visit 2 (Day Zero) - Vaccination Day

We will collect the questionnaire, stool sample and your 7-day food diary. We will check your temperature and general health to confirm that you are well enough to receive the vaccination. We will collect some blood from you before you are vaccinated, equivalent to 80 millilitres (this is a small volume - about 2 shot glasses), and this will distributed between 10 smaller tubes. Then we will vaccinate you. After the vaccination you will need to wait for 20 minutes so that we can ensure you don’t have a reaction to the vaccine. During this time, a dietician / study investigator may discuss the food diary with you directly, or via an internet / Skype link. This visit is the longest visit and might take between 40 and 60 minutes.

Visit 3 (Day 3) – 3 days after vaccination

We will take a blood sample which will be stored for analysis. Your blood test from this visit will be analysed depending on your results from the Visit 5 blood test. We will make sure you have remained well, and see if you have started, stopped or changed any medications. This is a brief visit.

Visit 4 (Day 7) – 7 days after vaccination

We will take a blood sample which will be stored for analysis. Your blood test from this visit will be analysed depending on your results from the Visit 5 blood test. We will make sure you have remained well, and see if you have started, stopped or changed any medications. At the end of this visit you will be given another stool collection kit and another food diary. This is a brief visit.

22 days after vaccination you will need to start your 7-day food diary planning for the last day of the food diary to be the same day as you come back and see us. We will send you a text reminder to start your diary.

25 days after your vaccination, you will need to plan to collect a stool sample in preparation for the 28 day visit. We will send you a text reminder for this.

Visit 5 (Day 28) – 28 days after vaccination

You will need to bring your stool sample and the 7-day food diary with you. We will then take another blood sample. We will make sure you have remained well, and see if you have started, stopped or changed any medications. This will be a brief visit.

Visit 6 (Day 180) – 6 months after vaccination
We will take another small blood sample at a very brief visit and we will make sure you have remained well, and see if you have started, stopped or changed any medications.

All samples taken during the course of the study will be destroyed after publication of the study results, unless you sign the optional future unspecified research participant information sheet.

**What are the possible benefits and risks to you of participating?**

**Study Procedure risks/ side effects**

- **Blood Samples**
  Side effects of having blood drawn are rare, and usually very minor. You may experience some discomfort during the taking of a blood sample and there is always the risk of bleeding, swelling and bruising at the site of the needle during sampling. All samples will be taken by trained staff. In some cases we may require extra samples, for example to re-do a test that could not be analysed.

  Your blood samples will be coded with a unique subject number and your name will not be used to identify the samples. The samples analysed locally will be destroyed by the laboratory once the results are reported unless you have agreed to them being stored for future research.

  You may hold beliefs about a sacred and shared value of all or any tissue samples removed. The cultural issues associated with storing your tissue should be discussed with your family/whānau as appropriate. There are a range of views held by Māori around these issues; some iwi disagree with storage of samples citing whakapapa and advise their people to consult prior to participation in research where this occurs. However it is acknowledged that individuals have the right to choose.

  - **Vaccination**

  There is no risk of the flu vaccine giving you flu; however it takes approximately 3 weeks for the flu vaccine to be fully protective and it is possible you may become infected with the flu during this period. It is also possible you may still become infected with a strain of influenza that is not covered by this flu vaccine.

  During clinical studies and in post-marketing surveillance, local and general signs and symptoms attributed to the vaccine have been recorded. These events have been categorised as follows:

  Local reactions (at the site of the vaccination).

  - Very common (frequency ≥ 10%): redness, swelling, pain.
  - Common (frequency ≥1 and < 10%: bruising, induration.

  Body as a whole.
- Very common (frequency ≥ 10%): headache.
- Common (frequency ≥ 1 and < 10%): fever, malaise (feeling generally unwell)
- Uncommon (frequency ≥ 0.1% and < 1%): shivering, fatigue, sweating, muscle pains, joint pains
- Very rare (frequency ≥ 0.01% and < 0.1%): nerve pain, numbness, convulsions, transient thrombocytopenia (blood disorder), vasculitis (inflamed blood vessel) transiently involving the kidneys, allergic reactions (such as angioedema) leading to shock.
- Generalised skin reactions including itching, hives and non-specific rashes have also been recorded

As with most biological products very rare post-vaccination neurological disorders such as encephalomyelitis, neuritis and Guillain-Barre syndrome (GBS) have been reported. Guillain-Barre syndrome (GBS) has been very rarely reported in temporal association with administration of influenza vaccines. In 1998, the US Public Health Advisory Committee on Immunization Procedures reported that a study of the 1992-93 and 1993-94 seasons found an elevation in the overall relative risk for GBS which represents an excess of an estimated one to two cases of GBS per million persons vaccinated.

If you are at all concerned that you have become unwell as a result of the flu vaccine, please get in touch with either study investigators or your usual healthcare provider.

Benefits

- You will receive a free flu vaccine as a part of participating in this study.
- You will find out how well you respond to the flu vaccine.
- You will have the opportunity to participate in ground breaking research!

There is no cost for you to take part in this study and you will be compensated for your time and travel. You will receive a total of $250; after visit 5 you will receive $125 then after visit 6 you will receive the remaining $125.

General Health Care

Your general health care remains with your family doctor while you are in the study and if you experience any problems you should contact your usual health care provider. As part of the informed consent process we will ask for your permission to inform your doctor of any unexpected findings. As part of the study you will be seen by study doctors and trained staff who will conduct all the study specific assessments. If during the study there are any unexpected findings relating to your health, then we will contact you and your GP.

What would happen if you were injured in the study?
If you were injured in this study, which is unlikely, you would be eligible to apply for compensation from ACC just as you would be if you were injured in an accident at work or at home. This does not mean that your claim will automatically be accepted. You will have to lodge a claim with ACC, which may take some time to assess. If your claim is accepted, you will receive funding to assist in your recovery.

If you have private health or life insurance, you may wish to check with your insurer that taking part in this study won’t affect your cover.

**What are the rights of participants in the study?**

Your participation is entirely voluntary (your choice). You do not have to take part in this study, and if you choose not to take part it will in no way affect your future health care. If you do agree to take part you are free to withdraw from the study at any time, without having to give a reason.

**Confidentiality and Data Privacy**

If you decide to participate, we will collect medical and personal information about you, as part of doing the study.

By agreeing to take part in this research, you will allow your medical information and results to be seen by people who check that the research was done ethically and appropriately, for example the ethics committee and study monitor.

Nothing which could personally identify you will be used in any reports on this study, or provided as part of future studies. Your personal information (such as name, sex, age and medical conditions) will be identified by a study specific patient identification number (i.e. coded), and not using your name.

Study records will be stored securely during the course of the study and after completion. After everyone has completed the study and the data verified, records will be archived for a minimum of 15 years. They will then be securely destroyed.

**What will happen after the study ends, or if you pull out?**

Once the study has finished and the data has been analysed the results will be made available to you on your request. You will have the opportunity to request a copy of the study results when you sign the informed consent form.

You may be asked to leave the trial, for the following reasons:
• In the Investigator’s opinion it would not be in your best interest to continue in the study
• Any safety concerns
• You do not follow instructions during the study visits

You may withdraw from the study at any time. If you would like to withdraw, please inform the study investigator. If you choose to withdraw and your blood samples and/or stool samples have not yet been analysed, you may ask the study investigator to destroy them. If your blood samples and/or stool samples have been analysed before you have withdrawn, you may ask the study investigator to withdraw this data from the study results. The study investigator will contact the Sponsor, who will organise the withdrawal of data and samples, as per your wishes. In the absence of any specific instructions form you, we will use the data and samples you have provided up until the point of your withdrawal.

Where can you go for more information about the study, or to raise concerns or complaints?

If you have any questions, concerns or complaints about the study at any stage, you can contact the study investigator:

Name: Nick Shortt
Phone: 04 805 0236
E-mail: guthealth@mrinz.ac.nz

For Maori health support, please contact:

Whānau Care Services, Cultural Care Centre, Level 2, Wellington Hospital
Phone: 0800 999442 or 04 806 0948
Email: wcs@ccdhb.org.nz

If you want to talk to someone who isn’t involved with the study, you can contact an independent health and disability advocate on:
Phone: 0800 555 050

Fax: 0800 2 SUPPORT (0800 2787 7678)

Email: advocacy@hdc.org.nz

You can also contact the health and disability ethics committee (HDEC) that approved this study on:

Phone: 0800 4 ETHICS (0800 4 384 427)

Email: hdecs@moh.govt.nz
Study Title: Feasibility study of the association between gut bacteria and immune response

Participant ID: __________

Please tick to indicate you consent to the following:

<table>
<thead>
<tr>
<th>Consent Item</th>
<th>Ticked</th>
</tr>
</thead>
<tbody>
<tr>
<td>I have read (or have had read to me in a language I understand) and understood the Participant Information Sheet.</td>
<td></td>
</tr>
<tr>
<td>I have been given sufficient time to consider whether or not to participate in this study.</td>
<td></td>
</tr>
<tr>
<td>I have had the opportunity to use a legal representative, whānau/ family support or a friend to help me ask questions and understand the study</td>
<td></td>
</tr>
<tr>
<td>I am satisfied with the answers I have been given regarding the study and I have a copy of this consent form and information sheet.</td>
<td></td>
</tr>
<tr>
<td>I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time without this affecting my medical care.</td>
<td></td>
</tr>
<tr>
<td>I consent to the research staff collecting and processing my information, including information about my health</td>
<td></td>
</tr>
<tr>
<td>I consent to my blood and stool samples to be sent overseas for analysis</td>
<td></td>
</tr>
<tr>
<td>If I decide to withdraw from the study, I agree that the information collected about me up to the point when I withdraw may continue to be processed</td>
<td></td>
</tr>
<tr>
<td>I consent to my GP/usual health care provider being informed of any significant abnormal results obtained during the study</td>
<td></td>
</tr>
<tr>
<td>I agree to an approved auditor appointed by the New Zealand Health and Disability Ethic Committees, or any relevant regulatory authority or their approved representative reviewing my relevant medical records for the sole purpose of checking the accuracy of the information recorded for the study.</td>
<td></td>
</tr>
<tr>
<td>I understand that my participation in this study is confidential and that no material, which could identify me personally, will be used in any reports on this study.</td>
<td></td>
</tr>
<tr>
<td>I understand the compensation provisions in case of injury during the study.</td>
<td></td>
</tr>
<tr>
<td>I know who to contact if I have any questions about the study in general</td>
<td></td>
</tr>
</tbody>
</table>
I understand my responsibilities as a study participant.

I understand my personal identifiable information will be stored on a secure, encrypted server based off-shore from New Zealand, having been sent electronically to the study sites.

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>I wish to receive a summary of the results from the study.</td>
<td></td>
</tr>
<tr>
<td>I give consent for study investigators to inform my GP/usual health care provider of my participation in this study</td>
<td></td>
</tr>
</tbody>
</table>

Declaration by participant:

I hereby consent to take part in this study

Participant name (print)

Participant signature Date

Declaration by member of research team:

I have given a verbal explanation of the research project to the participant, and have answered the participant’s questions about it.

I believe that the participant understands the study and has given informed consent to participate.

Name of person conducting informed consent discussion (print)

Signature of person conducting informed consent discussion Date
Appendix II: Lifestyle Questionnaire
Gut Microbiota and Influenza Vaccine

LIFESTYLE QUESTIONNAIRE

Personal Details

Participant ID: __________________

Initials: _______________

Today’s Date: _______________

Gender: ____________________

Height (cm): _______________

Weight (kg): _______________

Date of Birth: _______________

Country of birth: ____________

This Questionnaire can be filled out online using the link below

https://wrhssurvey.wufoo.com/forms/lifestyle-questionnaire-part-1/

If you are unable to complete the questionnaire online please use this paper copy instead. If you fill out the questionnaire online please still bring this blank paper copy with you to your next visit.
About you:

1.) What is your highest level of education?
   a. Did not complete High School / College
   b. High School or College completed
   c. Some University or Polytechnic study
   d. Diploma / Apprenticeship completed
   e. Bachelor’s Degree completed
   f. Some postgraduate study
   g. Postgraduate degree completed
   h. Prefer not to answer

2.) Which is your dominant hand?
   a. I am right handed
   b. I am left handed
   c. I am ambidextrous
   d. Prefer not to answer

3.) What is your ethnicity? (Choose any that apply to you)
   a. New Zealand European
   b. Māori
   c. Samoan
   d. Cook Island Māori
   e. Tongan
   f. Niuean
   g. Chinese
   h. Indian
   e. Other: __________________
   f. Prefer not to answer

4.) When did you last travel outside of New Zealand?
   a. In the last month
   b. In the last 3 months
   c. In the last 6 months
   d. In the last year
   e. I have not been outside of New Zealand in the past year.
   f. Prefer not to answer

If you answered a, b, c, or d please list the country/countries you visited below:

__________________________________________________________________________________
__________________________________________________________________________________
5.) When did you move to your current place of residence?
   a. Within the past month
   b. Within the past 3 months
   c. Within the past 6 months
   d. Within the past year
   e. I have lived in my current place of residence for more than a year.
   f. Prefer not to answer

6.) Are you related to any of the other participants in this study?
   a. Yes
   b. No
   c. Not sure
   d. Prefer not to answer

   If yes, Do you currently live with them? ________________

7.) How many people do you live with?
   a. None
   b. One
   c. Two
   d. Three
   e. More than three
   f. Prefer not to answer

8.) How many of the people that you live with are participating in this study?
   a. None
   b. One
   c. Two
   d. Three
   e. More than three
   f. Prefer not to answer

8a.) Supplementary questions to question 8
   What is your relationship to other people in the study who have voluntarily
told you of their participation (e.g. partner, children, roommates)? Note that
we will only use information that both parties provide:
1. Participant: ________________    This person is my: ________________
2. Participant: ________________    This person is my: ________________
3. Participant: ________________    This person is my: ________________

9.) Do you have a cat(s)?
   a. Yes,
      IF yes, how many cats do you have? ________________
      Please circle where they spend most of their time
      Indoor / outdoor / both indoor and outdoor
   b. No
   c. Prefer not to answer
10.) Do you have a dog(s)?
   a. Yes
      IF yes, how many dogs do you have? ___________
      Please circle where they spend most of their time
      Indoor / outdoor / both indoor and outdoor
   b. No
   c. Prefer not to answer

Your General Diet

11.) Do you eat meat/dairy products from animals treated with antibiotics?
   a. Yes
   b. No
   c. Not Sure
   d. Prefer not to answer

12.) What is your drinking water source at home?
   a. City
   b. Bore / Water tank
   c. Bottled
   d. Filtered
   e. Not sure
   f. Prefer not to answer

13.) I am allergic to __________ (mark all that apply)
   a. Peanuts
   b. Tree nuts (almond, cashew, macadamia, hazelnut etc)
   c. Shellfish
   d. Other, Please specify: _____________________________
   e. I have no food allergies that I know of.
   f. Prefer not to answer

14.) Do you eat a paleo, modified paleo, primal, Fodmap, Westen-Price, or other low-grain, low processed food diet?
   a. Yes, please specify__________________________
   b. No
   c. Prefer not to answer

15.) Are you lactose intolerant?
   a. Yes
   b. No
   c. Don’t know
   d. Prefer not to answer
16.) Are you gluten intolerant?
   a. I was diagnosed with Coeliac Disease
   b. I was diagnosed with a gluten allergy (anti-gluten IgG), but not Coeliac Disease
   c. I do not eat gluten because it makes me feel unwell
   d. No
   e. Don't know
   f. Prefer not to answer

17.) How would you classify your diet?
   a. Omnivore (eat meat, vegetables and fruits)
   b. Omnivore but do not eat red meat
   c. Vegetarian
   d. Vegetarian but eat seafood
   e. Vegan
   f. Prefer not to answer

18.) Do you follow any other special diet restrictions other than those indicated above?
   a. Yes (if yes, please explain below)

   ______________________________________________________________

   b. No
   c. Prefer not to answer

19.) Do you take a Vitamin D supplement?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

20.) How frequently do you take a probiotic?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer
21.) How frequently do you take a B-vitamin complex, folic acid or folate?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

22.) Are you taking a daily multivitamin?
   a. Yes
      IF yes, please bring these with you to your planned vaccination visit
   b. No
   c. Prefer not to answer

23.) Are you taking any other nutritional/herbal supplements?
   a. Yes
      IF yes, please bring these supplements to your planned vaccination visit
   b. No
   c. Prefer not to answer

**Your General Lifestyle and Hygiene**

24.) How often do you exercise?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

25.) Do you generally exercise indoors or outdoors?
   a. Indoors
   b. Outdoors
   c. Both
   d. Depends on the season
   e. None of the above
   f. Prefer not to answer

26.) Do you bite your fingernails?
   a. Yes
   b. No
   c. Prefer not to answer
27.) How often do you use a swimming pool/spa pool?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

28.) How often do you smoke cigarettes?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

29.) How often do you drink alcohol?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

30.) What type(s) of alcohol do you typically consume (select all that apply)?
   a. Beer/Cider
   b. Sour beers
   c. White Wine
   d. Red Wine
   e. Spirits/hard alcohol
   f. Not applicable
   g. Prefer not to answer

31.) How often do you brush your teeth?
   a. Never
   b. Less than once a day
   c. Once a day
   d. 1-2 times/day
   e. 2+ times a day
   f. Prefer not to answer

32.) How often do you floss your teeth?
33.) How often do you wear facial cosmetics?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

34.) Do you use deodorant or antiperspirant (antiperspirants generally contain 
aluminum)?
   a. I use deodorant
   b. I use an antiperspirant
   c. Not sure, but I use some form of deodorant/antiperspirant
   d. I do not use deodorant or an antiperspirant
   e. Prefer not to answer

35.) Approximately how many hours of sleep do you get in an average night?
   a. Less than 5 hours
   b. 5-6 hours
   c. 6-7 hours
   d. 7-8 hours
   e. 8 or more hours
   f. Prefer not to answer

36.) Do you use fabric softener when washing your clothes?
   a. Yes
   b. No
   c. Prefer not to answer

**Your General Health Background**

37.) Were you born via cesarean section (C-section)?
   a. Yes
   b. No
   c. Not Sure
   d. Prefer not to answer

38.) How were you fed as an infant?
   a. Primarily Breast Milk
   b. Primarily formula
   c. A mix of breast milk and formula
d. Not Sure
 e. Prefer not to answer

39.) Have you had chickenpox?
 a. Yes
 b. No
 c. Not Sure
 d. Prefer not to answer

40.) Have you had your tonsils removed?
 a. Yes
 b. No
 c. Not Sure
 d. Prefer not to answer

41.) Have you had your appendix removed?
 a. Yes
 b. No
 c. Not Sure
 d. Prefer not to answer

42.) Do you have seasonal allergies?
 a. Yes
 b. No
 c. Prefer not to answer

43.) Do you have any of the following non-food allergies?
 a. Drug (e.g. Penicillin)
 b. Pet
 c. Bee stings
 d. Pollen
 e. Other, Please specify: ________________________________
 f. None
 g. Prefer not to answer

44.) My weight has ________ within the last 6 months.
 a. Increased more than 5kg
 b. Decreased more than 5kg
 c. Remained stable
 d. Prefer not to answer

45.) When did you last take antibiotics?
 a. In the last week
 b. In the last month
 c. In the last 6 months
 d. In the last Year
 e. I have not taken antibiotics in the past year.
 f. I have never taken antibiotics
(If you answered a, b, c, or d please indicate which antibiotic you took and what you were treating below)

1. __________________________________________________________
2. __________________________________________________________
3. __________________________________________________________

46.) Have you ever taken antibiotics for a prolonged period (more than 3 months continuously or more than 12 times in a 12 month period)?
   a. Yes
   b. No
   c. Not Sure
   d. Prefer not to answer

47.) Complete this statement: ‘I have received a flu vaccine in the last….’ (select all that apply)
   a. Week
   b. Month
   c. For THIS (2016) flu season
   d. In 2015
   e. In 2014
   f. In 2013
   g. In 2012
   h. prior to 2012
   g. I have never had a flu vaccine

48.) Are you currently using some form of hormonal birth control?
   a. Yes, I am taking the “pill”
   b. Yes, I use an injected contraceptive (Depo Provera)
   c. Yes, I use a hormone implant (Implanon)
   d. Yes, I use the NuvaRing
   e. Yes, I use a hormonal IUD (e.g. Mirena)
   f. No
   g. Not applicable
   h. Prefer not to answer

49.) How many times do you have a bowel movement / stools in an average day?
   a. Less than one
   b. One
   c. Two
   d. Three
   e. Four
   f. Five or more
   g. Prefer not to answer
50.) Describe the quality of your bowel movements using the chart below:

![Bristol Stool Chart](chart.png)

- a. Type 1
- b. Type 2
- c. Type 3
- d. Type 4
- e. Type 5
- f. Type 6
- g. Type 7
- h. Prefer not to answer

51.) Complete this statement: ‘I have had a colonoscopy in the last……’

- a. Week
- b. Month
- c. 6 months
- d. Year
e. Never had a colonoscopy
f. Prefer not to answer

52.) Complete this statement: I have had colonic irrigation in the last….’
a. Week
b. Month
c. 6 months
d. Year
e. Never had colonic irrigation
f. Prefer not to answer
Your Detailed Dietary information

Meals
53.) Do you receive most (more than 75% of daily calories) of your nutrition from adult nutritional shakes (i.e. Ensure)?
   a. Yes
   b. No
   c. I eat both solid food and adult nutritional shake
   d. Prefer not to answer

54.) In an average week, how often do you consume meat/eggs?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

55.) In an average week, how often do you cook and consume home cooked meals? (Not ready-to-eat meals like boxed macaroni and cheese, ramen noodles, lean cuisine)
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

56.) In an average week, how often do you consume ready-to-eat meals (i.e macaroni and cheese, ramen noodles, lean cuisine)?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

57.) In an average week, how often do you eat food prepared at a restaurant, including takeaways?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer
58.) In an average week, how often do you eat at least 2 servings of whole grains (e.g. oat, barley, wheat, quinoa, rice, rye, corn, millet) in a day? One serving of whole grain is ½ cup cooked rice / grain / pasta / oatmeal, 1 slice whole grain bread, 1 cup 100% whole grain ready to eat cereal)
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

59.) In an average week, how often do you consume at least 2-3 servings of fruit in a day? (1 serving = ½ cup of fruit; 1 medium sized fruit; 120mL of 100% fruit juice.)
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

60) In an average week, how often do you consume at least 2-3 servings of vegetables, including potatoes in a day? (1 serving = ½ cup of vegetables/potatoes; 1 cup of leafy raw vegetables)
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

61.) How many different types of fruits and vegetables do you eat in a typical week? If you consume a soup containing peas, carrots and potatoes, each of these is considered a different vegetable.
   a. 1-2
   b. 3-4
   c. 5-7
   d. 8-9
   e. >10
   f. Prefer not to answer
62.) How often do you consume one or more servings of fermented vegetables or plant products a day? (1 serving = ½ cup of sauerkraut, kimchi or fermented vegetable or 1 cup of kombucha)?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

63.) In an average week, how often do you consume one or more servings of cultured dairy, fermented dairy, or yogurt a day? (1 serving = 1 cup of yogurt, 2 T of sour cream)
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

64.) In an average week, how often do you consume at least 2 servings of milk or cheese a day? (1 serving = 1 cup of milk; 40-60 grams of cheese)
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

65.) In an average week, how often do you consume milk substitutes (soy milk, lactose free milk, almond milk, etc.)?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

66.) How often do you eat frozen desserts (ice cream/gelato/milkshakes, sherbet/sorbet, frozen yogurt, etc.)?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer
67.) In an average week, how often do you eat red meat?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

68.) In an average week, how often do you consume higher fat red meats like prime rib, T-bone steak, hamburger, ribs, bacon, etc.?
   a. Never
   b. Rarely
   c. Occasionally (1-2 times/week)
   d. Regularly (3-5 times/week)
   e. Daily
   f. Prefer not to answer

69.) How many days in an average week do you consume chicken or turkey?
   a. Never
   b. Rarely
   c. Occasionally (1-2 days/week)
   d. Regularly (3-5 days/week)
   e. Daily
   f. Prefer not to answer

70.) How many days in an average week do you consume seafood (fish, shrimp, lobster, crab etc.)?
   a. Never
   b. Rarely
   c. Occasionally (1-2 days/week)
   d. Regularly (3-5 days/week)
   e. Daily
   f. Prefer not to answer

71.) How many days in an average week do you consume salted snacks (potato chips, nacho chips, corn chips, popcorn with butter, French fries etc.)?
   a. Never
   b. Rarely
   c. Occasionally (1-2 days/week)
   d. Regularly (3-5 days/week)
   e. Daily
   f. Prefer not to answer
72.) How many days in an average week do you eat sugary sweets (cake, cookies, pastries, donuts, muffins, chocolate etc.)?
   a. Never
   b. Rarely
   c. Occasionally (1-2 days/week)
   d. Regularly (3-5 days/week)
   e. Daily
   f. Prefer not to answer

73.) How many days in an average week do you cook with olive oil?
   a. Never
   b. Rarely
   c. Occasionally (1-2 days/week)
   d. Regularly (3-5 days/week)
   e. Daily
   f. Prefer not to answer

74.) How many days in an average week do you consume whole eggs (exclude just egg whites).
   a. Never
   b. Rarely
   c. Occasionally (1-2 days/week)
   d. Regularly (3-5 days/week)
   e. Daily
   f. Prefer not to answer

75.) How many days in an average week do you drink 473mL or more of sugar sweetened beverages such as non-diet soda or fruit drink/punch (not including 100% fruit juice)? (1 can of soda = 355mL).
   a. Never
   b. Rarely
   c. Occasionally (1-2 days/week)
   d. Regularly (3-5 days/week)
   e. Daily
   f. Prefer not to answer

76.) How many days in an average week do you consume at least 1litre of water?
   a. Never
   b. Rarely
   c. Occasionally (1-2 days/week)
   d. Regularly (3-5 days/week)
   e. Daily
   f. Prefer not to answer
77.) Please write anything else about yourself that you think may affect your personal microorganisms.

____________________________________________________________________________

____________________________________________________________________________

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____________________________________________________________________________
**Your Medical History**

Do you have any of the following medical conditions? If YES, please indicate if you were diagnosed by a doctor, another health professional (e.g. nurse, chiropractor, physiotherapist), or if you have diagnosed yourself with that condition.

<table>
<thead>
<tr>
<th>EXAMPLE</th>
<th>No</th>
<th>Diagnosed by a doctor</th>
<th>Diagnosed by another health professional</th>
<th>Self-diagnosed</th>
<th>Prefer not to answer</th>
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</thead>
<tbody>
<tr>
<td>Have you been diagnosed with <strong>asthma</strong>?</td>
<td>YES</td>
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<tr>
<th>Respiratory</th>
<th>No</th>
<th>Diagnosed by a doctor</th>
<th>Diagnosed by another health professional</th>
<th>Self-diagnosed</th>
<th>Prefer not to answer</th>
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<tbody>
<tr>
<td>Have you been diagnosed with <strong>asthma</strong>?</td>
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<tr>
<td>Have you been diagnosed with <strong>emphysema</strong>?</td>
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<tr>
<td>Have you been diagnosed with <strong>Chronic Obstructive Airways Disease (COPD)</strong>?</td>
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<tr>
<td>Have you been diagnosed with <strong>cystic fibrosis</strong>?</td>
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</table>
### Gastrointestinal

<table>
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<tr>
<th>Question</th>
<th>No</th>
<th>Diagnosed by a doctor</th>
<th>Diagnosed by another health professional</th>
<th>Self-diagnosed</th>
<th>Prefer not to answer</th>
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<tbody>
<tr>
<td>Have you been diagnosed with <strong>chronic bronchitis</strong>?</td>
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<td>Have you been diagnosed with any <strong>other respiratory disease</strong>?</td>
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<td><strong>Gastrointestinal</strong></td>
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<tr>
<td>Have you been diagnosed with <strong>small intestinal bacterial overgrowth (SIBO)</strong>?</td>
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<tr>
<td>Have you been diagnosed with <strong>irritable bowel syndrome (IBS)</strong>?</td>
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<tr>
<td>Have you been diagnosed with <strong>inflammatory bowel disease (IBD)</strong>?</td>
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<tr>
<td>Have you been diagnosed with <strong>Candida or fungal overgrowth in the gut</strong>?</td>
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<td>Have you been diagnosed with <strong>liver disease</strong>?</td>
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<td>Have you been diagnosed with <strong>Clostridium difficile infection</strong>?</td>
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<tr>
<td>Have you had <strong>bowel or stomach surgery</strong>?</td>
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<td>Please specify________________________________________________</td>
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<tr>
<th><strong>Cardiovascular</strong></th>
<th>No</th>
<th>Diagnosed by a doctor</th>
<th>Diagnosed by another health professional</th>
<th>Self-diagnosed</th>
<th>Prefer not to answer</th>
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<tbody>
<tr>
<td>Have you been diagnosed with <strong>congenital heart disease</strong>?</td>
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<tr>
<td>Have you been diagnosed with <strong>ischemic heart disease</strong>?</td>
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<tr>
<td>Have you been diagnosed with <strong>congestive heart failure</strong>?</td>
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<tr>
<td>Have you been diagnosed with <strong>rheumatic heart disease</strong>?</td>
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<tr>
<td>Have you been diagnosed with <strong>coronary artery disease</strong>?</td>
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<tr>
<td>Have you ever had a <strong>heart attack</strong>?</td>
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<tr>
<td>Question</td>
<td>No</td>
<td>Diagnosed by a doctor</td>
<td>Diagnosed by another health professional</td>
<td>Self-diagnosed</td>
<td>Prefer not to answer</td>
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<tr>
<td>Have you ever had a <strong>stroke</strong>?</td>
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<tr>
<td>Have you been diagnosed with any <strong>other heart condition and/ or cerebrovascular disease</strong>?</td>
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<td>Please specify</td>
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<tr>
<td><strong>Genitourinary</strong></td>
<td>No</td>
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<tr>
<td>Have you ever been diagnosed with a <strong>chronic renal (kidney) disease</strong>?</td>
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<td>Please specify</td>
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<tr>
<td><strong>Are you pregnant?</strong></td>
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<td>Please specify your due date</td>
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<tr>
<td><strong>Skin</strong></td>
<td>No</td>
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<tr>
<td>Have you been diagnosed with <strong>facial acne</strong>?</td>
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</tbody>
</table>
Are you taking prescription medication, over the counter medication or both?_______________________________________________________________

Have you been diagnosed with any other skin condition?

Please specify___________________________________________________

<table>
<thead>
<tr>
<th><strong>Neurological</strong></th>
<th>No</th>
<th>Diagnosed by a doctor</th>
<th>Diagnosed by another health professional</th>
<th>Self-diagnosed</th>
<th>Prefer not to answer</th>
</tr>
</thead>
</table>

Have you been diagnosed with migraines?

Have you been diagnosed with congenital myopathy?

Have you been diagnosed with cerebral palsy?

Have you been diagnosed with epilepsy or a seizure disorder?
<p>| Question | | | | | |
| --- | --- | --- | --- | --- | |
| Have you been diagnosed with <strong>hydrocephaly</strong>? | | | | | |
| Have you been diagnosed with <strong>motor neurone disease</strong>? | | | | | |
| Have you been diagnosed with <strong>multiple sclerosis</strong>? | | | | | |
| Have you been diagnosed with <strong>muscular dystrophy</strong>? | | | | | |
| Have you been diagnosed with <strong>myasthenia gravis</strong>? | | | | | |
| Have you been diagnosed with <strong>Parkinson’s disease</strong>? | | | | | |
| Have you ever been diagnosed with <strong>Alzheimer’s disease</strong>? | | | | | |
| Have you ever been diagnosed with <strong>Autism or Autism Spectrum Disorder</strong>? | | | | | |
| <strong>Psychiatric / Mental health</strong> | No | Diagnosed by a doctor | Diagnosed by another health professional | Self-diagnosed | Prefer not to answer |</p>
<table>
<thead>
<tr>
<th>Have you been diagnosed with <strong>depression</strong>?</th>
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<tbody>
<tr>
<td>Have you been diagnosed with <strong>bipolar disorder</strong>?</td>
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<td>Have you been diagnosed with <strong>schizophrenia</strong>?</td>
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<tr>
<td><strong>Endocrine</strong></td>
<td>No</td>
<td>Diagnosed by a doctor</td>
<td>Diagnosed by another health professional</td>
<td>Self-diagnosed</td>
</tr>
<tr>
<td>Have you been diagnosed with <strong>diabetes</strong>?</td>
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<tr>
<td>IF yes, please specify the type of diabetes:</td>
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<tr>
<td>Do you need to take Insulin for your diabetes:</td>
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<tr>
<td>Have you been diagnosed with a <strong>thyroid condition</strong>?</td>
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<td>IF yes, please specify the condition:</td>
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<tr>
<td><strong>Haematology</strong></td>
<td>No</td>
<td>Diagnosed by a doctor</td>
<td>Diagnosed by another health professional</td>
<td>Self-diagnosed</td>
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<tr>
<td>Have you been diagnosed with a <strong>blood disorder</strong>?</td>
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<td>IF yes, please specify___________________________________________________</td>
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Appendix III: Haemagglutination Inhibition Assay Protocol
Haemagglutination Inhibition Assay for detection of virus-specific antibodies in serum

Adapted from WHO Global Influenza Surveillance Network: Manual for the laboratory diagnosis and virological surveillance of influenza

Definitions

HAI – Haemaglutination Inhibition
HAU – Haemagglutination unit
RBC – (Chicken) red blood cells
RDE – Receptor destroying enzyme

Day 1

1. Make up solutions

Sterile PBS (0.01M, pH7.2)
Prepare a 1x solution of PBS
Test pH
Autoclave to sterilize
Store opened PBS for no longer than 3 weeks

RDE (II)
Reconstitute each vial with 20ml of sterile PBS
Use immediately or freeze in single use aliquots at -20°C

2. Treatment of reference sera to inactivate nonspecific inhibitors of HAI

a. Add 3 volumes of reconstituted RDE to 1 volume of serum (e.g. 150μl RDE + 50 μl serum) to a flat bottom 48 well microplate

b. Incubate 18-20hrs in a 37°C incubator

Day 2

b. Heat in a 56°C water-bath for 30 minutes to inactivate any remaining RDE.

c. Allow sera to cool to room temperature

d. Add 6 volumes of PBS. The final dilution of sera is therefore 1:10 in a 500μl volume (e.g. 150μl RDE + 50 μl serum + 300μl PBS)
3. Standardisation of RBCs
The final concentration of RBCs is 0.5%

a. Prepare a 1:10 dilution by adding 15 ml 5% RBC suspension to 135 ml PBS (pH 7.2) – pass RBC through cell strainer

b. Prepare a further 1:10 dilution by adding 1ml 0.5% RBC suspension to 9 ml PBS (pH 7.2)

c. Transfer 10 μl onto the haemacytometer channel and allow the cells to spread throughout the unit, being careful not to overfill the channel

d. Count the cells in 5 quadrants of the unit and calculate the cell#/ml of the 0.5% solution prepared in step 3.a. There should be 5x10⁷ cells/ml (+≤0.5 x10⁷ acceptable) in the 1:10 solution made step 3.a.

e. If out of range make another 1:10 and re-count. If still out of range use these counts to calculate RBC or PBS to add to make 5x10⁷/ml

f. Record RBC count/ml on HAU record sheet

g. Store at 4°C when not in use

4. Detection of nonspecific agglutinins in treated sera
See Figure 2.E-2 for schematic

Perform once on each serum sample

a. Add 25 μl PBS to rows B-H of a V-bottom well microplate

b. Add 50 μl PBS to the first well in column 12 (A12) for an RBC control

c. Add 50 μl of each serum sample + positive + negative control to row A.

d. Prepare serial 2-fold dilutions of the sera by transferring 25 μl from the first well of column to the successive wells in each column (i.e. A1 to B1; then B1 to C1; etc. up until G1 to H1). Discard the final 25 μl after row H

e. Add 25 μl PBS to all wells.

f. Add 50 μl standardized RBCs (see the standardization procedure for RBCs above) to all wells of columns. Mix by manually agitating the plates thoroughly

A total of 30 minutes is usually required – check regularly after this time
Record and interpret the results in accordance with FIGURE 2.E-3

*If the RBCs settle completely in the wells in a column containing diluted serum, that serum is acceptable for use in the HAI test. The presence of nonspecific agglutinins will be evident by any haemagglutination of the RBCs by the serum.*

If agglutination occurs the serum must be adsorbed with RBCs as follows:

h. To one volume of packed RBCs in a centrifuge tube add 20 volumes of RDE-treated serum (e.g. 10ul 5% RBC + 200ul RDE-serum). Mix thoroughly and incubate at 4°C for 1 hour, mixing at intervals to resuspend the cells

i. Centrifuge at 1200 rpm for 10 minutes. Carefully remove the adsorbed serum without disturbing the packed RBCs.

j. Check for the presence of nonspecific agglutinins in the serum, as described above. Repeat adsorption with RBCs until there is no haemagglutination associated with the serum.

5. **HAU determination**

See figure 2.E-4 for schematic

a. To a V-bottom microtitre plate add 50 μl PBS to wells 2 to 12 of each row (i.e. A2–A12; B2–B12; etc. up to H2–H12)

b. Add 100 μl of each different viral isolate to the first wells of rows A–C (i.e. A1–C1). For RBC control add 100 μl of PBS to H1

c. Prepare serial 2-fold dilutions of the virus by transferring 50 μl from the first well of rows A-G to the successive wells in each row (i.e. A1 to A2; then B1 to B2; etc. up until A11 to A12). Discard the final 50 μl after row 12

d. Add 50 μl of standardized RBCs to each well. Mix by manually agitating the plates thoroughly

e. Cover and incubate the plates at room temperature for **30 minutes**. Check the RBC control for complete settling of the cells.

f. Record haemagglutination titration end-point dilution

*The absence of haemagglutination can be confirmed by tilting the plates at a 45-degree angle for 20–30 seconds. If the settled RBCs “run” or form a teardrop at the same rate as the controls then that particular well is considered negative for agglutination. The haemagglutination titration end-point is defined as the highest dilution of virus that still causes complete haemagglutination. The haemagglutination titre is the reciprocal of this dilution. For example, if a virus causes complete haemagglutination up to a 1:256 dilution then the HA titre of*
the virus stock is 256. One unit of haemagglutination is contained in the end-point dilution of the HA titration. The unit of haemagglutination is an operational unit dependent upon the volumes used for haemagglutination titration. A haemagglutination unit is defined as the amount of antigen (virus) needed to agglutinate an equal volume of a standardized RBC suspension.

6. Preparation of viral isolate for HAI and back titration

a. Determine the volume of virus working solution needed for the HAI test. For example:
   Backtitration: 800ul
   HAI assay: 25ul/4HAU in 96 wells each plate, 6 plates per virus = 14400ul/2304HAU
   +20% = 17,280ul
   Make 18,000ul

b. In the HAI test 4 HAU/25ul are added to each well, equivalent to 8 HAU/50 μl. To calculate the virus dilution, divide the haemagglutination titer calculated in step 5 (which is based on 50 μl) by 8. For example, a haemagglutination titer of 160 divided by 8 is a 20-fold dilution

c. Prepare the dilution in PBS and record the dilution prepared on the sample record sheet

d. Perform a back titration to verify the correct units of haemagglutination by performing a second haemagglutination test described in step 5, using the virus dilution prepared in step 6.c

A haemagglutination titer of 4 haemagglutination units per 25 μl (equivalent to 8 units per 50μl) will haemagglutinate the first four wells of a row of a back-titration plate. If an antigen does not have a titer of 8 per 50 μl it must be adjusted accordingly by adding more antigen or by diluting. For example, if complete haemagglutination is present in the fifth well, the antigen has a titer of 16 and should be diluted 2-fold.

e. Once the correct unit is reached store the diluted virus at 4°C and use within the same day

7. Determining virus-specific antibody titer with HAI assay

See figure 2.F-1 for schematic

a. To a V-well microtitre plate add 25 μl PBS (pH 7.2) to the wells in rows B to H (B1–B12; C1–C12, etc. up to H1–H12). Add 25 μl PBS to RBC control A12.

b. Add 50 μl of each treated serum (already diluted to 1:10 in step 3) to the appropriate well in row A (A1–A11).

Run participant’s D0 and D28 serum in duplicate. Perform each participant’s samples in same experiment (on same plate not required). There should be an RBC control on each plate to indicate incubation time. One positive and one negative serum sample should be run each time the HAI assay is performed.

c. Prepare serial 2-fold dilutions of the treated sera by transferring 25 μl from the first to
successive wells of each column (i.e. A1–H1; A2–H2, etc.). Discard the final 25 μl after row H

d. Add 25 μl virus working solution to all wells (A1–H12) of plate, including RBC control
e. Mix the contents of the plates by manually agitating the plates thoroughly

f. Cover the plates and incubate at room temperature for 15 minutes
g. Add 50 μl standardized RBCs to all wells and mix thoroughly by manually agitating the plates thoroughly

h. Cover the plates and allow the RBCs to settle at room temperature for the appropriate time according to the RBC control

i. Record the HAI titer on the HAI record sheet as the reciprocal of the dilution at which haemagglutination was completely inhibited, monitor assay performance over time using the positive and negative serum controls