Changing Epidemiology of Leptospirosis in New Zealand, with a Focus on the Novel Strain of *Leptospira Borgpetersenii*

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Dedication

To the unwavering spirit and bravery of the people of Ukraine, to Kharkiv, the bravest of all cities.

In the face of adversity, your resilience and courage shine brightly, embodying the essence of determination and strength.

As Winston Churchill once said, "We shall not fail or falter; we shall not weaken or tire. Neither the sudden shock of battle nor the long-drawn trials of vigilance and exertion will wear us down."

Your steadfast resolve and unyielding bravery are a testament to the power of the human spirit and a beacon of hope for all who strive for freedom and justice.

"Never give in, never give in, never, never, never, never—in nothing, great or small, large or petty—never give in except to convictions of honour and good sense."

May your courage continue to inspire and guide us all.

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Abstract

In New Zealand, leptospirosis has been a common disease in dairy cattle since the 1940s. Six pathogenic *Leptospira* serovars from two species have been identified as endemic to New Zealand: *Leptospira borgpetersenii* serovars (sv.) Hardjobovis (Hardjo), Tarassovi, Ballum, Balcanica, and *Leptospira interrogans* sv. Pomona and Copenhageni. From these, sv. Pomona and Hardjo are the most commonly reported in cattle, and sv. Ballum, Tarassovi, and Copenhageni are less common. The estimated 99% of the national dairy herd is vaccinated against leptospirosis by vaccines containing antigens to Pomona and Hardjobovis and sometimes vaccines also include Copenhageni antigen. Vaccines for protecting dairy cows against Tarassovi were unavailable in New Zealand before December 2023. Historically, leptospirosis due to Tarassovi infection in New Zealand cattle was considered accidental and clinically unimportant.

Serosurveys of apparently healthy cattle in New Zealand showed that over the past fifty years, Tarassovi seroprevalence increased from 6% (50/300) at MAT ≥ 50 to 18% (698/3878) at MAT ≥ 48. More recently, a serology and urine shedding study from 2015-2016 evaluated the status of 4,000 dairy cows from 200 randomly selected farms, stratified by New Zealand's geographical location and herd size. The study found that on the animal level, 17% of the study animals showed evidence of past infection with Tarassovi, as defined by at least one sample reacting at MAT ≥ 48 for the study's purposes. Moreover, 96% (90/94) of PCR-positive urine samples were sequenced, and 68% (54/80) of those were found to have a novel allele in the sequenced region at the *glm*U loci. Thus, the use of molecular diagnostic tools, specifically molecular typing targeting a partial region of the *glmU* gene, allowed New Zealand researchers to identify a novel *L. borgpetersenii* strain, informally called strain (str.) Pacifica, in the urine of these cows. The 2015-2016 survey reported that dairy cows with Tarassovi titres were associated with urinary shedding, as determined by microscopic agglutination test (MAT) and polymerase chain reaction (PCR) testing, respectively. Because of this association, str. Pacifica is thought to belong to the serogroup Tarassovi. Additionally,

the DNA of str. Pacifica has been retrospectively detected in cattle and deer samples dating back as early as 2007.

Moreover, the 19-year (1999-2007) average annual incidence of notified human cases of Tarassovi leptospirosis was estimated at 12.59/100,000 in dairy farmers, compared to an overall annual average incidence of 2.01/100,000. These coincidental findings raised public health concerns.

Therefore, at least some cases of Tarassovi seropositivity, as identified by MAT and reported before 2021, could partially be attributed to str. Pacifica. To confirm str. Pacifica's serogroup, isolation by culture and complete genetic characterisation of an isolate are required. Since str. Pacifica was only recently detected, its epidemiology, morphology, maintenance, and pathogenicity in the host population, as well as its impact on animal and human health, were not well understood.

In this study, we investigated the possibility of isolating str. Pacifica from cow`s urine by running a series of laboratory experiments where laboratory-adapted strains were used as a proxy for *L. borgpetersenii* str. Pacifica in the absence of an isolate to better understand its growth requirements. Laboratory-adapted *Leptospira borgpetersenii* strains were seeded into different types of media, and *Leptospira* growth rates were evaluated (Chapter 3). As a result, we ruled out unsuitable media and growth conditions, and this work helped to select the best media and growth conditions for a follow-up field investigation, where freshly collected cow`s urine was seeded into selected media. Str. Pacifica was isolated from the urine of a shedding cow using HAN medium at 37°C and 5% CO_2. However, this medium failed to sustain str. Pacifica and the culture was lost (Chapter 4).

In addition, over the 2020-2021-2022 milking seasons, we collected and tested blood and urine samples from dairy farms, identified as str. Pacifica positive from the 2016 survey. Our results revealed that str. Pacifica was still maintained in the same dairy herds six years after initial detection. Moreover, we reported an estimated prevalence ratio (PR) of 7, indicating that the prevalence of shedders was seven times as high at the beginning than at the end of lactation in primiparous cows (Chapter 4). These

findings provide evidence that str. Pacifica is adapted to dairy cows in New Zealand, and the peak shedding in primiparous heifers occurs in early lactation.

Since the highest levels of str. Pacifica shedding were detected at the start of the milking season during peak milk production, which also coincides with a relatively short 3-month mating period, the associations between str. Pacifica and milk production and reproductive performance of milking cows were also investigated using herd test data, and serological and PCR test results of the 2016 survey. Statistical models, including linear, logistic, and generalised mixed models with fixed and random effects, as well as a shared frailty survival model, were used to evaluate the associations between str. Pacifica positivity and reproduction (Chapter 5) and milk reproduction (Chapter 6) in dairy cows. Results of the statistical analysis of the association between str. Pacifica positivity and reproduction (Chapter 5) of dairy herds showed that str. Pacifica delayed the time from calving to conception (HR = 0.84; 95%: CI 0.74-0.96), although there was no effect on the pregnancy rate (Chapter 5). An analysis of milk production data did not reveal any associations with str. Pacifica at either animal or herd level (Chapter 6). The absence of clinical signs and the lack of association with milk production and reproduction at both the animal and herd levels provides further evidence towards str. Pacifica being well adapted to dairy cows. Therefore, evidence from this thesis suggests that dairy cattle are the maintenance host for str. Pacifica in New Zealand. It is also important to note that str. Pacifica shedders can infect people, especially dairy farmers, milkers, and farm workers who are in regular contact with str. Pacifica-shedding animals. Therefore, the prevention of str. Pacifica transmission via vaccination or the use of appropriate personal protective gear should be prioritised.

CHAPTER 1

Chapter 1 General Introduction

1.1 Introduction

This general introduction briefly reviews leptospirosis, the epidemiology of the disease in New Zealand, and presents the aims and structure of this thesis. The thesis was formatted and presented as independent chapters suitable for peer-reviewed journal publications. Therefore, repetitions occur between chapters.

1.2 Background

Leptospirosis is a zoonotic infectious disease caused by pathogenic spirochaetes of the genus *Leptospira*. Globally, it affects approximately 1 million people annually and an estimated 60,000 perish (Costa *et al.* 2015). *Leptospira* is a diverse bacterial genus with more than 300 serovars (Picardeau *et al.* 2014) and seventy-one genomospecies currently identified (Korba *et al.* 2021), of which forty-two fall in the pathogenic clade (Vincent *et al*. 2019). Susceptible species include humans and other mammals, while amphibians, birds, and reptiles can also be infected (Picardeau 2017). The main route of infection is through direct contact with contaminated urine or indirectly through a contaminated environment (Haake and Levett 2014). *Leptospira* colonises the kidneys of the infected host and is excreted with urine into the environment, where it can survive for months under favourable conditions (Barragan *et al.* 2011).

While most mammal species are susceptible to infection, some have evolved to become maintenance hosts (Andersen-Ranberg *et al.* 2016). Levett (2001) defines a maintenance host as "a species in which infection is endemic and is usually transferred from animal to animal by direct contact". Most maintenance hosts have an asymptomatic course of the disease, and their infection with *Leptospira* is often lifelong and subclinical, guaranteeing the host's and bacteria's continued survival. In accidental hosts, the severity of the disease is greater than in maintenance hosts, although clinical signs can range from mild to severe. (Faine 1994). The severity of the disease also depends on the strain's virulence and the animals' immunological status, and young and old are more often diagnosed with clinical disease (Ellis 2015). Cattle can be maintenance hosts for some strains and accidental for others (Adler 2015). The

manifestation of leptospirosis in cattle depends on the infecting strain and can be presented in clinical and subclinical forms. Reported clinical effects of leptospirosis associated with the production of dairy cows include a sudden drop in milk production accompanied by fever, agalactia, or immobility (Hoare and Claxton 1972). Atypical mastitis, often affecting all four quarters at the same time, is also commonly reported (Higgins *et al.* 1980; Garoussi *et al.* 2017; Grippi *et al.* 2020). Reproduction failures attributed to clinical leptospirosis are often linked to high MAT titres of 2,000 to 50,000 (Emanuel *et al.* 1964) and may lead to early embryonic death (Oliveira *et al.* 2021) or mass late-term abortions (Kingscote and Wilson 1986), sometimes referred to as "abortion storms" (Carter *et al.* 1982; Elder *et al.* 1985). In young calves, the most reported symptom of clinical leptospirosis is haemoglobinuria or "redwater" (Taylor *et al.* 1980).

Subclinical leptospirosis in cattle associated with milk production is rarely reported but could present as subclinical mastitis, leading to a 10 to 30% drop in the annual expected milk volume (Dhaliwal *et al.* 1996). However, subclinical effects of leptospirosis associated with reproduction are more widely recognised and include fertility disorders and suboptimal performance, with prolonged calving to conception intervals and low conception rates being the most reported (Ayral *et al.* 2014; Mori *et al.* 2017). While clinical leptospirosis may be associated with high MAT titres, and conversely, lower MAT titres may indicate subclinical infection, the association of MAT titres with milk production may not have an apparent negative correlation, as reported by Dhaliwal *et al.* (1996), where no statistically significant difference was found between cows with low (MAT <30) and high (MAT >30) milk yields, possibly due to past exposure prior to current lactation

1.3 Leptospirosis in New Zealand

New Zealand has a unique and limited composition of mammals; the only endemic terrestrial mammals are two bat species, with the other 40 mammalian species being introduced over time (Hand *et al.* 2018; King and Forsyth 2021). New Zealand's geographical isolation and stringent biosecurity measures have limited the accidental introduction of *Leptospira*. The limited routes of introduction, combined with the small

range of available hosts, have resulted in a limited number of *Leptospira* species and serovars currently found in New Zealand. To date, two pathogenic species of *Leptospira* endemic to New Zealand have been identified: *Leptospira borgpetersenii* serovars (sv.) Hardjo, Ballum, Tarassovi, Balcanica, and *Leptospira interrogans* sv. Pomona and Copenhageni (Thornley *et al.* 2002). Different New Zealand mammalian species can be maintenance hosts for specific serovars (Wilkinson *et al.* 2023). Cattle are considered the maintenance hosts for Hardjo and Pomona; pigs for Pomona and Tarassovi; rats for Copenhageni; small, free-living mammals for Ballum, and sheep and deer are maintenance hosts for Pomona and Hardjo (Table 1.1). *Leptospira* are highly adaptive organisms and climatic and ecological changes may result in new host-serovar combinations (Hartskeerl *et al.* 2011).

Table 1.1 Table of Endemic *Leptospira* Serovars in New Zealand and Relevant Maintenance Hosts

Species	**Serovar**	**Serogroup**	**Maintenance Hosts (References)**
Leptospira borgpetersenii	Hardjo	Serjoe	Cattle, Deer, Sheep (Marshall and Manktelow 2002; Ayanegui-Alcerreca *et al.* 2007)
	Ballum	Ballum	Black Rat, Mouse, Hedgehogs (Hathaway *et al.* 1981)
	Tarassovi	Tarassovi	Pig (Marshall and Manktelow 2002)
	Balcanica	Serjoe	Brushtail Possum (Hathaway *et al.* 1981; Day *et al.* 1998)
Leptospira interrogans	Pomona	Pomona	Cattle, Pig, Sheep (Marshall and Manktelow 2002)
	Copenhageni	Icterohaemorrhagiae	Brown Rat, Mouse, Hedgehog (Hathaway *et al.* 1981)

In New Zealand, human leptospirosis is generally considered an occupational disease (Nisa *et al.* 2023). The work-related risk of contracting leptospirosis is higher in occupations with day-to-day contact with animals compared to those whose occupation does not include daily contact with animals (Marshall and Manktelow 2002). The first case of human leptospirosis in New Zealand was reported in 1952 when Pomona was isolated from a dairy farmer (Kirschner 1954). Since the 1980s, the most commonly detected serovars have been Hardjo, Pomona, Ballum, and Tarassovi, listed by their frequency (Wael *et al.* 2018). The number of reported leptospirosis cases in New Zealand peaked at 875 in 1971 (Anonymous 1991). However, following the introduction of the industry's dairy herd vaccination program in 1980, cases declined significantly from 234 per 100,000 in the 1970s to 4.5 per 100,000 in the early 1980s (Thornley *et al.* 2002). At that time, human leptospirosis was mainly associated with Pomona and Hardjo (Christmas *et al.* 1974). During 1990–1998, the annual incidence was 4.4 per 100,000; the next period, from 1999–2016, saw a significant decrease to 1.96 cases per 100,000 (ESR 2019). A growing concern is an increasing number of reported human cases attributed to non-vaccine serovars Ballum (Thornley *et al.* 2002) and Tarassovi (Wael *et al.* 2018; Nisa *et al.* 2020). There was an increase in notified human cases of leptospirosis serologically attributed to Tarassovi in New Zealand from 1999 to 2017, particularly noticeable among dairy farmers, with a reported incidence of 12.59/100,000 (Nisa *et al.* 2020). Currently, dairy cattle *Leptospira* infections associated with serovars Pomona and Hardjo are largely controlled via vaccination, with an estimated 99% of the dairy herds (Yupiana *et al.* 2019) complying with the industry's recommended vaccination scheme. Additionally, 27% of those dairy herds are vaccinated with a trivalent vaccine containing Copenhageni (Yupiana *et al.* 2019). A smaller proportion of beef cattle (estimated at 10%) are vaccinated against Hardjo and Pomona. A recently published cross-sectional serological study has estimated a seroprevalence in beef, sheep, and deer antibody titres (≥48) by MAT to Hardjo, Pomona, and Tarassovi. These, listed by frequency, were the most commonly detected in all three ruminants (Wilson *et al.* 2021).

1.4 Emerging *Leptospira* strain in New Zealand

Serosurveys of apparently healthy cattle in New Zealand showed that over the past fifty years, Tarassovi seroprevalence increased from 6% (50/300) at MAT ≥ 50 (Ryan and Marshall 1976) to 18% (698/3878) at MAT ≥ 48 (Wilson et al. 2021). More recently, a serology and urine shedding study from 2015-2016 evaluated the status of 4,000 dairy cows from 200 randomly selected farms, stratified by New Zealand's geographical location and herd size (Yupiana et al. 2019). The study found that on the herd level, 75% of the study herds showed evidence of a recent infection with Tarassovi, as defined by at least one sample reacting at MAT ≥ 48. Further statistical analysis showed a statistically significant association ($p < 0.01$) between urinary shedding and seropositivity to Tarassovi, and it was suggested that an elevated MAT titre (MAT ≥ 48) may indicate active urinary shedding. Moreover, 96% (90/94) of PCR-positive urine samples were sequenced, and 68% (54/80) of those were found to have a novel allele in the sequenced region at the *gyr*B and the *glm*U loci (Slack *et al.* 2009; Wilkinson *et al*. 2021). Therefore, the study reported the detection of a novel strain that serologically reacts to Tarassovi. However, genetically, it differs from Tarassovi and is currently informally referred to as strain (str.) Pacifica (Wilkinson et al. 2021). Although this is not an official name used in the *Leptospira* nomenclature, for consistency of reporting, the *Leptospira borgpetersenii* strain Pacifica will also be referred to as str. Pacifica throughout this thesis.

It is possible that some of the human cases serologically attributed to Tarassovi prior to 2021 (Ryan and Marshall 1976; Nisa *et al.* 2020; Wilson *et al.* 2021) could be attributed to Pacifica, as, at the time, serological tests were the preferred testing method and a molecular test method capable of distinguishing Tarassovi and str. Pacifica was only described and published in 2021 (Wilkinson *et al.* 2021).

1.5 Thesis Aims and Structure

Previous research projects identified the presence of a novel strain of *Leptospira* in New Zealand livestock, with a notable reported prevalence in dairy herds (Yupiana *et al.* 2019). Due to the recent detection of the novel strain, the epidemiology, morphology,

transmission mechanism, maintenance in the host population, and associations between novel *Leptospira* and the host's health are not understood well.

1.5.1 Thesis Aims

This PhD project aims to:

1. Approximate the best-suited laboratory isolation, growing, and maintenance conditions for str. Pacifica, using other laboratory-adapted strains as its proxy.
2. Conduct an epidemiological investigation of str. Pacifica in New Zealand dairy herds and investigate possible associations of str. Pacifica with the production and reproduction of dairy cows.

The results of these studies will lead to a better understanding of str. Pacifica's epidemiology and growth requirements and may enable further researchers to culture Pacifica, characterise it by whole genome sequencing (WGS), and advance diagnostic methods and vaccine development to prevent str. Pacifica leptospirosis.

1.5.2 Thesis Structure

This PhD thesis consists of seven chapters, including four analytical chapters.

- Chapter 1: General introduction
- Chapter 2: Literature review focused on *Leptospira borgpetersenii* serovar Tarassovi and *Leptospira borgpetersenii* strain Pacifica
- Chapter 3: Evaluation of Growth Media and Culture Conditions for *Leptospira borgpetersenii*

  T y p e t e x t h e r e
- Chapter 4: Epidemiology of *Leptospira borgpetersenii* strain Pacifica in New Zealand Primiparous Dairy Cows during 2020-2022

- Chapter 5: Investigation of the Association Between *Leptospira borgpetersenii* strain Pacifica and Reproductive Performance of Dairy Cows in New Zealand

- Chapter 6: Exploring the Association Between *Leptospira borgpetersenii* Strain Pacifica and Milk Production of New Zealand Dairy Cows

- Chapter 7: General Discussion

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CHAPTER 2

Chapter 2 Literature Review

2.1 Introduction

Leptospirosis is a global zoonotic disease affecting a wide range of mammals and some non-mammalian species (Picardeau 2017). The World Health Organization (WHO) estimates that 0.1–1 people per 100,000 living in temperate climates and ten or more people per 100,000 living in tropical climates are affected by leptospirosis each year worldwide (WHO 2011).

In New Zealand, the comparison of two periods (1999 - 2007) and (2008 - 2017) of *Leptospira* notifications showed that the incidence of Tarassovi in humans increased (IRR: 1.75, 95% CI: 1.13–2.78) and was particularly noticeable in dairy farmers at 12.59/100,000, whereas the overall incidence rate was 2.01/100,000 (Nisa *et al.* 2020). A previous study analysed 2016 survey data from 4,000 dairy cows across 200 farms, finding that over 50% of *Leptospira*-shedding cows were Tarassovi seropositive (Yupiana *et al.* 2019). However, genomic amplification showed that 68% (54/80) of sequences belonged to a novel *Leptospira borgpetersenii* strain, informally called strain (str.) Pacifica (Wilkinson *et al*. 2021). Presently, human leptospirosis in New Zealand is diagnosed by serological methods that cannot discriminate between str. Pacifica and Tarassovi. Therefore, it is unknown which strain is causing Tarassovi leptospirosis in humans. Leptospirosis is considered an occupational hazard in New Zealand, with sporadic infections mainly registered among workers in regular direct contact with animals or those working in contaminated environments. Thus, the emergence of the novel Tarassovi strain, specifically str. Pacifica as a leading cause of human leptospirosis among dairy farmers has been identified as a public health concern (Yupiana et al. 2019; Benschop et al. 2021; Nisa et al. 2023) and underscores the critical need for a comprehensive understanding of its significance.

2.2 Scope, Aims, and Reporting of the Results

2.2.1 Scope

This work is focused on a novel Tarassovi strain Pacifica, but only a few publications by New Zealand researchers reporting it are currently available, as the method to differentiate Pacifica from Tarassovi was only published in 2021 (Wilkinson *et al.* 2021). More recently, detailed host-serovar associations in farmed and wild mammals were investigated in 2023 (Wilkinson *et al.* 2023). Therefore, due to limited current knowledge regarding the epidemiology, host specificity, transmission, and geographical distribution of str. Pacifica in New Zealand and other countries, the scope of the literature review included all reports of identification by serology, culture, or molecular methods of *Leptospira* serologically identified as Tarassovi as a proxy for strain Pacifica.

The main reason for this is that in the absence of additional information, given the likely similarity in lipopolysaccharide (LPS) and the role that plays in pathogenesis, we are hypothesising that historical cases of leptospirosis serologically attributed to serogroup Tarassovi, and compatible subclinical/clinical signs could include both Tarassovi and Pacifica infections.

The geographical distribution of Pacifica may be limited to specific regions; for example, serovar Pomona-Kennewicki is an important serovar among cattle in North America and Australasia but has not been reported in Europe (Hamond *et al.* 2019). Therefore, geographically diverse reports of serogroup Tarassovi are also included in this review to identify its distribution.

2.2.2 Aims

The aims of this literature review were:

1. To identify all reported hosts, including humans, investigate the host specificity disease manifestations of *Leptospira* serogroup Tarassovi.

2. To investigate the global distribution and the geographic range of pathogenic *Leptospira* serogroup Tarassovi.

2.3 Methodology of Literature Search

It is important to note that this is not a systematic review; thus, a systematic review methodology was not followed here. The literature search was conducted in August-October 2021; the same search was repeated in August 2023, and several newly published relevant publications were added. Three specific inclusion criteria defined our literature searches:

a) First reports and identifications of serogroup Tarassovi.

b) Isolation of Tarassovi and serosurveys with positive Tarassovi identification by culture and/or MAT and/or PCR methods.

c) Hosts and approximate geographical distribution of Tarassovi.

d) Tarassovi as an occupational risk factor in humans.

The following steps were taken to identify publications of interest.

1. Database search terms were defined and included names of serovars of the Tarassovi serogroup, including additional search terms outlined in Table 2.1 below.

2. Three databases were searched: Web of Science, Scopus, and Google Scholar. Search terms were slightly modified to fit each database requirement.

3. There were no restrictions on the year and language of publication.

4. 'Grey literature' and non-peer-reviewed reports were included in this review.

5. All serosurveys that reported positive Tarassovi with various cut-off MAT titres were included.

Table 2.1 Search Terms Used for Literature Search in Databases

Search Terms	Search String Description
History of *Leptospira* Tarassovi	Search string for articles on *leptospir** AND *[animal* OR *mammal*] AND *Tarassovi* OR [*serovar* OR *serogroup* OR *serotype* OR *Mitis* OR *Hyos* OR *Perepelitsin* OR *Weil* OR *Redwater* OR *Spirochete*]
Tarassovi Isolation in non-human hosts	*Leptospir** AND *animal* [OR *mammal* OR *reptile* OR *bird* OR *avian* OR *amphibian* [AND *Atchafalaya* [OR *Atlantae* OR *Bakeri* OR *Bravo* OR *Chagres* OR *Corredo*res OR *Darien* OR *Gatuni* OR *Gengma* OR *Guidae* OR *Hyos* OR *Kanana* OR *Kaup* OR *Kisuba* OR *Langati* OR *Malaysia* OR *Mengpeng* OR *Mitis* OR *Mogden* OR *Navet* OR *Ngavi* OR *Sulzerae* OR *Tarassovi* OR *Topaz* OR *Tunis* OR *Vughia* OR *Yunxian* OR *serovar* OR *serogroup* OR *serotype]*
Tarassovi as an occupational risk factor in humans	*Leptospir** AND *animal* OR *mammal* OR *reptile* OR *bird* OR *avian* OR *amphibian* OR *human* OR *man AND Tarassovi OR serovar* OR *serogroup* OR *serotype* OR *Mitis* OR *Hyos* AND *Occupation* OR *Work*

Footnote: The symbol * is a wildcard symbol enabling the detection of any suffix.

2.3.1 How Historical and Current Results are Reported

Over the years, the nomenclature of Tarassovi expanded from a single strain to multiple serologically homologous serovars belonging to various genomospecies; the same species have been reported and classified differently. Thus, the reader should be aware that the *Leptospira* classification used between the 1930s and 1980s was based mainly on serology and may not reflect contemporary *Leptospira* classification. Therefore, serogroup was the only common identifier used throughout the years for all Tarassovi and can be used as a proxy for literature search, although the serogroup name was changed a few times as discussed in 2.4.2.

In this literature review, regardless of the nomenclature and orthography used in the original publications, sg. and sv. Tarassovi, as well as strain names, will always be capitalised. Additionally, species will be denoted as spp., serogroup as sg., serovar as sv., and strain as str.

MAT titres and cut-off values were included as reported by authors when available. However, while this literature review broadly evaluates the serogroup Tarassovi, extra attention was given to published reports of findings related to *Leptospira borgpetersenii* serovar Tarassovi and *Leptospira borgpetersenii* strain Pacifica as per current classification.

2.4 *Leptospira* Classification and Taxonomy

Leptospirosis is caused by pathogenic bacteria from the *Leptospira* genus, phylum of Spirochaetes, order Spirochaetes, and the family Leptospiraceae (Paster *et al.* 1991). *Leptospira* are grouped into over 300 serovars and 50 serogroups by serological classification. The "serovar" is the basic taxon used to classify *Leptospira* serologically and is based on structural differences in their outer membrane LPS (Cerqueira and Picardeau 2009). Variability in the LPS is important because it is partially responsible for the ability of *Leptospira* to establish chronic infection in the kidneys, as surface LPS antigens are reported to have an important role in primary host-pathogen interaction invasions and adhesion (Ko *et al.* 2009; Haake and Levett 2015; Xia *et al.* 2017).

Serovars that share cross-agglutinating antigens are then classified into serogroups (Faine 1994). Although serogroups have no official taxonomic status, they remain used as a practical way of grouping serovars (Wolff 1954). The most widely used laboratory test for serogroup identification is the microscopic agglutination test (MAT), considered a "gold standard" test by the WHO. The antigen panels used in micro-agglutination serology include serovars as indicators of relevant serogroups applicable to the tested population (Barragan *et al.* 2017). MAT principles and limitations are explained in detail by Levett (2003).

Genomic *Leptospira* classification is based on DNA heterogeneity and relies on molecular techniques such as DNA sequences of conserved genes or multilocus sequence genotyping (Thibeaux *et al.* 2018). A recent study undertook an extensive comparative genomic investigation of *Leptospira* species, which led to a new nomenclature and classification of the genus *Leptospira* by comparative genomics (Vincent *et al.* 2019). Currently, approximately 71 genomospecies (Korba *et al.* 2021)

are described by molecular methods and within the *Leptospira* genus, with two major clades recognised, those being pathogenic (P) and saprophytic (S). These clades are subdivided into two subclades, P1, P2, S1, and S2 (Vincent *et al.* 2019). Molecular testing to allow differentiation between different *Leptospira* spp. clades can be based on the detection of genes present in pathogenic *Leptospira,* such as *gyrB* (Slack *et al.* 2009), *lipL32 (*Blanchard *et al.* 2021*)*, *lfb1* (Merien *et al.* 2005), *35ligA*, *ligB2* (Palaniappan *et al.* 2005), and *glmU* (Wilkinson *et al.* 2021*).*

Although both serological and genomic classification systems are commonly used, they correlate poorly (Thibeaux *et al.* 2018). An example of poor correlation between serological and genomic classification can be drawn from the nomenclature of serogroup Tarassovi, in which 26 serovars belong to five different genomospecies, *L. alexanderi, L. borgpetersenii, L. kmetii, L. santarosai and L. weilii* (Yasuda *et al.* 1987; Ramadass *et al.* 1992; Gravekamp *et al.* 1993; Brenner *et al.* 1999). The relationship between species and serovars is complex, and the fact that different species are serologically homologous could be explained by horizontal gene transmission of the LPS gene between species, but the exact mechanism of this transfer is unknown (Bulach *et al.* 2006; Achtman 2008). However, Nieves *et al.* (2023) reported that the *rfb* cluster in the LPS gene strongly correlates with *Leptospira* serovar designation.

2.4.1 *Leptospira borgpetersenii* Serovar Tarassovi

Leptospira nomenclature was developed over time, and since *Leptospira* was first isolated in 1915 by Ido and Inado, many serovars and serogroups of these bacteria have been discovered. However, because most of the work has been done by researchers in different countries and these discoveries were not always communicated on time, *Leptospira* classification was "a work in progress" for many decades, mainly because of the constant development of sufficient catalogue for strain identification and availability of antisera collections around the world. Sometimes, the isolated bacteria remained unnamed or classified under the incorrect name until the classification of reference strains became available for serological testing and comparison. For example, in the 1940s, Savino and Rennella (1950) examined sera from 300 cattle in

Argentina. They found that 55 animals were seropositive to *Leptospira* serovar, unknown to them due to the cross-reactivity of antigens, and named it *Leptospira* Suis. Nevertheless, L. Suis was later identified as L. Pomona when the L. Pomona reference strain, initially isolated in Australia, became available for comparison. Since then, the scientific nomenclature of *Leptospira* species has developed over time, and the present nomenclature includes genus, species, serogroup, serovar, and strain of bacteria.

2.4.2 Historical Nomenclature of Serogroup Tarassovi

L. borgpetersenii sv. Tarassovi strain Perepelitsin is currently used as a reference strain of serogroup Tarassovi. The first report of Tarassovi isolation was published by Tarasov, the scientist who isolated Tarassovi in 1938 (Varfolomeeva 1958) from a man in the Far East region of the USSR. In the period from 1938 to 1958, this serological type of *Leptospira* was named L. Perepelitsin (1938), T 384 (1940), PK-20 (1941), DVA (1941), L. Mitis (1942), L. Hyos (1944) and L. Tarassovi (1951).

In 1940, two years after the initial isolation of *Leptospira* Perepelitsin by Tarasov, the Australian researcher Johnson (Johnson 1942) independently reported the isolation of the fifth Australian strain, *Leptospira* Mitis, presently identified as *L. borgpetersenii* sv Tarassovi str Perepelitsin, from the blood of a timber worker in Innisfail, North Queensland. Following this report, serovar Mitis was named Mitis (Johnson), and both names Mitis and Perepelitsin were used for some time.

In 1952, Dr S. T. Cowan (Cowan 1952) submitted a request to the *Leptospira* Subcommittee of Bacteriological Nomenclature and Taxonomy to clarify the status of the species name *Leptospira* Mitis. After Johnson named Mitis, he discovered that the exact name of Mitis had already been claimed by Mino (Cowan 1952) for the strains isolated in Northern Italy, later identified as Bataviae. Therefore, Dr. S. T. Cowan proposed to rename the Mitis to Hyos (presently identified as L. *borgpetersenii* sv Tarassovi str Perepelitsin) as in 1944, Savino and Rennella (1945) isolated the *Leptospira* serologically identical to Mitis from pigs and men in Argentina and named it Hyos.

Following Dr S.T. Cowan's request, serovar Mitis Johnson, also known as Mitis, has been renamed Hyos; however, the name Mitis Johnson remained as strain name within Hyos serovar nomenclature. Additionally, the names Hyos and Perepelitsin co-existed for about ten years. Two strains were thought to be different until 1955 when the *Leptospira* Perepelitsin strain became available for comparison with those strains of Hyos (formerly L. Mitis) isolated respectively in Argentina, Australia, and Finland. The results of agglutination and cross-immunity tests concluded that the USSR strain is identical to Hyos and Mitis, thus confirming the work of Kmety et al. (1956). Once Hyos and Perepelitsin were recognised as serologically identical, Varfolomeeva (1958) proposed to rename it to Tarassovi, according to the rule of the oldest legitimate epithet for this strain. Finally, 20 years after the initial isolation, this serovar was classified under bacterial nomenclature as *L. interrogans* sv. Tarassovi, which is not correct under the present classification. Until the late 1980s, strains Mitis Johnson and Perepelitsin of *L. borgpetersenii* sv. Tarassovi were used as reference strains for serogroup Tarassovi until 1982 when strain Perepelitsin was recognised as the only reference for serovar Tarassovi (Amsterdam Medical Centre 2022). Presently, this strain is identified as *Leptospira borgpetersenii* serogroup Tarassovi serovar Tarassovi strain Perepelitsin.

2.4.3 Serological Classification of *Leptospira* spp. Serogroup Tarassovi

The first *Leptospira* belonging to the serogroup Tarassovi was discovered in 1938, and by 1967, the serogroup had grown considerably and consisted of 10 serovars. Serovars Chagres and Gatuni, included in the 1967 list of recognised serovars, were unpublished, and their reference strains were quoted as LT 924 and LT 839, respectively. These have now been changed to 1913 K and 1473 K according to the designations given in the original publication (Sulzer *et al.* 1975). In the approved list of serovars of 1988, the Tarassovi serogroup was extended by adding seven serovars: Tunis, Kaup, Vughia, Navet, Kanana, Darien and Mogdeni. Since then, four new serovars have been isolated in China, namely Banna, Gengma, Mengpeng, and Yunxian, and described as belonging to the Tarassovi serogroup. The *Leptospira* Research Centre in the Netherlands studied two further strains, M 39090, and LT 82, and found them to represent new serovars. The serovar names that had been

suggested were Langati and Sulzerae. Approximately 29 pathogenic antigenically related serovars are currently described within the Tarassovi serogroup (Figure 2.1). New serovars are periodically described and added to the list (Corney *et al.* 2008). As an alternative to serological identification, molecular typing methods have become increasingly popular for identifying serovars because molecular methods are less technically demanding than serological and could provide better discrimination of strains than serological typing methods (Hartskeerl *et al.* 2011).

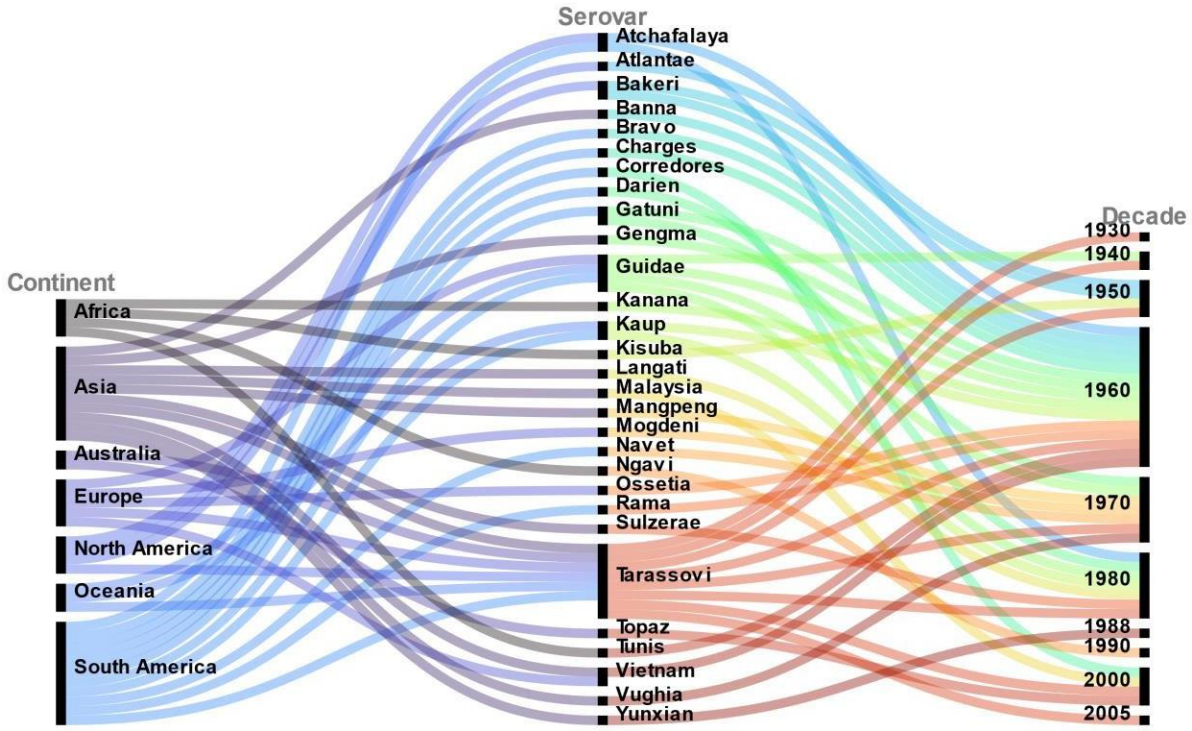

Figure 2.1. Breakdown of the serogroup Tarassovi classification by continent of discovery, serovar and decade of discovery, shown as a parallel sets plot. The thickness of the coloured lines in black boxes indicates the number of reports included in the set.

2.4.4 Genomic Classification of Serogroup Tarassovi

Over the years, isolates of serovars from serogroup Tarassovi were genetically identified, described, and attributed to five different genomospecies (Amsterdam

Medical Centre 2022). Detailed descriptions of serovars, their corresponding genomospecies, origins and referencing publications can be found in Table 2.2

Table 2.2 Descriptions of serovars of serogroup Tarassovi, their corresponding genomospecies, origins and referencing publications.

Serovar	**Reference Strain**	**Species**	**Isolated from**		**Country of Origin**	**Year of Publication**	**Date Recognised**	**Publication**
Atchafalaya	LSU 1013	*L. santarosai*	opossum	*Didelphis*	USA	1963	1967	(Roth *et al.* 1963a)
Atlantae	LT 81	*L. santarosai*	opossum	*Didelphis*	USA	1960	1957	(Galton *et al.* 1957)
Bakeri	LT 79	*L. santarosai*	opossum	*Didelphis*	USA	1957	1967	(WHO 1967)
Banna	A 31	*L. alexanderi*	human	*Homo Sapiens*	China	1985	1990	(Zhang *et al.* 1985)
Bravo	Bravo	*L. santarosai*	human	*Homo Sapiens*	Panama	1966	1967	(WHO 1967)
Chagres	1913 K	*L. santarosai*	spiny rat	*Rattus praetor*	Panama	1982	1967	(WHO 1967)
Corredores	JICH 05	*L. santarosai*	human		Costa Rica	2013		(Valverde *et al.* 2013)
Darien	637 K	*L. santarosai*	opossum	*Didelphis*	Panama	1982		(Sulzer *et al.* 1982)
Gatuni	1473 K	*L. santarosai*	opossum	*Didelphis*	Panama	1982	1967	(WHO 1967)
Gengma	M 48	*L. borgpetersenii*	pig	*Sus scrofa*	China	1985	1960	(Zhang *et al.* 1985)
Guidae	RP 29	*L. borgpetersenii*	pig	*Sus scrofa*	Brazil	1960	1965	(Wolff and Bohlander 1960)

Serovar	Reference Strain	Species	Isolated from		Country of Origin	Year of Publication	Date Recognised	Publication
Kanana	Kanana	*L. borgpetersenii*	gerbil	*M. unguiculatus*	Kenya	1981	1986	(Dikken *et al.* 1981)
Kaup	LT 64-68	*Unknown*	bandicoot	*Perameles gunni*	Papua New Guinea	1971	1986	(Morahan 1971)
Kisuba	Kisuba	*L. borgpetersenii*	human	*Homo Sapiens*	The Congo (DRC)	1956	1965	(Van Reil *et al.* 1956)
Langati	M 39090	*L. weilii*			China	1985		Unknown
Malaysia	Bejo-Iso9	*L. kmetyi*	soil		Malaysia	2009		(Slack *et al.* 2009)
Mengpeng[1]	A 82	*Unknown*			China	1985	1990	(Zhang *et al.* 1985)
Mogden	Compton 746	*L. weilii*	sewage		England	1987	1977	(Cinco *et al.* 1980)
Navet	TRVL 109873	*L. santarosai*	human	*Homo sapiens*	Trinidad	1978	1986	(Green *et al.* 1978)
Rama	316	*L. santarosai*	opossum		Nicaragua	1966	1986	(WHO 1967)
Sulzerae	LT 82	*L. santarosai*	human	*Homo sapiens*	China		1990	(Marshall 1992)

Serovar	Reference Strain	Species	Isolated from		Country of Origin	Year of Publication	Date Recognised	Publication
Tarassovi	Perepelitsin	*L. borgpetersenii*	human	*Homo sapiens*	U.S.S. R	1941	1966	(WHO 1967)
Topaz	94-79970/3	*L. weilii*	bovines	*Bos Taurus*	Australia	2008		(Corney *et al.* 2008)
Tunis	P 2/65	*L. borgpetersenii*	pig	*Sus scrofa*	Tunis	1969	1986	(Bakoss 1969)
Vughia	LT 89-68	*L. weilii*	human	*Homo Sapiens*	Vietnam	1971	1986	(Tsai and Sulzer 1971)
Yunxian	L 100	*L. borgpetersenii*	pig	*Sus scrofa*	China	1988	1988	(Li *et al.* 1988)

[1]Serovar Menpeng is tentatively included in the serogroup Tarassovi. Based on the information provided on the Leptospirosis Reference Centre website https://leptospira.amsterdamumc.org

2.5 Seroprevalence, Isolation, and Manifestations of Serogroup Tarassovi in Animals

This section covers Tarassovi seroprevalence and reports of isolation by culture from various mammalian and non-mammalian species. The manifestations related to each species are listed in chronological order by year of publication where appropriate. The last sections of the review cover Tarassovi isolation and seropositivity in humans, as well as occupational risks and manifestations.

Reports of isolation by a culture of serovars of serogroup Tarassovi include a wide range of hosts living on all human-inhabited continents. Table 2.2 details isolation years, hosts, and their geographical origins. Most commonly, serogroup Tarassovi has been cultured from mammalian species, such as humans (Galton *et al.* 1957; Van Riel 1964; Tsai and Sulzer 1971b; Cinco *et al.* 1996; Valverde *et al.* 2013b), pigs (Nicolescu *et al.* 1970; Ryan and Marshall 1976; Tercilla and García 2005; Zhang *et al.* 2012) as well as from some free-living mammals such as opossums (Galton *et al.* 1957; Roth *et al.* 1963b; Sulzer *et al.* 1975) spiny rat (Sulzer *et al.* 1975), bandicoot (Morahan 1968) and gerbil (Dikken *et al.* 1981b). Tarassovi has been isolated less commonly from cattle; there are three publications describing these, specifically by (Feresu *et al.* 1998; Corney *et al.* 2008, Hamond *et al.* 2022), one publication by Carlos *et al.* (1970) discussing Tarassovi isolation from water buffalos. In addition, there have been reports of isolation of *Leptospira* serogroup Tarassovi from non-mammalian species, such as the giant marine toad in Grenada (Schüffner 1934) and slider turtle in the USA (Glosser *et al.* 1974). Interestingly, the isolation of sg. Tarassovi from sewage (Coghlan and Kmety 1987) and soil (Slack *et al.* 2009) have also been reported.

2.5.1 Cattle (*Bos Taurus*)

After the almost simultaneous isolations of Tarassovi by researchers in Argentina, Australia, and the USSR, epidemiological surveys and attempts to culture Tarassovi were conducted to establish levels of seroprevalence and shedding of Tarassovi in cattle. Savino and Rennella (1945) tested the sera from 300 cattle in Argentina and found that 55 animals reacted positively with Hyos, and eight reacted positively to Hyos

and Suis. The same authors also collected the kidneys of 142 cattle and attempted to culture *Leptospira* from them but were unsuccessful.

From 1942 to 1962, several Australian serosurveys of apparently healthy animals estimated Tarassovi prevalence in cattle but attempts to culture it were unsuccessful. For example, Johnson from Australia in 1942 reported that agglutinins against both *L.* Pomona and *L. Miti*s were found in 30 cattle sera (Johnson 1942). However, the author noted that although cattle have shown serological evidence of Hyos infection, no exact MAT titre was mentioned. Another Australian study by Peterson (1951) reported possible positive serological identification of Hyos from approximately 80 cattle from six herds with a MAT titre of ≥ 300 considered positive, but this strain was lost before definitive culture results could be obtained. Then, in 1963, Australian author Winks (1962) published the results of a serosurvey on cattle, where 1,766 serum samples were collected from beef and dairy cattle at the North Queensland abattoir. Of the total number of sera tested, 877, or 47.8% were positive for Hyos, 502 samples or 28.4% were positive for Pomona, and 259, or 14.6% of animals were seropositive to more than one serovar.

A "red water" leptospirosis outbreak in calves was reported by Wellington, Ferris, and Stevenson (1953) in Australia, and this particular case has mainly been attributed to Pomona; however, some titres to Hyos were also detected. As part of the epidemiological investigation, authors noted that pigs on the property were kept in the same pens as calves for some years. No clinical leptospirosis in pigs was observed before this outbreak. Thus, pigs as a source of infection were ruled out, as reported by the authors to the best of their knowledge at the time. However, a new bull from a farm with a history of leptospirosis was introduced to the herd before the outbreak, and the authors concluded that introducing a new bull was the cause of the Hyos leptospirosis outbreak on the farm (Wellington *et al.* 1953). Please note that none of the studies above reported which MAT cut point was used for discrimination between positive and negative results.

More recently, New Zealand, South American, African, and Mexican researchers (2011-2020) reported Tarassovi seroprevalence in apparently healthy cattle ranging from 0.3% to 53.6%. A 2016 survey of 200 dairy herds reported that 17% of New Zealand's adult dairy cows had exposure to *Leptospira* Tarassovi (Yupiana *et al.* 2019). In this 2016 survey, Tarassovi (results were considered positive if MAT was ≥48) was the only serovar significantly associated with PCR-positive urine, and 68% (54/80) of PCR-amplicon sequences were genotypically attributed to str. Pacifica (Yupiana *et al.* 2019, Wilkinson *et al*. 2021). Moreover, a study within the State of Piauí, north-eastern Brazil, reported a 0.3% (95% CI: 0% - 0.9%) seroprevalence to Tarassovi positive at (MAT≥100) in 329 on-farm tested cattle from 33 farms (Campos *et al.* 2017) whilst a cross-sectional study of 500 slaughtered cattle in Uganda reported 11.6% of cattle reacting to serovar Tarassovi serogroup Tarassovi at MAT ≥100 cut-off point (Alinaitwe et al. 2020). Finally, the highest reported Tarassovi prevalence in cattle was 53.6% (MAT ≥ 100), as per Cardenas-Marrufo *et al.* (2011) study evaluating 476 cattle from 34 herds in Yucatan, Mexico.

Three studies reported Tarassovi isolation from cattle. The first was the isolation of a new *L. borgpetersenii* serovar Ngavi (Feresu *et al.* 1998) from oxen in Zimbabwe; however, no further details were reported. The second was a report of the successful isolation of serovar Topaz, belonging to the serogroup Tarassovi. It was isolated from cattle in Australian North Queensland and classified into *L. welii* genomospecies (Corney *et al.* 2008). The third was reported by Hamond *et al.* (2022) reported the isolation of *L*. *borgpetersenii* serovar Tarassovi strain Perepelitsin from the urine of dairy cows in Iowa, USA. This was the only reported isolation of *Leptospira* Tarassovi from bovine species in the USA. In this case, the herds` poor reproductive history, specifically failure to breed and maintain the pregnancy, prompted a veterinary investigation and led to the successful isolation of *L*. *borgpetersenii* serovar Tarassovi strain Perepelitsin.

The five studies discussed below did not observe any clinical symptoms in the shedding and seropositive animals for Tarassovi. In 1962, (Winks, 1962) reported that cattle infected with Hyos showed no clinical signs. However, they expressed concern about

the potential for human infection, as cattle were already known to be a source of *Leptospira* infection in humans. Similarly, in 1976, (Ryan and Marshall 1976) cited several authors who reported cases of Tarassovi infection in cattle across Europe, but there was no indication that the infection caused severe clinical disease. Feresu *et al.* (1998) reported isolating Tarassovi from the kidneys of two cattle, but it is unclear whether any clinical signs of leptospirosis were observed. When *Leptospira* Weilii, serovar Topaz, was isolated from a heifer in Australia in 2008 (Corney *et al.* 2008), no clinical signs of leptospirosis were reported in the animal. Similarly, a dairy cow from which a Tarassovi isolate was obtained in Iowa in 2022 showed no clinical signs of leptospirosis during a two-week quarantine. However, the herd from which this cow came was reported to have unspecified reproductive problems (Hamond *et al.* 2022).

Nevertheless, several studies reported underlying reproductive problems in animals seropositive to Tarassovi. For example, a study investigating a laboratory diagnosis by a combination of serological (cut-off for MAT was ≥ 10), post-mortem, and molecular diagnostic methods of bovine abortions (n=116) caused by *Leptospira* in non-maintenance hosts in Belgium identified Tarassovi strain Perepelitsin as a causative agent by matching dam serology with aborted fetuses PCR results in one of 65 cases of bovine abortions with an identifiable *Leptospira* serogroup (Grégoire *et al.* 2020). Additionally, a study from India investigating seropositivity to *Leptospira* in cattle with known reproductive problems found that 11.3% (42/373) of animals were seropositive to Tarassovi (MAT titre ≥ 100, or four-fold or greater rise in MAT titre between acute and convalescent sera was considered positive). Of these, 38 animals had reproductive problems, and the remaining four were healthy. This study enrolled a total of 45 dairy farms in different regions of India, with animals that had a history of reproductive disorders, such as abortions, anoestrous, and endometritis; none of these animals were vaccinated against leptospirosis during the study period of 2015-2016 (Balamurugan *et al.* 2018). However, the seroprevalence reported from the studies above may not be comparable, as MAT cut points ranging from MAT ≥ 10 to MAT ≥ 100 were used to discriminate positive and negative results.

2.5.2 Buffaloes (*Bubalus bubalis*) and Zebu (*Bos Indicus*)

Studies have investigated the prevalence of Tarassovi in various bovine species, including water buffaloes, Zebu, and African buffaloes, reporting prevalence rates ranging from 36% to 95%. Two studies conducted in the Philippines, four decades apart, have reported estimated prevalence rates of Tarassovi in water buffaloes. The first study, conducted by Carlos *et al.* (1970), reported a prevalence rate of 65% (N=27) followed by subsequent isolation (N=3). The second study, conducted by Villanueva *et al.* (2016), reported a prevalence rate of 36% (N=746) in water buffaloes (MAT ≥ 40). Meanwhile, in Thailand, a study investigating *Leptospira* serovar distribution in humans, domestic species, and livestock indicated a seroprevalence of Tarassovi at MAT ≥ 100 cut-off point in water buffaloes. The study found that 38.2% (N=1,937) of water buffaloes were seropositive in the north-eastern region, while 33.3% (N=878) were seropositive in the eastern region. According to the authors, water buffaloes may be maintenance hosts of serovar Tarassovi (Chadsuthi *et al.* 2017). A seroprevalence study conducted on African buffaloes (*Syncerus caffer*) free-ranging across Hluhluwe-iMfolozi in KwaZulu-Natal parks in South Africa found that the overall seroprevalence to *Leptospira* was 21% (21/98, 95% CI: 14–31%). The study also revealed that 95% (20/21) of positive results reacted to Tarassovi at MAT ≥ 100 as a cut-point (Goosen *et al.* 2021).

In 2019, two studies reported high seroprevalence of Tarassovi in Zebu. The first study (Yatbantoong and Chaiyarat 2019) tested 512 serum samples from cattle living in Salakphra Wildlife Sanctuary, Thailand. The overall seroprevalence was 92.2%, and from those, 92.9% of animals were seropositive to Tarassovi, and the individual sera were considered positive if agglutination was present at dilutions of 1/50 or more. The second study reported that overall seroprevalence to *Leptospira* in Madagascar's Malagasy cattle (Zebu) was 59.3% (95% CI; 52.0-66.2%) by MAT, where samples with a titre of 100 were considered seropositive. At the same time, seropositivity specifically to Tarassovi was 40.2% (33.3-47.5%) (Schafbauer *et al.* 2019).

2.5.3 Sheep (*Ovis aries*) and Goats (*Capra aegagrus hircus*)

Sera and renal tissues were collected from 110 small ruminants (sheep and goats) at a southern Greece slaughterhouse and tested by MAT, where samples were considered positive at the titre ≥ 100, PCR, and culture. Overall seropositivity by MAT was 25.5% (28/110). Of these, 36% (10/28) of animals were seropositive to Tarassovi, and a further 39.5% (15/38) (of individual kidney samples were PCR positive, but no positive cultures were reported (Bisias *et al.* 2015). The seroprevalence study from the State of Piauí, north-eastern Brazil, evaluated the seroprevalence of Tarassovi (at MAT cut-off titre of ≥ 100) in sheep (N=114) and goats (N=91) and found no Tarassovi reactions in these animals (Campos *et al.* 2017). Wilson *et al.* (2021) investigated the seroprevalence of *Leptospira* spp. in small ruminants (sheep) in New Zealand and reported that 14.0% (95% CI=12.4–15.8) were seropositive to Tarassovi by MAT (≥ 48).

Clinical signs of *Leptospira* spp. infection in sheep and goats is rarely reported and investigated because it is generally not expected to cause clinical disease in small ruminants compared to large ruminants, pigs, and dogs (Ellis *et al.* 1994). Small ruminants, goats in particular, are considered low-value species in some countries, however, this varies from country to country. A study of *Leptospira* seroprevalence among small ruminants in Greece did not report any clinical signs but showed that 27.3% (30/110) of sheep and goats had various degrees of kidney microscopic lesions, as identified by histopathology. In this study, kidneys were collected from 25.5% (28/110) of animals seropositive to *Leptospira* spp., from which 35.7% (10/28) were seropositive to Tarassovi (Bisias *et al.* 2015).

2.5.4 Deer (*Cervus elaphus*)

In New Zealand's red and fallow deer, a low prevalence of Tarassovi has been observed, with 26% (92/360) of animals seropositive at the titre ≥ 10 to *Leptospira borgpetersenii* serovar Tarassovi (Flint *et al.* 1988). Additionally, another New Zealand-based seroprevalence study reported a 3.6% (95% CI =2.7–4.8) prevalence of serovar Tarassovi by MAT (≥ 48) in farmed deer (Wilson *et al.* 2021). However, no successful isolation of Tarassovi from deer has ever been reported in New Zealand (Ayanegui-

Alcerreca *et al.* 2007). The most recent retrospective study of samples collected during 2009-2010 utilised molecular typing methods using *glmU* and *gyrB* PCR and identified 2% (2/97) of New Zealand-farmed deer positive for Pacifica (Wilkinson *et al.* 2023).

Clinical cases of leptospirosis in deer attributed to serovar Tarassovi are not reported in the available literature. In support of this, Ayanegui-Alcerreca *et al.* (2007), in the review of leptospirosis in farmed deer, noted that most clinical cases in deer were confirmed as leptospirosis have been associated with serovar Pomona; however, it is unknown if all reviewed studies included Tarassovi antigens in MAT panel.

In conclusion, although studies frequently reported geographically diverse seroprevalence of Tarassovi in cattle, disease manifestation reports are uncommon, and only three studies described Tarassovi isolation directly from cattle urine. Similarly, there are some reports of Tarassovi seroprevalence in small ruminants and deer, but disease manifestation reports are rarely reported as in sheep and goats or not observed or published for deer.

2.5.5 Swine (*Sus scrofa*)

Initial reports of Weil's-like disease observed in swine were reported as early as 1937 (Keast *et al.* 1956), and the first culture of *Leptospira* spp. obtained from the sick piglets was classified serologically as Icterohaemorrhagie. Already in 1945 and 1946, two strains of Hyos were isolated from pigs slaughtered in Brisbane (Ryley 1956). The author also noted that Hyos infections in men were reoccurring primarily due to contact with pigs and cattle. In Argentina, Savino and Rennella (1950) carried out an extensive investigation of Tarassovi in pigs, and the authors isolated what they reported as two species (that under the present nomenclature would probably be classified as serovars) of *Leptospira* from pigs that were recently slaughtered. The authors cultured strains of *Leptospira* from the kidneys of 178 animals. Of these, 38 were found to be Hyos, and four were Pomona (L. suis). Of 201 swine sera collected from the same group of animals, 13% (N=26) contained antibodies for Hyos (Savino and Rennella 1950). On the other side of the globe, in Vietnam, a seroprevalence study of *Leptospira* in 1,959 fattening pigs identified seroprevalence to serovar Tarassovi Mitis at 2.20% (1.55–2.84)

as the most prevalent serovar in seropositive animals at MAT ≥100 (Lee *et al.* 2017). In New Zealand, a survey of pig sera was carried out in 1954-1955 (Russell and Hansen 1958), and from 1125 sera samples, 4% had Hyos titres of 200 or higher. The authors concluded that Hyos was widespread among the New Zealand pig population. Two decades later (Ryan and Marshall 1976) reported the isolation of Tarassovi from slaughtered pigs in New Zealand, where eighty animals were sampled during a serological survey. Tarassovi was isolated from one animal, however, it is not clear whether it was isolated from the urine of a live animal or the kidney of a culled pig. The authors did not report specific MAT titres but noted that these were "high", and the positive MAT cut-off was ≥ 50 (Ryan and Marshall 1976). At the same time, in 1976, Davos reported the first isolation of Tarassovi in Australia from aborted sows (N=12); titres to L. Tarassovi ranged from MAT≥ 400 to MAT≥6400. Tarassovi culture from one urine showed growth after five and a half weeks of incubation (Davos 1977).

Three decades later, *L. borgpetersenii* serogroup Tarassovi serovar Tarassovi was isolated for the first time in Cuba from aborted swine fetuses in 2005, and two independent isolates were obtained from fetal liver tissues (Tercilla and García 2005). The same study reported overall seropositivity in swine to *Leptospira* spp. at 70%, and Tarassovi had the highest prevalence (exact figures were not reported). A survey conducted in Italian Tuscany to evaluate the possible genital *Leptospira* infection of wild boars also reported the successful isolation of *L. borgpetersenii* serovar Tarassovi from the kidneys of these animals. Interestingly, the authors of this study utilised both culture and molecular methods but did not use serology (Cilia *et al.* 2020).

Penso and Rosa, in 1938, as reported by Bohl (1952), were the first researchers to document clinical signs of leptospirosis in swine: "In swine, Tarassovi leptospirosis begins with a mild digestive disturbance, fetid diarrhoea, moderate fever and a state of excitation, in which true epileptic convulsions occasionally appeared. Sometimes, the sick animals would run aimlessly, showing excessive salivation, now and then bleeding from the nose, and generally also showing pronounced neck stiffness. In a few cases, the meningeal symptoms predominated (Bohl 1952)". In addition to these symptoms, abortion, and abortion storms were also widely reported; for example, (Kemenes 1967)

reported an abortion storm in sows in Hungary, where 18% (62/350) of pregnant sows were aborted for three months. Still, the paired sera tests did not show the titre rise in animals seropositive to Tarassovi. An Australian veterinarian attributed an abortion in sows to Tarassovi, as all the sows aborted had high MAT titres to Tarassovi, ranging from 400 to 1600 (Davos 1977). Tercilla and García (2005) observed a 33% (40/120) abortion rate in asymptomatic sows. In this study, Tarassovi culture was obtained from two aborted fetuses, and live bacteria were observed by Dark Field Microscopy (DFM) in one of the aborted fetuses' liver, kidney, and aqueous humour. MAT titres to Tarassovi in sows ranged from 100 to 102,400; the sample was considered positive if MAT was ≥ 100.

To summarise, Tarassovi in swine is recognised as a disease of clinical importance, causing mild disease, but sometimes identified as a cause of abortion and abortion storms. Historically, seroprevalence to Tarassovi was reported in both domestic and wild pigs, and New Zealand, Australian, and Cuban researchers reported successful Tarassovi isolation by culture.

2.5.6 Rats and Other Small Mammals

An Australian study by Emanuel *et al.* (1964) investigated the epidemiology of *Leptospira* in a range of small free-living as well as in 253 domestic mammal hosts on the Atherton Tableland and adjoining areas of North Queensland. For this study, 1,498 samples were collected from mice (*Mus musculus*), rats (*Rattus rattus*), and marsupials (*Marsupialia*). *Leptospira* spp. from serogroup Hyos was isolated from 4 animals: a giant white-tailed rat (*Uromys caudimaculatus*) (n=1) and an Australian allied rat (*Rattus fuscipes*)(n=3). Of three bandicoots (*Peramelemorphia*) and sixteen rodents seropositive to Hyos, five were shedding Hyos, and four had antibody titres less than 100; in the whole series, nine had titres of 100, three of 300, and four of 1,000 or more. In addition, twenty-nine dairy cattle had titres to Hyos of 100 or greater. MAT titres of 100 or greater were considered positive. In summary, the authors noted that wild species are most likely to be the accidental hosts of Hyos, while cattle could be the maintenance hosts of this serovar. Additionally, the authors stated that antibody levels

in wild animals shedding and seropositive to Hyos appeared lower than other serotypes in seropositive and shedding wildlife, but the differences were not statistically significant. Hathaway *et al.* (1981) reported two brown rats, one black rat, two possums, and one hedgehog seropositive to Tarassovi at MAT >24. These animals were sampled from forest, farmland, and urban New Zealand locations, and *Leptospira* isolates obtained by authors did not include Tarassovi.

A study of *Leptospira* infection in rat species in Colombia reported that 7/254 samples collected from *Rattus norvegicus* captured in a farmers' market in Medellin were seropositive to Tarassovi (MAT titre >50). In this study, authors obtained 12 isolates not characterised to the serovar level (Agudelo-Flórez *et al.* 2009). In a *Leptospira* seroprevalence study of 241 rats from Mekong Delta, Vietnam, two were seropositive to *L. borgpetersenii* sv. Tarassovi and rat serum were considered positive when a titre of ≥ 20 or higher was obtained with at least one strain (Loan *et al.* 2015). A study evaluating seroprevalence in rats trapped from selected areas of metropolitan Manila, Philippines, reported that 2.8% (3/106) of rats were positive (MAT ≥ 20) for Tarassovi at titres ≥ 160, 320, and 560 (Villanueva *et al.* 2010). Although authors reported the isolation of *L. interrogans serovars* Manilae and Losbanos and *L. interrogans serogroups* Grippotyphosa and Javanica, no Tarassovi isolates were reported. In a Brazilian study (Paixão *et al.* 2016) of *Leptospira* in rats captured in a wild animal rehabilitation centre in Sao Paulo, 14% (2/14) of rats were seropositive to Tarassovi (MAT ≥100). The authors also reported the successful isolation of *Leptospira* spp. from seven renal and hepatic tissue samples. However, the authors did not identify isolates at a serogroup level. A more recent New Zealand *Leptospira* seroprevalence study of invasive mammals and farm environments reported one of eight hedgehogs (*Erinaceus europaeus*) and one of the eighteen common brushtail possums (*Trichosurus vulpecula*) Tarassovi positive (MAT ≥48) (Moinet 2020).

There were no reports in the reviewed literature of clinical disease manifestations associated with serovar Tarassovi in rats and mice. However, some studies used golden hamsters (*Mesocricetus auratus*) and grivet monkeys (*Chlorocebus aethiops*) as animal models for Tarassovi infections. For example, golden hamsters (n=2) were

inoculated intraperitoneally with 0.5 ml of Tarassovi culture isolated from an aborted fetus at concentrations of $8x10^7$ cells/ml (Tercilla and García 2005). On day 5, the death of hamsters was recorded, so researchers confirmed the virulence of the isolated strain. However, the author does not leave a detailed report of clinical manifestations of Tarassovi infection in these hamsters.

Similarly, grivet monkeys were experimentally infected with Tarassovi, as Marshall *et al.* (1980) reported. These animals showed no clinical signs of disease. However, severe meningoencephalitis was demonstrated histologically in animals euthanased 26 and 33 days after infection. The meningeal and perivascular reactions were exclusively lymphocytic. Mild focal degeneration and cellular infiltration lesions were also present in the kidneys and femoral musculature.

In conclusion, although Tarassovi seroprevalence and isolation by culture are commonly reported in free-living small mammals, no clinical signs are reported in rats; however, experimental Tarassovi infections in golden hamsters were lethal, and in monkeys, Tarassovi infection resulted in meningoencephalitis Thus, small mammal species susceptibility plays a major role in Tarassovi manifestation.

2.5.7 Horses (*Equus ferus caballus*)

Savino and Rennella (1945) examined sera from 146 horses and found eight positive reactions with Hyos in Argentina. Attempts to culture *Leptospira* from the urine of 115 horses were negative. Wellington et al. (1953) reported that a mare aborted and showed a rising titre for Mitis. A short paper reported the results of a seroprevalence study in 12 horses in the Philippines, noted that one animal had a Tarassovi MAT titre ≥ 100, and the other had a MAT titre ≥ 100 to two serovars, Tarassovi and Batavia. However, no Tarassovi isolates were obtained (Carlos *et al.* 1971). A seroprevalence study of leptospirosis in South African horses reported that seropositivity to Tarassovi (MAT ≥ 100) was 7.7% (95% CI: 5–10%). Another cross-sectional study investigated the prevalence of *Leptospira* in Bardigiano horses in the province of Parma, Italy. Of 137 horses, 90 (67.2%; 95% CI: 63.2–71.1) were seropositive to *Leptospira.* Of those, 28.4% of horses were seropositive to Tarassovi, where a MAT titre of ≥ 100 was

deemed positive (Vera et al. 2019). In New Zealand, a cross-sectional survey of horses identified that seroprevalence to Tarassovi in racing and thoroughbred horses (n=499) was 5% (95% CI 3–7%) at MAT cut-off ≥50 (Bolwell *et al.* 2020).

Leptospira infection does not always result in systemic disease in horses, and it is believed that horses play a minor role in spreading *Leptospira* compared to other livestock and wild animals. One of the first reports of Mitis infection in horses was published by Wellington *et al.* (1953). Abortion in the broodmare was thought to be caused by the Mitis; authors reported that a mare aborted and showed rising MAT titres at 320 for Mitis. Then, serum samples taken 169 days later showed titres to Mitis at 80 and Pomona at 1280; therefore, authors could not conclude whether abortion was due to Mitis or Pomona infection. One of the more recent studies of *Leptospira* in horses highlights that the Tarassovi manifestation of leptospirosis in horses is recurrent uveitis, usually associated with serovar Pomona but not Tarassovi (Frellstedt and Slovis 2009). This finding is supported by Lucchesi *et al.* (2002) molecular work, where authors reported that a DNA sequence of serovar Pomona related to the antigen mimicry with equine cornea showed that although this particular sequence was present on other strains, it was not detected in *L. biflexa* serovar Patoc strain Patoc and *L. borgpetersenii* serovar Tarassovi strain Perepelitsin. Thus, the authors concluded that Tarassovi might lack the DNA sequence regions responsible for antigen mimicry and may not be able to cause recurrent uveitis, which confirms observations made by (Frellstedt and Slovis 2009).

A study by Vera *et al.* (2019) of 134 small breed Baragiano horses noted that although 34% (31/90) of animals were found seropositive to Tarassovi (MAT ≥ 100), authors concluded that the anamnestic data suggested that the infection in Bardigiano horses was subclinical in most of the cases.

To summarise, according to this literature review, Tarassovi seropositivity is uncommon in horses, and it appears that no clinical Tarassovi leptospirosis was reported in horses.

2.5.8 Dogs (*Canis lupus familiaris*)

Over the years, studies from different countries reported seroprevalence to Tarassovi in dogs ranging from 4% to 23.6%. One of the reported isolations of Hyos from dogs was published by Herrer et al. (1958). However, this report contains no additional details, such as a MAT cut-off titre or the total number of dogs. In New Zealand, Hyos was isolated from a dog for the first time in 1958, as Mackintosh et al. (1980) and Robinson et al. (1982) reported. In the same paper, the authors reported the seroprevalence of different strains of *Leptospira* in farm dogs. In this study, 74% (26/35) of animals were found seropositive to *Leptospira* spp., but no dogs were seropositive (MAT ≥24) to Tarassovi. The seroprevalence study of dogs in Brazil (de Castro *et al.* 1966) showed that 23.6% of tested dogs (n=268) had a titre against Tarassovi; however, this study did not investigate the shedding of bacteria and did not attempt to isolate it. Barmettler et al. (2011) reported seroprevalence of 4% (2/52) to Tarassovi (MAT ≥ 10) in dogs admitted to the University of Bern Vetsuisse Faculty Small Animal Clinic in 2007 and 2008. A smaller proportion of dogs, seropositive to Tarassovi was reported in the seroprevalence study in rural dogs in national parks in Uganda, where 10.7% of dogs (N=105) were seropositive to Tarassovi at MAT titre of 200 or greater (Millán *et al.* 2013).

Mackintosh et al. (1980) reported that a pack of hounds in New Zealand showed no clinical signs while seropositive to Tarassovi. The seroprevalence of Tarassovi was confirmed by MAT, followed by isolation of Tarassovi, which was typed as serovar Tarassovi (strain Precious) by the WHO Reference Laboratory, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA. Later, this Tarassovi isolate was inoculated into five seronegative dogs. They became infected and developed leptospiruria, which persisted for over seven months in all five animals, but none showed any clinical signs. After two months, titres ranged from 96 to 192, and after five months, titres ranged from less than 24 to 96. A seroprevalence study in dogs in Brazil (de Castro *et al.* 1966) did not report the clinical signs in sampled dogs. A more recent study (Kurilung *et al.* 2017) reported the isolation of *L. welli*, serovar Topaz, of

serogroup Tarassovi from four healthy dogs with no apparent clinical manifestations of disease in Thailand.

More recently, in New Zealand, a study reporting routine test results for ten rural dogs with azotaemia and/or abnormal hepatic enzymes showed that 70% (7/10) of tested dogs had Tarassovi ranging from MAT ≥ 50 to ≥ 1600, and MAT titres ≥ 50 were considered positive (Thompson 2018). The authors suggested that in addition to three serovars (Pomona, Hardjo, and Copenhageni), the Tarassovi antigen should be considered for addition to the laboratory test panel.

In conclusion, there were cases of reported seroprevalence to Tarassovi in dogs, and Tarassovi isolation by culture was reported by two authors, according to published reports. These publications highlight the dogs' susceptibility to Tarassovi leptospirosis.

2.5.9 Non-mammals

Over the past six decades, reported seroprevalence to Tarassovi in non-mammal species ranged from 6.12 to 91%. A USA researcher investigated *Leptospira* prevalence in turtles (*Testudines*), lizards (*Lacertilia*), and snakes (*Serpentes*) (Andrews *et al.* 1965) and reported that while 26% (18/69) seropositive turtles and 11% (1/9) snakes were positive (MAT ≥ 100) to Hyos, no positive cultures were obtained. Another report from the USA, Georgia, showed that 91% (41/46) of sera collected from turtles were positive to serogroup Tarassovi at MAT ≥ 50. Researchers obtained 26 isolates from kidneys and cloacal suspensions of turtles and recovered *Leptospira* isolates from hamsters inoculated with pond water where turtles lived. Six isolates were serologically identical to serovar Tarassovi (Glosser *et al.* 1974).

More recently, a study (Dezzutto *et al.* 2017) investigating *Leptospira* epidemiology in Italian turtles (2010-2011) reported a high prevalence of 63% (10/16) to Tarassovi, a MAT cut-off of ≥ 50 was used to identify the serological positives. Similar seroprevalence to Tarassovi was reported in the 31 spot-legged turtles (*Rhinoclemmys punctularia*) living in Rodrigues Alves Botanical Garden-Amazon Zoobotanical Park, located in Belem, Para State, Brazil, 35% (11/31) of turtles were seropositive to

Leptospira spp., and eight out of eleven were seropositive to Tarassovi. A much lower prevalence was reported by an Italian study (Bonacina *et al.* 2021) where 6.1% (95% CI: 0.01–12.7) of red-eared terrapins (*Trachemys scripta elegans) (*N= 49) were seropositive to serovar Tarassovi (strain Johnson). The authors conclude that turtles were the likely carriers of the Tarassovi strain. Despite not showing signs of illness due to its low pathogenicity, the presence of antibodies in turtles may indicate the prevalence of this strain in the surrounding animal environment. Higher Tarassovi prevalence but in a small sample size was reported by (Lindtner-Knific *et al.* 2013), where authors investigated the seroprevalence of *Leptospira* spp snakes, lizards, including Gekko (*Gekkonidae*) and turtles imported to Slovenia. In short, 50% (3/6) of snakes, 26% (8/31) of lizards, and 44% (4/9) of turtles were seropositive to serovar Tarassovi. However, in this study, several animals were seropositive to multiple serovars. Additionally, authors reported using strain Mitis Johnson as a reference antigen to serovar Tarassovi. Still, according to an Amsterdam reference laboratory, the only Tarassovi reference strain recommended for seroidentification is strain Perepelitsin (Amsterdam Medical Centre 2022). This study also did not report any culturing attempts. Therefore, reported seroprevalence to Tarassovi in non-mammals varies significantly and may be influenced by location, infected serovar species, animal species, MAT cut-off titre, and even reference strain used in the MAT. None of the authors of non-mammal seroprevalence studies reported observation of clinical symptoms in tested animals.

In conclusion, while seroprevalence was reported in various non-mammal species, only one study reported the successful isolation of Tarassovi from turtles. However, most researchers hypothesised that non-mammal species seroreactivity was due to their reservoir status and may be a good indicator of circulating strains in particular ecosystems.

2.6 Isolation and Manifestations of Serogroup Tarassovi in Humans

The first isolation of Tarassovi in 1938 from a person whose surname was Perepelitsin was reported in 1940 by Tarasov (Kiktenko *et al.* 1969), but no other details of the

patient or researcher are available. Australian researcher (Johnson 1942) reported the isolation of Mitis from the blood of a timber worker in Innisfail, North Queensland. After these first independently reported cases, Tarassovi isolations were commonly reported worldwide by independent researchers and then by passive surveillance reporting systems. Table 2.2 details some cases of Tarassovi isolation from humans.

However, the isolation by a culture of serovar Tarassovi from humans is no longer a common practice in the clinical diagnosis of leptospirosis. Since then, the focus in public health has shifted towards disease prevention through various transmission reduction practices. This shift has been influenced by the implementation of improved diagnostic methods, the introduction of antibiotic treatments, mass vaccinations of animals worldwide, and the identification and mitigation of risk factors. The commonly used diagnostic methods now are MAT and PCR tests, and their results are recorded in public health surveillance systems. These systems communicate data outputs as reports of de-identified disease notifications, binary results (PCR test), or infecting serovars (MAT). Thus, the isolation of known serovars from individuals is only reported in unique circumstances in the literature. In contrast, most scientific reports focus on the prevalence, identifications by molecular methods, and risk factors of known serovars.

Many early researchers (Johnson 1950; Kirschner 1954; Mackintosh *et al.* 1980) observed that Tarassovi causes a mild disease in infected individuals. In New Zealand, only three antigens, Pomona, Canicola, and Icterohaemorrhagiae, were used for leptospirosis diagnoses in humans until 1954. However, when Kirschner (1954) suspected leptospirosis in patients displaying clinical symptoms, whose sera did not react with three antigens used at the time, he added Mitis into a test panel. Following this addition, Kirschner reported that three patients were seropositive to Mitis with titres up to 2,000 while seronegative to the other three serovars. A centralised nationwide *Leptospira* diagnostic service was started in September 1954 by the National Health Institute, Wellington, and from 774 human sera samples tested over the next two years, six were positive for Tarassovi at titre >300. Johnson et al. (1950), in a study of 72 patients infected with Pomona or Mitis, stated that 32 patients complained of conjunctivitis or photophobia and that four developed iritides or iridocyclitis. Another

case report presented two clinical cases of symptomatic aseptic meningitis due to *L. borgpetersenii* serovar Tarassovi strain bakeri in a Sri Lankan hospital (Bandara *et al.* 2021). This paper described neuroleptospirosis with significant neurological involvement at presentation in the absence of the typical clinical feature of leptospirosis in both cases. Neuroleptospirosis has varied manifestations, including encephalitis, intracranial haemorrhage, movement disorders, and other symptoms. However, the most common presentation is aseptic meningitis (Panicker *et al.* 2001). Thus, Tarassovi infections are still common in humans, and some lead to clinical disease and could be manifested by neurological symptoms.

2.6.1 Occupational Risks Associated with Serogroup Tarassovi and Seroprevalence Studies

One of the widely acknowledged risk factors associated with leptospirosis is occupation. One of the first documented reports of occupational leptospirosis caused by Hyos was provided by a study in Switzerland's St. Gallen area in 1946, 1947, and 1948 (Wiesmann 1949). The author showed the importance of occupation regarding the type and incidence of human leptospirosis. This paper summarised the occupations of the patients diagnosed with leptospirosis and seropositive to Hyos: a total of 16 people whose occupations were recorded as cheesemakers (N=7), swine herdsmen (N=4), farmers (N=3) and butchers (N=2). Most of those reported infections were due to *Leptospira* Mitis occurring in individuals with swine contact.

An Australian researcher reported that there appears to be a definite correlation between the disease caused by Mitis in man and its association with cattle and pigs (Johnson 1942). In 1968, a case of swineherd's disease associated with Hyos was reported by Janbon and Bentran in France (Gsell 1990). A report from the Netherlands of the first case of leptospirosis caused by Hyos in 1968 was of a pig slaughterman from Amsterdam reported to have severe leptospirosis, and the probable cause of the disease was contact with hog's urine during animal slaughter (Minkenhof *et al.* 1968). In 1961, a serosurvey of 307 people working in piggeries and 32 slaughterers in Italy showed that 30% were seropositive to Pomona and Hyos (Bajocchi *et al.* 1961). In the

first epidemiological survey in Sao Paulo, Brazil, de Castro *et al.* (1966) examined the sera of 372 butchers across Sao Paulo. Of these, 3% (11) were seropositive to *Leptospira*, and one person was positive at titre at 200 to Hyos (de Castro *et al.* 1966). In New Zealand, the first evidence of Tarassovi in men was provided by (Kirschner 1954), who reported three cases of leptospirosis by Hyos in farmers with pig and cattle contact. Later, (Blackmore *et al.* 1981) reported Tarassovi infections in New Zealand pig abattoir workers, especially those involved in killing and dressing animals in abattoirs.

The pioneering study by Waitkins (1986) established occupational exposure as a predisposing factor for leptospirosis among British farmworkers, with 24% of all cases reported employed in the farming industry. Since then, more evidence has been collected on occupational risks, and now, it is accepted by most researchers that high-risk occupations are those having contact with animals.

Thornley et al. (2002) stated that from 1990–1998, 87.1% of New Zealand`s reported cases were from high-risk occupations. Nisa *et al.* (2020) reported that from 1999–2007, New Zealand dairy farmers had the highest incidence of Tarassovi (12.59/100,000). The spatial analysis showed that the incidence of Tarassovi was the highest in the Waikato, Taranaki, and Northland District Health Board regions, which are considered the biggest dairy farming regions in New Zealand. A seroprevalence study in 400 randomly selected people in Yucatan, Mexico, showed that Tarassovi serovar was predominant at 17.5% among seropositive (MAT ≥ 100) participants (Vado-Solis *et al.* 2002). However, their occupations were not disclosed, although authors reported that rodent contact was a significant risk factor.

2.7 Conclusion

Our literature review shows two distinct periods of research interest in *Leptospira* spp. serogroup Tarassovi. The first period began after its discovery and lasted until the 1960s. The second period began in the late 1990s when advanced DNA and molecular analysis tools became accessible.

During the first period, many serovars of the Tarassovi serogroup were isolated, and their epidemiology and maintenance hosts were described to an informative extent. Although Tarassovi seropositivity was reported in the range of different animals, its definitive affinity to certain species could not be detected in this literature review due to publication bias. Naturally, production animals were investigated more frequently compared to free-living mammals or non-mammalian species, thus resulting in a greater number of publications. As a result, it was widely accepted that Tarassovi could cause abortions and clinical manifestations in pregnant sows, and the Tarassovi antigen was included in the *Leptospira* vaccine for swine. As for cattle, despite reported seroprevalence, ruminants were not acknowledged maintenance hosts for Tarassovi, which was considered to cause mild leptospirosis in animals. This literature review found a few reports of production and reproduction effects in cattle attributed to Tarassovi in the early years from the 1930s to the 1960s. Initial seroprevalence studies in horses established that Tarassovi might cause an abortion, but in general, it would not be a cause of systemic disease. Seroprevalence studies reporting successful isolations of Tarassovi from non-mammal species, such as turtles, amphibians, and even snakes, lead researchers to think that Tarassovi might originate from livestock and, when shed into the environment, could be accidentally picked up by non-mammals. Researchers of Tarassovi in free-living mammals reached similar conclusions that small free-living mammals were unlikely to be maintenance hosts of Tarassovi. They suggested that it might be harboured by stock and passed to free-living mammals. Many seroprevalence studies of this period reported Tarassovi titres alongside Pomona titres, and after the introduction of vaccination against Pomona (Webster and Reynolds 1955), Tarassovi-only titres were more commonly reported. However, the whole range of antigens used for MAT was rarely reported, therefore, the full extent of co-infections is unknown.

The second period of interest in Tarassovi was primarily focused on seroprevalence and molecular studies. However, some new serovars from the serogroup Tarassovi were also cultured, mostly from people. Human cases were rarely documented unless acute disease symptoms were present and remain largely uninvestigated. However, more animal species were surveyed, including large free-living mammals and significant

seroprevalences to Tarassovi in some zebu and buffalo populations were established. Additionally, serosurveys reported notable seroprevalence ranging from 0.3 to 53.6% to Tarassovi in cattle, predominantly living in warm temperate and subtropical climates, such as Thailand, Mexico, Brazil, India, New Zealand, Kenya, and Uganda. In non-mammals such as turtles, snakes, and other herpetofauna, significant Tarassovi titres were also reported.

Globally, Tarassovi seroprevalence in cattle may indicate that ruminants play an important role in the zoonotic transmission of Tarassovi serovar to humans. For example, a Mexican study evaluated the seroprevalence of *Leptospira* spp. in people, pigs, dogs, rodents, bovines, and opossums, showed that Tarassovi was the predominant serovar in people and bovines, and no other reservoirs were seropositive to Tarassovi, excluding pigs at 5% (Vado-Solis *et al.* 2002).

In conclusion, this literature review indicated that Tarassovi is endemic in large ruminants in many populations. However, its transmission routes are unclear and other reservoir hosts may also be involved. Clinical signs are rarely apparent with Tarassovi infection; most likely, that disease is subclinical in infected animals, but no specific effects are reported. To date, it is evident that *Leptospira* of serogroup Tarassovi could be isolated from ruminants as shown by (Corney *et al.* 2008; Hamond *et al.* 2022); however, *Leptospira* from serogroup Tarassovi has not been isolated from ruminants in New Zealand.

This project will attempt to fill in knowledge gaps associated with serogroup Tarassovi in dairy cattle in NZ, emphasising the following areas: epidemiology of strain Pacifica, the effect of seropositivity to Tarassovi and Pacifica shedding on the production and reproduction of dairy cattle and will attempt to culture *L. borgpetersenii* serovar Tarassovi strain Pacifica from dairy cows.

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CHAPTER 3

Chapter 3 Evaluation of Growth Media and Culture Conditions for *Leptospira borgpetersenii*

3.1 Introduction

Leptospirosis is an underreported, re-emerging zoonotic disease distributed worldwide (Haake and Levett 2014) that affects all mammalian and some non-mammalian species (Picardeau 2017). Leptospirosis is caused by pathogenic bacteria from the family Leptospiraceae (Paster *et al.* 1991). Six pathogenic *Leptospira* serovars from two species have been identified as endemic to New Zealand: *Leptospira borgpetersenii* serovars (sv.) Hardjobovis (Hardjo), Tarassovi, Ballum, Balcanica and *Leptospira interrogans* sv. Pomona and Copenhageni. In New Zealand, leptospirosis is considered an occupational disease, and livestock are recognised as the primary source of human leptospirosis (Thornley *et al.* 2002; Nisa *et al.* 2020). Livestock vaccination remains one of the main interventions to prevent transmission between livestock and people (Thornley *et al.* 2002; Yupiana *et al.* 2019). Approximately 99% of dairy herds are vaccinated against Pomona and Hardjo, and 27% of dairy herds are also vaccinated against Copenhageni (Yupiana *et al.* 2021). However, vaccines for protecting dairy cows against Tarassovi were unavailable in New Zealand at the time of this evaluation.

Vaccine development requires live *Leptospira* cultures, which are usually obtained by isolation from samples collected from a clinically affected host, i.e., samples containing live bacteria inoculated into specially formulated media or a susceptible healthy laboratory mammal to propagate the growth of bacteria (Faine 1982). Pure culture isolation of pathogenic *Leptospira* spp. from urine can be challenging due to an intermittent urinary shedding pattern (Monti *et al.* 2023), and the maintenance of isolate could be challenging due to their fastidious growth requirements (Nally 2012) and sample contamination with environmental bacteria or normal microbiota (Chakraborty *et al.* 2011).

Over time, specific media were developed for *Leptospira* propagation. For example, Ellinghausen Jr and McCullough (1965) developed a medium where serum, previously

used in all media types, was replaced by albumin and other supplements, such as vitamin B12 and Tween (polysorbate). This medium was reported to effectively support the growth of 13 different serotypes of *Leptospira interrogans* (Herman 1965). Two years later, this medium was modified by (Johnson and Harris 1967) and named the Ellinghausen-McCullough-Johnson-Harris medium, abbreviated as EMJH. Another example is the *Leptospira* transport medium (LTM), a simplified variant of EMJH medium that can be prepared with different concentrations of agar (Faine 1982; Guedes et al. 2020). This medium protects *Leptospira* during transportation from changes in pH tr, which can lead to bacteria degradation. Solid and semi-solid media are reported to be effective for isolating *Leptospira* as these media are less prone to contamination.
Leptospira do not disperse throughout these media but concentrate in one area, forming a dense growth zone with an optimum oxygen concentration for *Leptospira* growth, often referred to as a Dinger's disk or a Dinger's zone (Lawrence 1951). Once the appearance of a disk is noted, the disk can be transferred to another tube. The time between passages depends on the genomospecies and may range from one week to six months.

In any medium formulation, supplementation of enrichment broth with cell culture provides additional nutrients that support cell growth and maintenance. While growth conditions may vary depending on the specific *Leptospira* requirements, the most similar conditions to the host's physiological environment are typically at 38°C and 5% CO_2. These conditions mimic the host`s internal environment, promoting optimal *Leptospira* function and growth (Hornsby et al. 2020).

As it is almost impossible to avoid contamination of urine samples collected under field conditions, researchers must take special precautions to suppress the growth of contaminants by adding antimicrobials to the media. The most common single antimicrobial is 5' – fluorouracil (5FU). 5FU acts in several ways but principally as a suppressor of DNA replication in fast-growing cells (Focaccetti et al. 2015). However, it does not inhibit the growth of most Gram-negative bacteria and fungi. Therefore, a combination of several antimicrobials is often required to suppress contamination (Chakraborty et al. 2011). The most common combination of antimicrobials used to

suppress the growth of contaminants is abbreviated as STAFF (Chakraborty *et al.* 2011). It consists of sulfamethoxazole (S), trimethoprim (T), amphotericin (A), fosfomycin (F), and 5FU (F). These antimicrobial agents were selected by testing the susceptibility of 17 strains, including eight *Leptospira* isolates recently obtained from field samples against 15 antimicrobial agents. Currently, STAFF is the most popular choice of antimicrobials used for *Leptospira* propagation.

The scientific literature describes various *Leptospira* cultivation methods, media types, filtration, serial dilutions, CO_2 concentrations, temperatures, selective agents, and supplements. Nevertheless, the reported rate of pure *Leptospira* cultures obtained using those methods is variable—the genomospecies and serovar of the cultivar impact the ability to isolate and cultivate *Leptospira* successfully. Different genomospecies may have additional growth requirements directly related to their DNA composition. For example, Bulach *et al.* (2006) reported that the genome of L. *borgpetersenii* is ~700 kb smaller than that of *L. interrogans*. The authors concluded that *L. borgpetersenii* might be undergoing genome reduction and, therefore, may evolve towards more direct host-to-host transmission. Specifically, the authors postulated that the reduction of parts of the genome responsible for the metabolism and solute transport of *L. borgpetersenii* likely limits the range of acceptable nutrients and impairs its ability to survive in water and its virulence (Bierque *et al.* 2020). Therefore, cultivating *L. borgpetersenii* may require a finessed selection of suitable nutrients and its survival ability and virulence outside a host organism may be limited to a very short period.

Similarly, several authors listed below suggested that media composition should be tailored to support the growth of a particular species, as some might have specific nutritional requirements or tolerances. For example, a study reported isolating a new species of pathogenic *Leptospira* from a Southern Right Whale (*Eubalaena australis*) cultivated in medium supplemented with 5% seawater. Therefore, the authors concluded that these species are resistant to and can grow in seawater (Grune Loffler *et al.* 2015). Another example of a medium tailored to the needs of a particular *Leptospira* serovar is media developed by Ellis (1984). It targeted the isolation of *Leptospira* interrogans serovar Hardjo from cattle, and its components include polysorbate 40, lactalbumin hydrolysate, superoxide dismutase, and rabbit serum.

Over the past two decades, the emergence of serovars, which are not included in commercially available vaccines, known as non-vaccine serovars, has been reported in New Zealand (Thornley *et al.* 2002; Nisa *et al.* 2020; Moinet *et al.* 2023; Wilkinson *et al.* 2023). Specifically, the emergence of Tarassovi in dairy cows was reported (Yupiana *et al.* 2019). Moreover, 68% (54/80) of urine samples from the cows that were shedding were sequence-typed at Massey University's Molecular Epidemiology and Public Health Laboratory (^{m}Epilab) using amplicon sequencing of the *glmU* gene. Sequence data indicated that the *Leptospira* in the urine was a strain of *Leptospira borgpetersenii*, referred to as *Leptospira borgpetersenii* strain (str.) Pacifica (Wilkinson *et al.* 2021). Further analysis showed a statistically significant association ($p < 0.01$) between urinary shedding and seropositivity to Tarassovi, and it was suggested that a MAT titre of ≥ 48 may indicate urinary shedding. Because of this two-fold association, Pacifica is thought to belong to the serogroup Tarassovi (Wilkinson et al. 2023). Therefore, when identified by serology only, Tarassovi infections in New Zealand animals and humans could at least partially be attributed to Pacifica because other Tarassovi serovars are recognised in New Zealand. However, isolation by culture is required to confirm Pacifica's serotype, and complete genetic characterisation of an isolate will show how closely str. Pacifica is related to Tarassovi. This characterisation is also important in understanding this strain's epidemiology and disease transmissibility.

In New Zealand, *Leptospira* serovar Tarassovi has been isolated and sequenced three times: from a pig (strain T74) (Ryan and Marshall 1976), a dog (strain Precious) (Mackintosh *et al.* 1980) and a human (strain Gill) (J. Collins-Emerson, *personal communication,* September 30, 2023). These isolates were subsequently found to be similar to the internationally recognised reference strain *L. borgpetersenii* serovar Tarassovi strain Perepelitsin by whole genome sequencing (WGS, BioProject PRJNA666522). These isolates, as well as *Leptospira borgpetersenii* serovar Tarassovi strain Perepelitsin, have been maintained and propagated in EMJH under ^{m}Epilab conditions for a number of years. Therefore, ^{m}Epilab has the expertise and well-developed protocols for isolating and maintaining *Leptospira borgpetersenii*. However, the researchers were not successful in isolating Tarassovi or str. Pacifica from the urine of New Zealand cattle (Yupiana *et al.* 2019; Wilkinson *et al.* 2023). The failure to culture

str. Pacifica is suggestive of bacterium-specific requirements. There is one published work that reported isolating *L. borgpetersenii* serovar Tarassovi from a dairy cow in Iowa, USA (Hamond *et al.* 2022a), using a novel Hornsby-Alt-Nally (HAN) media formulation (Hornsby *et al.* 2020) under modified growth conditions. Therefore, we hypothesise that media for the isolation of *Leptospira borgpetersenii* strain Pacifica from the urine of dairy cows require modification.

The overarching aim of this experiment was to prepare and select the most suitable medium(a) for cultivating str. Pacifica from cattle urine samples collected on the farm. This was done by inoculating selected *Leptospira borgpetersenii* strains used as a proxy for str. Pacifica and observing the bacteria's growth rates under specific conditions. The following sections describe the rationale for selecting *Leptospira* spp. for testing the media, the media tested, and the growth conditions assessed.

3.2 Materials and Methods

3.2.1 *Leptospira* Strains

The selection of serovars/strains used in this experiment to test different media was based on the hypothesis that members of *L. borgpetersenii* genomospecies may have similar nutrient and growth requirements. Thus, cultures of *L. borgpetersenii* could be used to test different media as a proxy for str. Pacifica. The following criteria were applied to the selection of serovars for testing the growth media:

I. Serovars belonging to genomospecies *L. borgpetersenii*

II. Field strains of *L. borgpetersenii* that may be less adapted to laboratory conditions.

III. Serovars from serogroup Tarassovi, as Pacifica shedding animals, are also seropositive to Tarassovi.

IV. The most challenging *L. borgpetersenii* to cultivate in ^{m}Epilab.

According to the criteria above, four strains of *L. borgpetersenii* available at ^{m}Epilab were chosen for media testing. These comprised three field strains, specifically, *L. borgpetersenii* serovar Tarassovi str. "Precious" isolated from a New Zealand dog (Mackintosh *et al.* 1980), *L. borgpetersenii* serovar Hardjo str. 902 and 905 isolated from New Zealand sheep (Fang *et al.* 2014), and a laboratory-adapted str. of *L. borgpetersenii* serovar Tarassovi strain Perepelitsin originally obtained from the Leptospirosis Reference Centre, Queensland, Australia. Although *L. borgpetersenii* serovar Ballum is also closely genetically related to Pacifica, it was not considered for this experiment, as Ballum is known for its relative ease of propagation. In contrast, Pacifica is regarded as a more difficult strain to propagate (Edwards 2019; Yupiana *et al.* 2019), and Hardjo is also recognised as a strain with fastidious propagation requirements (Putz *et al.* 2022).

3.2.2 Standardisation of Inoculum

Before the investigation, the selected strains were propagated in EMJH supplemented by 5-fluorouracil at 28°C with shaking at 30 RPM. These cultures were subcultured monthly for use and quarterly for medium-term maintenance.

Then, prior to media inoculation with selected *Leptospira* strains, the densities of *Leptospira* stock cultures of the four selected strains were assessed using the Petroff-Hausser counting chamber by dark field microscopy. The inoculation volume needed to achieve a starting concentration of approximately 10^6 *Leptospira* /ml was calculated, and 100 µl of prepared cultures of Tarassovi and Hardjo were added to each medium type. Vials without cultures were used as negative controls.

3.2.3 Assessment of *Leptospira* Growth and Growth Conditions

In this experiment*, Leptospira* growth was assessed by the following criteria:

I. Growth rates or highest concentration reached

II. Ability to resist contamination

III. Ability to develop a positive growth curve.

The growth of *Leptospira* was assessed in liquid and semi-solid culture media. Cultures were inoculated with a 1:10 dilution, i.e., 1 ml of maintenance stock culture to 10 ml of fresh EMJH medium and incubated for one week at 28 °C according to laboratory protocols. Liquid cultures were assessed visually at the frequency noted in Table 3.2 via dark field microscopy to evaluate their viability and motility by placing one drop from the culture on a glass slide. The density of motile and intact *Leptospira* was then manually calculated using a Petroff-Hausser counting chamber using the dark field microscope. Growth in semi-solid media was also assessed visually and scored as having no visible growth (-), visible growth, which was confirmed by dark field microscopy (+), or the presence of a Dinger zone confirmed by dark field microscopy (D). The estimated total number of bacteria was recorded in an Excel spreadsheet. During the experiment, seeded vials were kept inside incubators on the shaking platform at an average temperature of 28°Cat 30 RPM in all stages (Table 3.2).

3.2.4 Media Preparations

The main aim of media and supplement selection was to identify the most suitable media and supplements for cultivating str. Pacifica from cattle urine samples collected on farms from live animals. Specific selection criteria are listed below.

I. Types of media propagating *Leptospira borgpetersenii* from bovine urine collected under field conditions.

II. Supplements that prevented contamination of media from bacteria naturally occurring in the urine samples.

III. Supplements that promote *Leptospira* growth

IV. Media, when seeded, would be potentially suitable for transportation for more than 4 hours.

Based on these criteria, the following three media were chosen for the experiment: EMJH medium, HAN medium, and *Leptospira* transport medium (LTM). These media have been successfully used to isolate and cultivate *L. borgpetersenii (*Ellinghausen Jr

and McCullough 1965; Chideroli et al. 2017; Hornsby et al. 2020; Hamond et al. 2022b*).* Additionally, the STAFF supplement (Chakraborty *et al.* 2011) was selected to prevent the growth of contaminants. EMJH-5-FU and EMJH-STAFF have also been successfully used to cultivate *Leptospira* in ^{m}EpiLab.

3.2.5 Media Preparation Protocols

In this experiment, eight types of EMJH, two types of LTM, and the HAN medium were tested (Table 3.1). Media ingredients and protocols are included in Appendix 1. The media preparation involved four generic steps: stock or basal medium solution preparation, supplement addition, and mixture filtering. The first step of EMJH medium preparation was the preparation of the BSA stock solution as previously described by Ellinghausen and McCullough (1965) and modified by Johnson and Harris (1967). Then, one portion of the basal stock solution was supplemented by BSA enrichment prepared in-house, while the other was supplemented by commercially prepared enrichment (Table 2 in Appendix). The first mix was denoted as EMJH, and the second was EMJH_COMM (Table 3.1). Next, separate portions of EMJH variants (Table 3.1) were supplemented each by 10% rabbit serum (RS) or 10% newborn calf serum (NCS) (Faine 1982; Ellis 1984).

LTM media was prepared as described by Faine (1982) and modified by Guedes *et al.* (2020), and HAN media was prepared as described by Hornsby *et al.* (2020); however, in HAN media, the hemin was replaced by ferrous sulphate because both are considered to have the same function. The difference is that hemin is an organic compound and ferrous sulphate is an inorganic compound. The reason for replacement was logistical and supply issues, as hemin needed to be shipped from overseas during the COVID-19 pandemic, and the delivery date could not be estimated at the time and ferrous sulphate was readily available.

All media types were prepared with and without STAFF (Table 3.1). All ingredients and media preparation protocols are listed in Tables 1, 2, and 4 of the Appendix.

All media (Table 3.1) were filtered and enriched according to the preparation protocols and were tested for contamination as follows: media was aliquoted in tubes, kept at controlled temperatures of 28°C and 38°C for one week, and observed for contamination growth. If the media passed the contamination test and where contamination growth was observed, 9 ml of each media type were aliquoted into 30 ml screw-top glass sterilised vials. Glass vials were replaced in subsequent experimental stages 1a, 2, 3, and 4 with plastic sterile yellow-top BD vacutainer® tubes. Media that were not in use were stored at 4°C.

3.2.6 Glassware and Water Preparation

Sterilising glassware and water and sourcing quality Bovine Albumin Serum (BSA) are crucial steps in media preparation for *Leptospira* cultivation. In this instance, glassware used for media preparation, which was previously washed and autoclaved, was rinsed again by hand in distilled water and autoclaved again at [121ºC at 100 kPa (15 psi) for 15 min] to eliminate any residues on glass that may negatively affect *Leptospira* growth.

Then, double distilled or double ionised water was sterilised by autoclaving at 121°C for 20 minutes at 100 kPa (15 psi), as recommended (Faine 1982). Meticulous water preparation prevents saprophytic *Leptospira*, naturally found in water sources, from contaminating media designated for cultivating pathogenic *Leptospira*. Filter sterilisation of water is not advised for media preparation because saprophytic *Leptospira* can pass through the filter.

Lastly, using high-quality Bovine Albumin Serum (BSA) fraction V as a fatty acid source (Francis 2010) is also important, as BSA quality can directly impact *Leptospira's* propagation by negatively affecting its growth. To ensure BSA suitability, a new batch of BSA was pre-tested by seeding the media with *L. borgpetersenii* serovar Hardjo as the most challenging laboratory strain to grow and assessing its growth rates. The list of all reagents used in this experiment is available in Appendix 1.

Table 3.1 Types of Media and Abbreviations Used in the Media Experiment to Determine the Best Conditions for the Growth of *Leptospira borgpetersenii* spp.

EMJH	**EMJH_COMM**	**LTM**	**HAN**
EMJH	EMJH with commercial enrichment (DIFCO™) =EMJH_COMM	LTM	HAN
EMJH supplemented with STAFF antimicrobials =EMJH_STAFF	EMJH with commercial enrichment and 10% rabbit serum =EMJH_RS_COMM	LTM supplemented with STAFF antimicrobials =LTM_STAFF	HAN supplemented with STAFF antimicrobials = HAN_STAFF
EMJH supplemented with10% rabbit serum =EMJH_RS			
EMJH supplemented with10% rabbit serum and STAFF =EMJH_RS_STAFF			
EMJH supplemented with 10% newborn calf serum = EMJH_NCS			
EMJH supplemented with 0.1% Noble agar and STAFF=EMJH_AGAR_STAFF			

3.2.7 Experimental Design

Four stages of media experiments (1,1a, 2, 3, and 4) were designed to evaluate the performance of liquid media types by evaluating the growth curves of *L.borgpetersenii* seeded in these media (Table 3.2). The media with the highest number of bacteria, the best growth curves, and the ability to resist contamination were then selected for use in the next stage. Therefore, the design was informed by the results of the previous stages.

In addition to the liquid media experiment with four distinct stages, a separate experiment during stage 3 was also conducted to compare the ability of two semi-solid media, EMJH_STAFF_AGAR and LTM (Table 3.1) to support the growth of *L. borgpetersenii* short and long-term. For this experiment, each media type was seeded in triplicate with selected *L. borgpetersenii* strain (Table 3.2)

Table 3.2 Stages of experiment, liquid media types, *Leptospira* strains, frequency of checks and the purpose of each stage.

Stage	**Media**	***L. borgpetersenii***	**Checking Frequency**	**Duration**	**Purpose**
Stage 1	*EMJH, EMJH_AGAR_STAFF, EMHJ_COMM, *EMJH_NCS, EMJH_RS_STAFF, *EMJH_RS_COMM, EMJH_STAFF	sv. Hardjo str.905, sv. Tarassovi str. Perepelitsin	Every day after inoculation	7 days	To compare the ability of all EMJH subtypes (Table 3.1) to support the growth of Tarassovi and Hardjo strains.
Stage 1a	EMJH, EMJH_AGAR_STAFF, EMHJ_COMM, EMJH_NCS, EMJH_RS_STAFF, EMJH_RS_COMM, EMJH_STAFF	sv. Hardjo str.905, sv. Tarassovi str. Perepelitsin	Every day after inoculation	7 days	To compare the ability of all EMJH subtypes (Table 3.1) to support the growth of selected Tarassovi and Hardjo strains. New EMJH_NCS was prepared. Repeat of the stage 1.
Stage 2	EMJH_STAFF, EMJH_RS_STAFF	sv. Hardjo str.905, sv. Hardjo str.205, sv. Tarassovi str. Precious	Every third day after inoculation	18 days	To compare the ability of the best media selected from stage 1, EMJH_STAFF and EMJH_RS_STAFF, to support the growth of the different strains Hardjo and Tarassovi, different from stages 1 and 1a.

Stage	Media	*L. borgpetersenii*	Checking Frequency	Duration	Purpose
Stage 3	EMJH_STAFF, EMJH_RS_STAFF, LTM_STAFF	sv. Hardjo str.905, sv. Tarassovi str. Perepelitsin	Every day after inoculation	18 days/12 months for LTM	To compare the ability of the two best EMJH types selected from both stages 1 and 2 to LTM to support the growth of the selected *Leptospira* strains.
Stage 4	EMJH_STAFF, EMJH_RS_STAFF, HAN_STAFF	sv. Hardjo str.905, sv. Tarassovi str. Perepelitsin	Every day after inoculation	18 days	To compare the ability of the two best EMJH types selected from both stages 1 and 2 to HAN media to support the growth of the selected *Leptospira* strains.

*Supplemented by 5FU

3.2.8 Data Analysis

The 95% confidence intervals for the average of triplicates of estimated bacteria numbers were calculated using epiR functions and plotted using the ggpot2 package. Analyses were performed using R version 4.1.0 (2021-05-18) 'Camp Pontanezen (R Core Team, 2021) with the packages epiR (Stevenson et al. 2018), tidyr (Wickham et al. 2019) and ggplot2 (Wickham 2011).

3.3 Results

3.3.1 Stages 1 and 1a

The purpose of stages 1 and 1a was to compare the ability of all EMJH subtypes (Table 3.1) to support the growth of Tarassovi and Hardjo. Stage 1 and 1a experiments showed that EMJH_STAFF and EMJH_RS_STAFF were the best to propagate *Leptospira borgpetersenii*, while EMJH_NCS did not support its growth. Figures 3.1 and 3.2 show the average triplicate of the estimated daily concentration of bacteria in each type of EMJH media for inoculation in stages 1 and 1a. EMJH was the best for Hardjo propagation, with the highest concentration of *Leptospira* in stages 1 and 1a *Leptospira*/ml Hardjo in EMJH (Table 3.1), whereas the highest concentration of Tarassovi was $1.4x10^9$ *Leptospira*/ml. Two samples of EMJH_RS_COMM seeded with serovar Hardjo str.905 were lost to contamination four days post-inoculation despite using an aseptic technique in a biohazard cabinet. Additionally, while 5FU was added to all stage 1 media, 12% (5/42) of media presented occasional contamination while cultivated under laboratory conditions. Therefore, additional steps were taken after stage one to avoid contamination in the following stages, such as the replacement of 5FU by STAFF in all media and the replacement of multiple-use glass screw-top vials with aseptic single-use screw-top plastic vials.

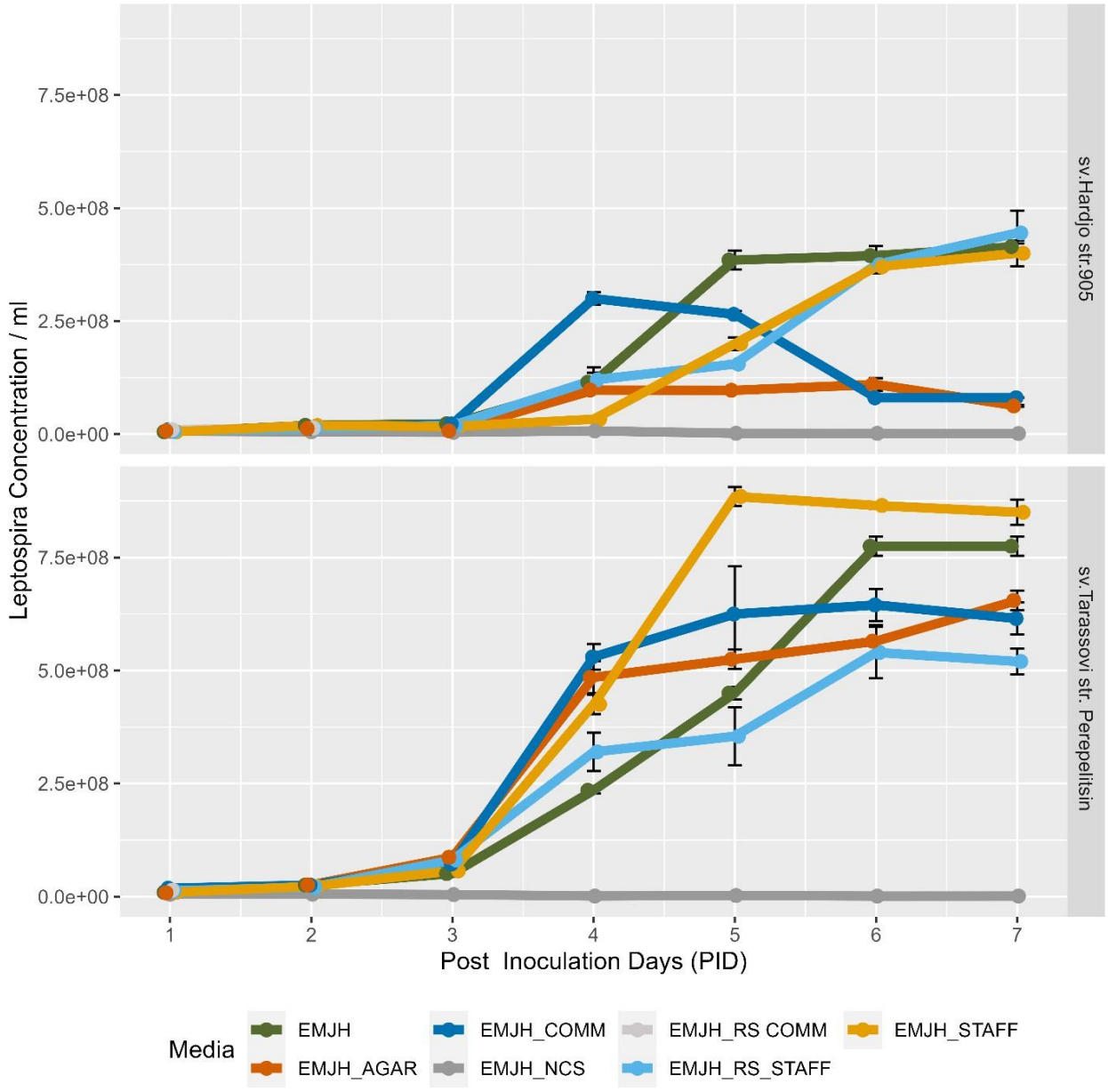

Figure 3.1 Growth curves of *Leptospira* in stage 1. Serovars Hardjo (str.905) and Tarassovi (str. Perepelitsin) were incubated at 28 °C and enumerated in the Petroff-Hausser counting chamber after seeding into EMJH_STAFF (Orange), EMJH (Green), EMJH_COMM (Dark Blue). Growth curves are averages of triplicate cultures. Standard error bars are shown, representing the average of triplicate results. The post-inoculation day (PID) of the media is indicated on the X-axis, and the concentration of *Leptospira*/ml is indicated on the Y-axis.

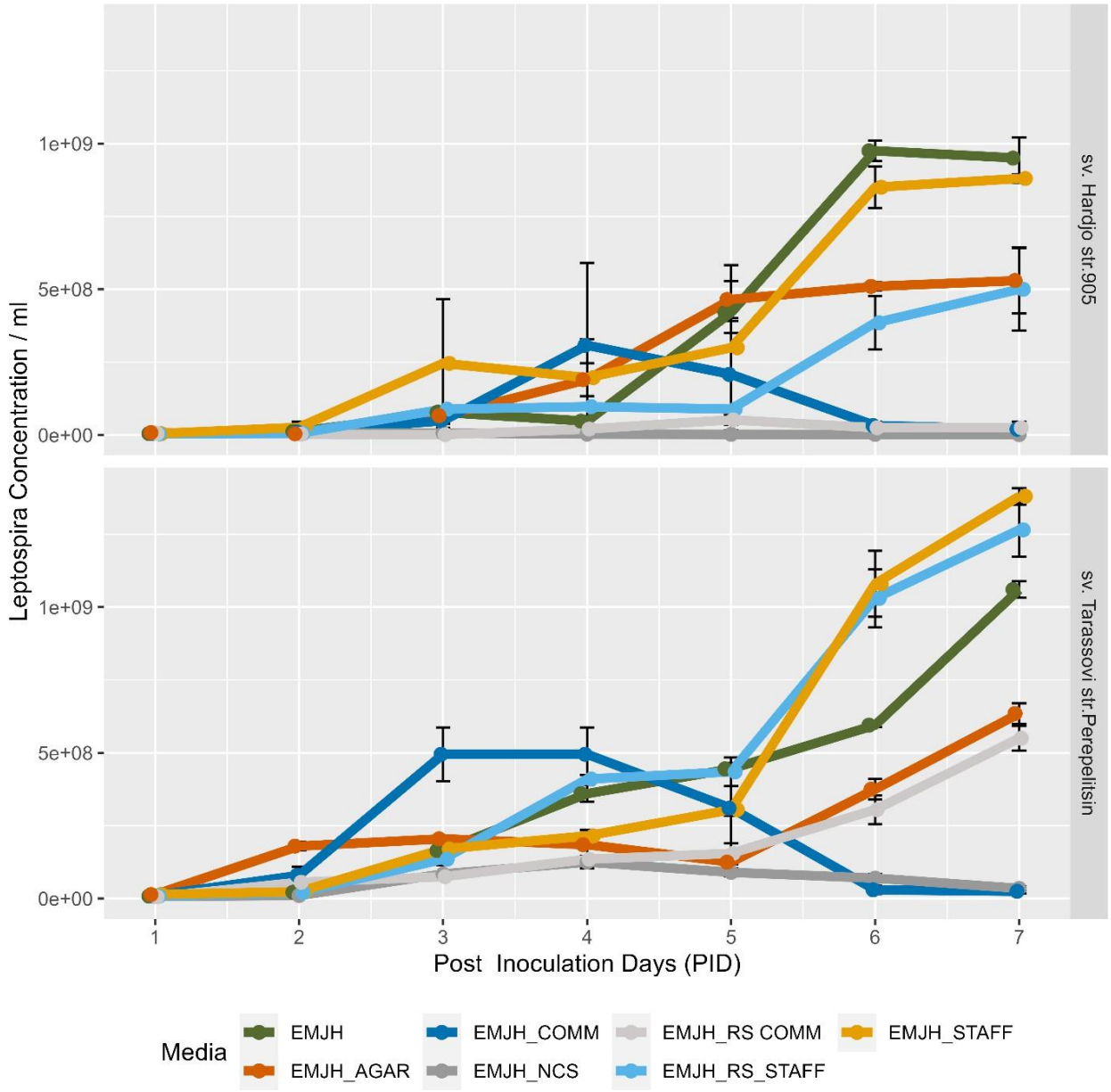

Figure 3.2 Growth curves of *Leptospira* in stage 1a. Serovars Hardjo (str.905) and Tarassovi (str. Perepelitsin) were incubated at 28 C° and enumerated in the Petroff-Hausser counting chamber after seeding into EMJH_STAFF (Orange), EMJH (Green), EMJH_COMM (Dark Blue). Growth curves are averages of triplicate cultures. Standard error bars are shown, representing the average of triplicate results. The post-inoculation day (PID) of the media is indicated on the X-axis, and the concentration of *Leptospira*/ml is indicated on the Y-axis.

3.3.2 Stage 2

The stage 2 experiment included one round of inoculation into the best media for propagation of *L. borgpetersenii* identified in rounds 1 and 1a, specifically, EMJH_STAFF and EMJH_RS_STAFF. The average of the triplicate results of stage 2 showed that, overall, EMJH_ STAFF was better than EMJH_RS_STAFF at supporting the growth, as determined by the highest concentration gained, ability to resist contamination, and the growth curve of all *Leptospira* strains (Figure 3.3). It was also evident that strains 205 and 905 reached their maximum concentrations at different times, and strain 205 had the highest estimated number of *Leptospira* in EMJH_STAFF in comparison to strain 905, whereas Tarassovi Precious reached its highest concentration on EMJH_RS_STAFF. The growth curve of sv. Hardjo str. 905 was more similar to the sv. Tarassovi growth curve, therefore, sv. Hardjo strain 905 was selected to use at the following stages (Figure 3.3).

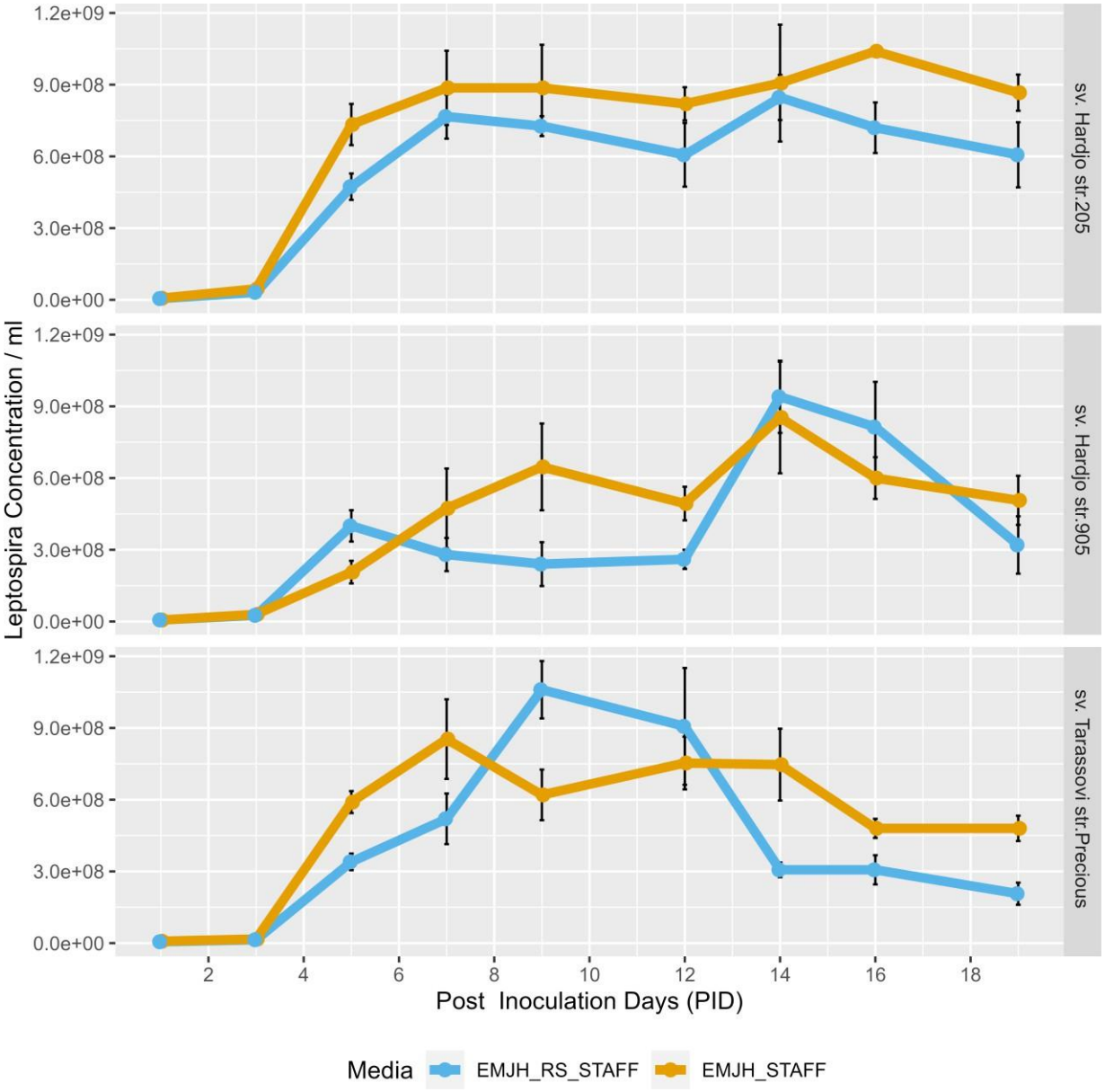

Figure 3.3 Growth curves of *Leptospira* in stage 2. Serovars Hardjo (str. 205, str. 905) and Tarassovi (str. Precious) were incubated at 28°C and enumerated in the Petroff-Hausser counting chamber after inoculation into EMJH_RS_STAFF (Blue) and EMJH_STAFF (Orange). Growth curves are averages of triplicate cultures. Standard error bars are shown, representing the average of triplicate results. The post-inoculation day (PID) of the media is indicated on the X-axis, and the concentration of *Leptospira*/ml is indicated on the Y-axis.

3.3.3 Stage 3

The purpose of stage 3 was to compare the ability of the two best EMJH types selected from stage 2 to LTM to support the growth of the selected *Leptospira* strains. The results of stage 3 showed that EMJH_STAFF supported the growth of *Leptospira* very well, and LTM_STAFF media showed the second-best ability to maintain the growth of both strains, followed by EMJH_RS_STAFF (Figure 3.4). The latter did not support the bacteria propagation to high concentrations. The maximum concentration of serovars Hardjo and Tarassovi was $3x10^8$ *Leptospira/*ml. The stage three experiment showed that EMJH_STAFF and LTM_STAFF support *Leptospira* propagation equally well.

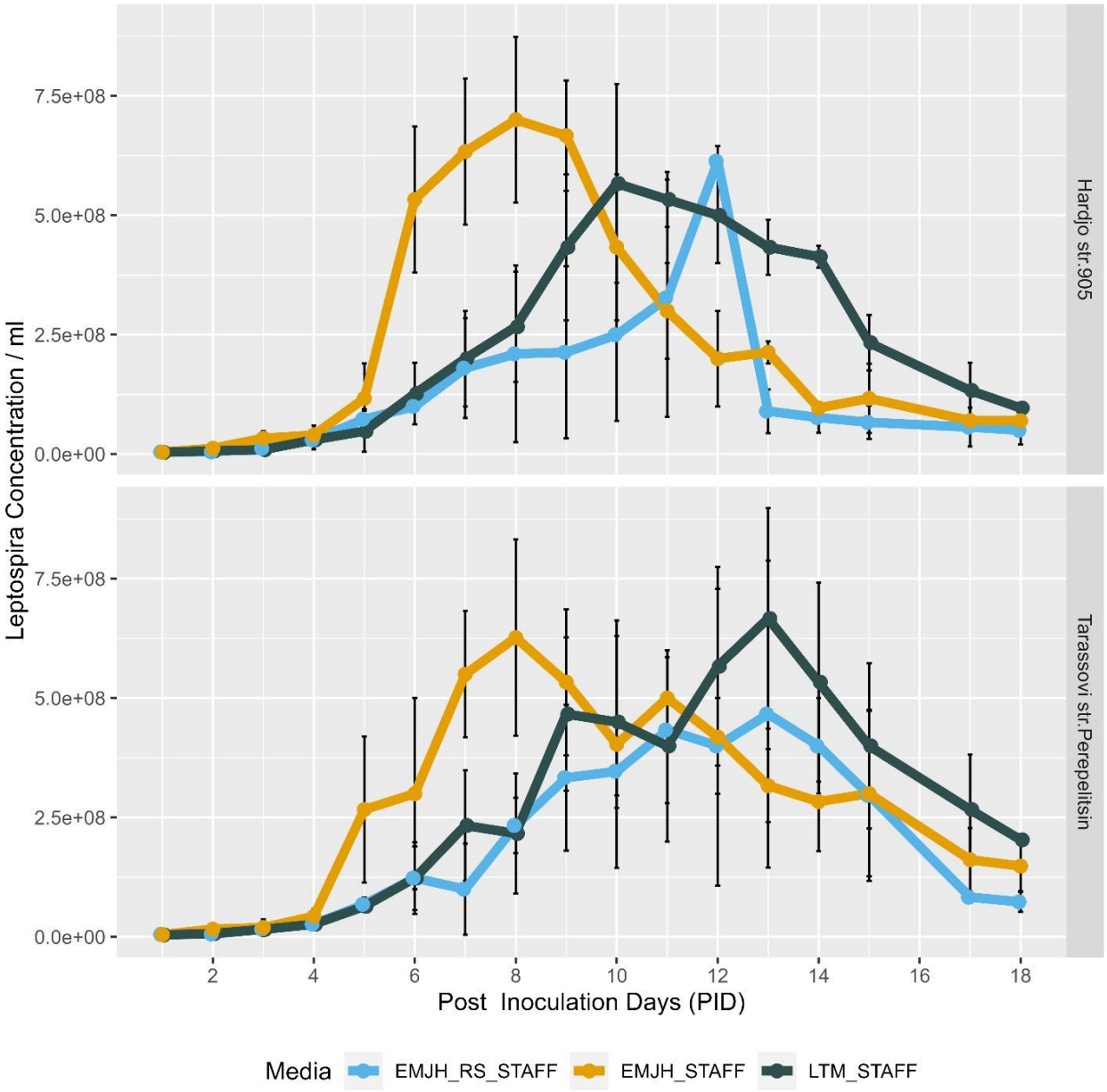

Figure 3.4 Growth curves of *Leptospira* in stage 3. Serovars Hardjo (str.905) and Tarassovi (str. Perepelitsin) were incubated at 28 °C and enumerated in the Petroff-Hausser counting chamber after inoculation into EMJH_RS_STAFF (Blue), EMJH_STAFF (Orange) and LTM_STAFF(Teal). Growth curves are averages of triplicate cultures. Standard error bars are shown, representing the average of triplicate results. The post-inoculation day (PID) of the media is indicated on the X-axis, and the concentration of *Leptospira*/ml is indicated on the Y-axis.

3.3.3.1 Semi-solid media

Semi-solid EMJH_STAFF and semi-solid LTM _STAFF were prepared for long-term maintenance testing. A duplicate Tarassovi culture in EMJH_STAFF and LTM_STAFF was kept for 12 months at 28 °C to assess the ability of these semi-solid media for long-term maintenance of Tarassovi. The results are presented in Table 3.3. The concentrations of Tarassovi indicate that Tarassovi cultures could still be detected in LTM_STAFF twelve months after the media was seeded.

Table 3.3 Long-term cultivation of *Leptospira borgpetersenii* serovar Tarassovi. Cultures were visually assessed for growth at post-inoculation months (PIM) 1, 3, 6, 9 and 12. Each concentration is the average of duplicate samples.

Serovar	**Medium**	**PIM1**	**PIM3**	**PIM6**	**PIM9**	**PIM12**
Tarassovi 1	EMJH STAFF	5E+06	2E+07	2E+06	2E+04	-
Tarassovi 1 Duplicate	EMJH STAFF	6E+06	2E+07	2E+06	2E+04	-
Tarassovi 2	LTM STAFF	4E+06	2E+07	7E+07	5E+06	1E+04
Tarassovi 2 Duplicate	LTM STAFF	4E+06	3E+07	9E+07	2E+06	3E+04

A comparison of LTM_STAFF (which is a semi-solid medium) and EMJH_AGAR_STAFF showed that LTM_STAFF was better at supporting the growth of serovars Hardjo and Tarassovi, as the appearance of a distinct Dinger zone was observed in duplicate LTM_STAFF samples inoculated with Tarassovi and Hardjo within 9- and 11-days post-inoculation (Table 3.4), while in EMJH_AGAR_STAFF Dinger zone was detected in only in 2 samples inoculated with Hardjo on 15th-day post-inoculation Table 3.4).

Table 3.4 Serovars Hardjo and Tarassovi cultures in semi-solid media. Cultures were visually assessed for growth at post-inoculation day (PID) 7 and post-inoculation week (PIW) 2, 3 and 4. Cultures were scored as having no visible growth (-), visible growth (+), and Dinger Zone detected (D).

Serovar	Medium	PIW1	PIW2	PIW3	PIW4
Tarassovi 1	LTM_STAFF	–	+	+ D	+ D
Tarassovi 1 Duplicate	LTM_STAFF	–	+ D	+ D	+ D
Hardjo	LTM_STAFF	–	+	+ D	+ D
Hardjo 1 Duplicate	LTM_STAFF	–	+	+ D	+ D
Tarassovi 2	EMJH_AGAR_STAFF	–	–	–	–
Tarassovi 2 Duplicate	EMJH_AGAR_STAFF	–	–	–	–
Hardjo 2	EMJH_AGAR_STAFF	–	–	+	+ D
Hardjo 2 Duplicate	EMJH_AGAR_STAFF	–	–	+	+

3.3.4 Stage 4

The purpose of stage 4 was to compare the ability of EMHJ, LTM, and HAN to support the growth of *Leptospira borgpetersenii*. The results of stage 4 showed that two batches of HAN media prepared separately a month apart failed to grow any strains of laboratory adapted *Leptospira*, while both EMJH_STAFF and LTM_STAFF performed well. The Tarassovi and Hardjo concentrations in EMJH_STAFF that peaked earlier showed a sharp drop in concentration compared to LTM_STAFF. LTM_STAFF yielded the highest concentration of Tarassovi at $7x10^8$ *Leptospira*/ml, while EMJH_STAFF yielded the highest concentration of Hardjo at $5x10^8$ *Leptospira* /ml (Figure 3.5).

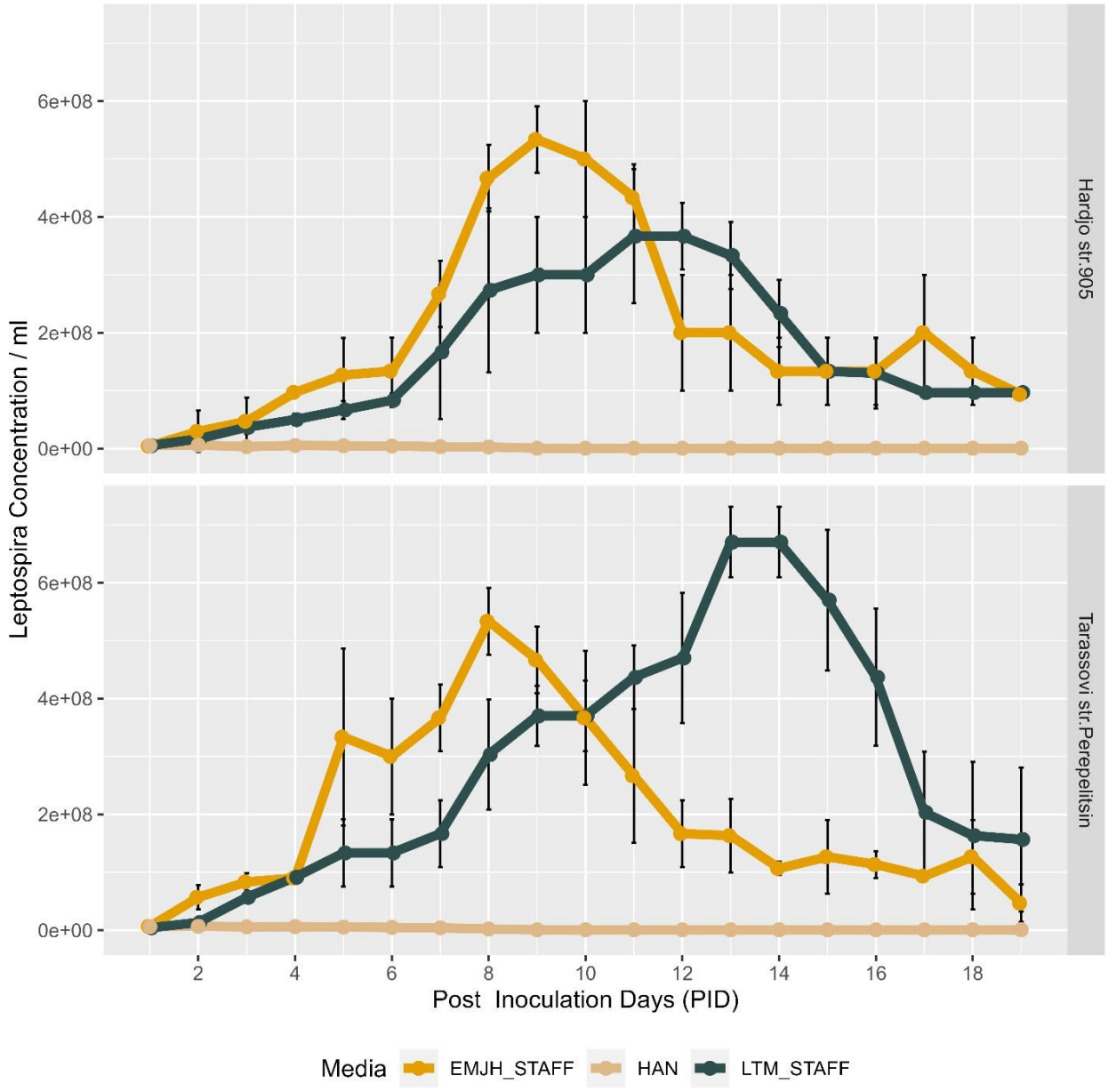

Figure 3.5 Growth curves of *Leptospira* in stage 4. Serovars Hardjo (str.905) and Tarassovi (str. Perepelitsin) were incubated at 28°C and enumerated in the Petroff-Hausser counting chamber after inoculation into EMJH_STAFF (Yellow), HAN (Brown) and LTM_STAFF (Teal) media. Growth curves are averages of triplicate cultures. Standard error bars are shown, representing the average of triplicate results. The post-inoculation day (PID) of the media is indicated on the X-axis, and the concentration of *Leptospira*/ml is indicated on the Y-axis.

3.4 Discussion

The experiments conducted in this study aimed to identify and select the best media suitable for cultivating *Leptospira borgpetersenii spp*. as a proxy for *Leptospira borgpetersenii* str. Pacifica*.* Twelve media were tested at 28°C, and the results showed that three media types, EMJH RS_STAFF, EMJH_STAFF, and LTM, were superior to the other media in supporting the growth and maintenance of laboratory cultures. The addition of STAFF effectively prevented media contamination, and HAN media failed to sustain the growth of *L. borgpetersenii* tested in this study. Interestingly, EMJH_COMM, which other researchers successfully used (Loureiro *et al.* 2022), did not support *Leptospira* propagation under the conditions tested in our laboratory. The results of the experiment will be discussed by the media type.

3.4.1 EMJH Media

Overall, in this experiment, EMJH performed well. Eight different types of EMJH media were used in this experiment (Table 3.1); three were made with commercially made enrichment, and five were made with in-house prepared enrichment. The two best media types throughout the experiment were EMJH RS_STAFF and EMJH_STAFF.

3.4.1.1 Regular EMHJ

The performance of the regular EMJH referred to as EMJH in Table 3.1, was not the best in this study and also differed slightly between the current study and similar studies. For example, Guedes *et al.* made EMJH supplemented with in-house prepared enrichment following the method described by (Faine 1982), which is also the reference method for EMJH prepared for this study (Guedes *et al.* 2022). However, there were some differences in the amounts of reagents used between the two studies, the most notable being the amount of Tween 80. Guedes *et al.* (2022) added 1.2 ml/litre of Tween 80, while we added ten times more, 12 ml/litre. Tween 80 is a source of essential fatty acids (Faine 1994; Adler 2015), and its low concentration in media could be proportional to low *Leptospira* growth rates (Stalheim 1966). Therefore, the slower growth of *Leptospira* (5-8 days to reach ~2 x10^8 *Leptospira* /ml) in the study by Guedes and co-authors in EMJH original compared to our EMJH may be attributed to the low

concentration of Tween 80. At the same time, in our experiment, it took six days for *Leptospira* to reach a concentration of ~2 × 10^6 *Leptospira* /ml.

3.4.1.2 EMJH and Rabbit Serum

EMJH media supplemented by 10% rabbit serum and STAFF showed a great ability to support the short-term growth of Tarassovi. Concentrations of Tarassovi peaked at 2x10^9 *Leptospira/* ml, while the Hardjo's highest concentrations only reached 9x10^8 *Leptospira/* ml. Overall, the concentrations of Hardjo ranged from 5x10^8 to 9x10^8 *Leptospira/* ml at stages 1, 1a and 3 (Figures 3.1,3.2,3.4), and Tarassovi concentrations varied from 4.8 x 10^8 to 2x10^9 *Leptospira/* ml in the same stages (Figures 3.1,3.2,3.4).

It is unsurprising to see these results, as rabbit serum is a popular choice for the source of fatty acids it also contains native haemoglobin, which along with thiamine helps in *Leptospira* growth (Faine 1982). Some researchers also suggest that rabbit serum is also a source of Vitamin B12 in the media (Philip *et al.* 2018). However, when the EMJH protocol was first introduced, Tween 80 was listed as a source of fatty acids, Vitamin B12 was listed as a separate ingredient, and rabbit serum was suggested as an optional add-on component for EMJH. Nevertheless, rabbit serum is still commonly used in different types of media in different concentrations, at 2% (Te Brugge and Louw 1985; Corney *et al.* 2008), 3% (Wuthiekanun *et al.* 2014), 10% (Miraglia *et al.* 2008) and 15% (Johnson and Gary 1963). It is generally accepted that rabbit serum improves *Leptospira* growth, but it can be replaced by bovine or ovine serum (Babudieri 1961). Improved *Leptospira* growth rates were reported in media supplemented with 1% rabbit serum without other supplements such as Tween or BSA (Stalheim 1966). Similarly, Johnson and Gary (1963) showed that adding 10% and 15% of ultrafiltrated rabbit serum to basal media supported and markedly stimulated the growth of Pomona. More recently, liquid, and semi-solid EMJH supplemented by 2% rabbit serum was used for the isolation of *Leptospira weilii* serovar Topaz, a member of the Tarassovi serogroup directly from bovine urine (Corney *et al.* 2008). Rabbit serum is also used to supplement solid media *Leptospira* Vanaporn Wuthiekanun agar (LVW). It has been reported that the optimal LVW solid media contained 1% Noble agar with 10% RS medium (Wuthiekanun *et al.* 2014).

On the negative side, rabbit serum was reported to precipitate phosphates from media, and these precipitates are shown to decrease the clarity of media and mask bacteria under dark field microscopy (Adler 2015). Indeed, our EMJH_RS media was less clear under the microscope and more challenging to read when compared to EMJH. Additionally, an interesting observation regarding the use of rabbit serum was reported by Johnson and Harris (1967), where saprophytic leptospires maintained in the laboratory for a number of years were killed by low rabbit serum concentrations (2 to 10%). Additional investigations by Jonson and Harris (1967) of their assay yielded mixed findings. On the other hand, saprophytic *Leptospira*, recently isolated from surface waters, were frequently completely resistant to as much as 50% serum. At that time, researchers had no explanation for the observed difference (Johnson and Harris 1967).

Moreover, a recent study reported the successful propagation of *Leptospira biflexa* serovar Patoc in EMJH supplemented by 10% rabbit serum; thus, media supplemented by rabbit serum can successfully propagate both pathogenic and saprophytic *Leptospira* (Niloofa *et al.* 2021). Therefore, supplementation of media by rabbit serum might increase its susceptibility to colonisation by saprophytic *Leptospira*, which could be found in on-farm collected samples due to an increased risk of contamination from the environment or animals.

3.4.1.3 EMHJ and Newborn Calf Serum

Like rabbit serum, different concentrations of fetal bovine serum (FBS) or newborn calf serum (NCS) are often used as an optional media supplement. In this experiment, we used NCS as a cost-effective alternative to FBS. Results of this experiment showed that EMJH supplemented by 10% NCS failed to support *Leptospira* growth. After no *Leptospira* growth was detected in the initial batch of EMJH_NCS (Figure 2.2), another batch of EMJH_NCS was prepared and used to propagate Hardjo and Tarassovi cultures in stage 1a. However, the second batch also failed to grow *Leptospira*, and no viable bacteria were found in triplicate media samples by the end of the experiment. The same batch of EMJH was used for all EMJH-based media, including both batches of

EMJH_NCS; thus, the most likely reason for the failure to grow *Leptospira* by EMJH_NCS was NCS.

The failure of the EMJH_NCS medium to support *Leptospira* growth could be attributed to the possible presence of endotoxins, which are known to suppress bacterial proliferation. NCS (newborn calf serum) and FBS (fetal bovine serum) are both utilised as supplementary sources of fatty acids in cell culture media, but NCS typically contains higher levels of endotoxins, IgG, and overall protein content compared to FBS, therefore its regarded as "less pure" supplement (Cheever et al. 2017). The composition of these sera can vary depending on several factors, including the local strains of *Leptospira* that cattle or calves have been exposed to, either naturally or through vaccination. This exposure can lead to the presence of specific antibodies in the serum. Additionally, the age of the animals from which the serum is harvested plays a role, as sera from foetuses or young calves will contain maternal antibodies derived from vaccinated dams, which may also be present in commercial NCS. Despite all batches of NCS and FBS passing quality control, anecdotal evidence suggests that different serum batches can have varying effects on cell cultures. This variability is also observed with BSA (bovine serum albumin), where batches that appear equivalent may not be equally effective for *Leptospira* propagation. NCS was chosen for this study because it is more readily available and less expensive as it is harvested from calves less than 14 days old, whereas FBS is more costly and difficult to obtain as it is derived from foetuses.

EMJH_NCS_STAFF and EMJH_FBS_STAFF media may not be suitable for as primary media both isolation and maintenance of *Leptospira.* For example, Chideroli and co-authors reported successful isolation of *L. borgpetersenii* directly from the urine of infected bovines in EMJH using multiple passages of isolates alternating in three types of EMJH, where one of three media types was supplemented with FBS (Chideroli et al., 2017). Thus, it appears that additional investigation of EMJH_NCS or EMJH _FBS may help clarify if L. borgpetersenii can be cultivated directly in EMJH supplemented with newborn calf serum or fetal bovine serum under ^{m}Epilab conditions, or if these media may be only used as a passage media to adapt field strains of *Leptospira* to medium or may not be suitable to use for this purpose at all.

3.4.1.4 EMJH and commercial supplement

Distinct differences were found between the performance of EMJH supplemented with an in-house prepared BSA enrichment, and EMJH prepared with a commercially procured enrichment, as the latter did not support *Leptospira* growth. In contrast, a similar study investigated the ability of three types of EMJH (adapted enrichment, original enrichment, and commercial enrichment) and reported good performance of EMJH supplemented with commercially prepared enrichment (Guedes *et al.* 2022). The authors reported that it took *Leptospira* 3-5 days to reach a concentration of $\sim 2 \times 10^{8}$ *Leptospira*/ml in media supplemented with commercial enrichment, which was the shortest time of all tested media; the authors also did not report any contamination. Differences in the performance of EMJH enriched with commercial enrichment between the two studies could be attributed mainly to the variety of *Leptospira* used for EMJH enriched with a commercially prepared supplement, as Guedes *et al.* (2022) used 24 different *Leptospira* serovars, including *L. borgpetersenii* while the current study used two, *L. borgpetersenii* sv. Tarassovi str. Perepelitsin and sv. Hardjo str. 905.

Additionally, the source of BSA is also important, and it is very common for researchers to try various batches of BSA on their *Leptospira* cultures before purchasing the best-performing lot (Hornsby *et al.* 2020). The reason for variability between BSA batches performance may lay in varying amounts and types of long-chain fatty acids between BSA lots that may or may not match the specific growth requirements of particular *Leptospira* species. Therefore, it is possible that BSA in the commercially prepared enrichment used in this experiment was not fully compatible with *L. borgpetersenii*.

In this study, it was also found that EMJH_COMM was not suitable for propagating *L. borgpetersenii* sv. Tarassovi str. Perepelitsin and sv. Hardjo str. 905 under ^{m}Epilab conditions. However, if this was a batch-related issue, an alternate lot of commercial supplements may demonstrate better results. Nevertheless, in this experiment, *Leptospira* growth in this media was slow (Figures 3.1 and 3.2).

3.4.2 LTM

Results of our experiment showed that LTM_STAFF was one of the two best performing media used in this trial, the other being EMJH_RS_STAFF. In the absence of expensive supplements such as rabbit serum, NCS, FBS, or commercially manufactured EMJH supplements, LTM can be used as a less expensive medium for the propagation and long-term storage of *L. borgpetersenii* spp., as shown in this experiment. LTM was able to propagate *L. borgpetersenii* serovars Tarassovi and Hardjo, and concentrations of *Leptospira* in LTM reached $6x10^8$ *Leptospira*/ml for Tarassovi and $4x10^8$ *Leptospira*/ml for Hardjo. However, these results apply to the lab-adapted strains we used as a proxy for Pacifica, and *Leptospira* obtained from freshly voided urine may have different growth requirements.

In this experiment, LTM was evaluated by two methods. First was a manual enumeration of bacteria using the Petroff-Hausser counting chamber in stage 2, where LTM_STAFF was compared with liquid EMJH-STAFF media (Figures 3.4 and 3.5). In the second evaluation method, the growth of *Leptospira* in LTM_STAFF and semi-solid EMJH_AGAR_ STAFF was compared by direct DFM and visually by identification of the absence or presence of Dinger Zone. Additionally, LTM showed good resistance to contamination as no LTM cultures were discarded due to contamination in this experiment.

One of the observed characteristics of LTM_STAFF was that *Leptospira* Hardjo and Tarassovi propagated in this media reached their peak concentrations two weeks after seeding, or one week later than *Leptospira* seeded in EMJH_STAFF. The Dinger Zones appeared in all LTM_STAFF media samples two weeks after inoculation (Table 3.3). Similar observations were seen in other studies. For example, while it is common for *Leptospira* cultivated in liquid EMJH to reach its peak concentrations in 3-5 (Guedes *et al.* 2020) or ~ 10 days (Hornsby *et al.* 2020), in semi-solid media colonies and the Dinger zone can appear on the tenth (Hornsby *et al.* 2020) or in two weeks (Bromley and Charon 1979). However, it should be noted that the studies referenced above used different serovars and strains of *Leptospira* in their experiments; for example, (Hornsby

et al. 2020) used *L. borgpetersenii* serogroup Sejroe serovar Hardjo type bovis strain HB15B203 and *L*. *interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni. Guedes *et al.* (2022) used fourteen different serovars belonging to serogroups Australis, Autumnalis, Bataviae, Ballum, Canicola, Icherohaemorrhagiae, Cynopteri, Grippothyphosa, Hebdomadis, Javanica, Mini, Panama. Different concentrations of starting *Leptospira* cultures also may have affected the time to when the Dinger zone was observed. For example, Hornsby *et al.* (2020) used a starting concentration of 10^8 L*eptospira* /ml, the current study used 10^6 *Leptospira* /ml, while Guedes *et al.* (2020) seeded media with 3.7×10^4 *Leptospira* /ml; therefore, it appears that in this case in the cultures with highest concentrations took longest for Dinger Zone to appear,

Another unexpected characteristic of LTM_STAFF was its ability to maintain *Leptospira* long-term. LTM media is generally acknowledged as a short-term storage solution for cultures; however, our experiment indicated that it could also support cultures long-term. After one-year post-inoculation at 28 °C, LTM media cultures still contained viable *Leptospira* at a concentration of $3x10^4$ (Table 3.3). Another type of media, specifically, LWV agar, was reported to have a long-term ability to maintain *Leptospira* (Wuthiekanun *et al.* 2014). The authors were able to preserve *Leptospira* for 12 months at room temperature; however, the dryness of agar was also observed, which may have negatively affected *Leptospira* survival times. Interestingly, LWV agar is a solid media, and its composition is dissimilar to LTM; it contains 10% Rabbit serum, sodium pyruvate, and 10% Nobel agar, while LTM includes 1% Nobel Agar, 10% BSA, salts, Tween 80, and the Phenol Red pH indicator. Besides, LTM cultures were kept at 28º C, while LWV agar sat at room temperature. However, despite some differences in composition and concentration of reagents, both media showed the same ability to preserve *Leptospira* for 12 months.

3.4.3 HAN Media

In this experiment, HAN media failed to support the growth of *L. borgpetersenii*. When the first batch of HAN media failed to grow any *Leptospira* at 28° C (Figure 3.5), the second batch of HAN media was prepared and seeded with Tarassovi and Hardjo cultures alongside other media types. This was done for repeatability purposes and to exclude any human error that may have affected the first batch. However, the second batch of HAN media also failed to grow *Leptospira* (Figure 3.5), and no viable bacteria were detected on the 18th day post-inoculation. While the results of these two inoculation rounds are consistent, the failure of HAN media could not be attributed to a single definitive cause. In a protocol for a novel HAN media published in 2020, authors described HAN media as suitable for direct isolation of *Leptospira* from the host (experimentally infected golden hamsters) at either 28°C or 37 °C in both semi-solid and liquid compositions (Hornsby *et al.* 2020). HAN media contains essential amino acids and other reagents also found in EMJH. In 2022, the successful isolation of *L. borgpetersenii* serovar Tarassovi str. Perepelitsin from the urine of dairy cows using HAN media at 38°C and 5% $C0_2$ was reported (Hamond *et al.* 2022b), and similarly, the isolation of *L. borgpetersenii* in HAN media at 38°C and 5% $C0_2$ from the urine of dairy cows in Puerto Rico was also reported (Hamond *et al.* 2022a).

In our experiment, the only modification to the HAN media preparation protocol was hemin replacement with its analogue, ferric sulphate, as both compounds have the same functionality and are used interchangeably for *Leptospira* media preparation (Loureiro *et al.* 2015; Hornsby *et al.* 2020; Guedes *et al.* 2022; Hamond *et al.* 2022b). Apart from the hemin replacement, no other modifications were made. Hornsby *et al.* (2020) used cultures of L*. interrogans* serovar Copenhageni and *L. borgpetersenii* serovar Hardjo, and in the current experiment, we used *L. borgpetersenii* serovars Tarassovi and Hardjo, therefore serovar Harjo was used in both experiments. Hence, the HAN failure was unlikely caused by inoculation of a non-compatible species of *Leptospira*. However, the reported successful isolations of *L. borgpetersenii* from the urine of dairy cows in HAN media both used 38°C and at 5% $C0_2$ growth conditions and did not use STAFF(C. Hamond, *personal communication,* November 5, 2022);

therefore, HAN media's failure to support *Leptospira* growth in this study could be attributed to the use of STAFF or temperature conditions or maybe some other unknown factors. For future research, *L. borgpetersenii* growth in the HAN media at 38°C and 5% $C0_2$ with no STAFF supplement will need to be evaluated, as this particular combination of media and growth conditions was proven to be successful for direct isolations of *L. borgpetersenii* from cows' urine.

3.4.4 Effect of Antimicrobial Agents: STAFF and 5FU

The results of our experiment showed that in stage 1, EMHJ_RS_STAFF was superior to other types of EMJH by all three media selection criteria. This media, supplemented by antimicrobials STAFF and 5FU, showed an excellent ability to resist contamination and propagated both Tarassovi and Hardjo to concentrations of ~10^9 *Leptospira*/mL; this media also supported *Leptospira* growth for at least two weeks.

Currently, two popular antimicrobial combinations are used to suppress contaminants in *Leptospira* media. First is STAFF (Chakraborty *et al.* 2011), and second is an antibiotics combination by Schönberg (1981). For this experiment, STAFF was selected as the preferred option, and the antimicrobial composition recency mainly determined this selection. STAFF combination was introduced relatively recently compared to Schoenberg's combination. Since STAFF testing and validation in 2011, contaminating bacteria may not have developed resistance to this particular combination. However, given the length of time, since Schönberg's (1981) published antimicrobial composition, some bacteria may have developed antibiotic resistance to those antimicrobials. Nevertheless, the use of the antimicrobial formulation as described by Schönberg was necessary for the successful isolation of *Leptospira* serogroup Serjoe serovar Hardjo (Chideroli *et al.* 2017).

STAFF is a more commonly used "antibiotic cocktail' for suppressing contaminants in EMJH media used for the isolation of *Leptospira* from the urine of cows (Zacarias *et al.* 2008; Loureiro *et al.* 2015). In this study, all media was supplemented by STAFF in stages 2, 3, and 4 to increase media resistance to contamination, and as a result, supplemented media outperformed media not supplemented by STAFF. similar findings

were reported by a study where media supplemented by STAFF was successfully used to culture 23 isolates from 50 urine samples collected directly from the bladders of 50 slaughtered cattle was published by Loureiro and Lilenbaum (2020). Isolates were typed as *L. borgpetersenii* serovars, Hardjo, and Serjoe. The authors also noted that while 91% of media tubes presented occasional contamination, only 1.6% presented moderate contamination. Therefore, while occasional contamination is common and all precautions are usually taken to avoid it, expectedly, the occasional contamination levels appeared to be much higher in field samples. However, it has also been reported that STAFF completely inhibited the growth of *L. borgpetersenii* in EMJH in the study where field strains, including *L. borgpetersenii*, were seeded in different types of media, and their growth was estimated daily for fifteen days (Loureiro *et al.* 2022). Therefore, while the EMJH+STAFF combination is commonly used and was one of the top performers in this study, it may not be equally effective for all *Leptospira;* specifically, STAFF may slow or suppress *Leptospira borgpetersenii* growth, but this is not has been observed in the current study, probably due to the adaptation of laboratory cultures to STAFF.

Another commonly used contaminant suppressor is 5FU, and in this study, all media was supplemented with 5FU to increase resistance to contamination. While most researchers report successful isolation and cultivation of *Leptospira* supplemented by 5FU (Thibeaux *et al.* 2018; Cordonin *et al.* 2020), Mohamad Safiee *et al.* (2020) reported improved rates of *Leptospira* isolation in EMJH without 5FU from blood samples taken from febrile patients in North-Eastern Malaysia. In their study, the authors compared leptospiral growth rates in EMJH with and without 5FU and reported a day lag in cultures grown in EMJH 5FU. This study may have found 5FU less useful because authors could control sample collection and inoculation conditions; blood samples were collected in a hospital environment and inoculated under laboratory conditions shortly after. Another reason for not seeing a day lag was that specimens collected from febrile patients may have had a higher bacterial load. When collected from acute patients, blood samples are presumed sterile or may only contain infectious agents. Therefore, 5FU may have been unnecessary in this case as aseptic sampling conditions and sample type already minimised primary contamination sources. From

these examples, it is evident that researchers presently use 5FU as a single add-on antimicrobial or STAFF as a combination of five antimicrobials to isolate *Leptospira* from the urine of dairy cows successfully; however, these may not be efficient for all types of *Leptospira*, as shown by Hammond et al. (2022). While the success of culturing *Leptospira* is directly related to the sample source, incubation temperature, and media components, control of contaminating microorganisms from the environment or animal's microbiota is also important (Cameron 2014).

3.5 Study Limitations

The present study's aim was to examine the ability of different types of media to propagate *Leptospira borgpetersenii* spp. as a proxy for str. Pacifica. Firstly, the most important limitation of this experiment was that we used two laboratory-adapted strains of *Leptospira* in this study. We also included two field strains, but one can argue that they can be acknowledged as laboratory adapted as they were propagated under laboratory conditions for several years. Therefore, all *Leptospira* cultures used in this experiment can be considered laboratory-adapted strains and may have an affinity to specific media, supplements, and growth conditions.

Secondly, a media-related limitation was a possible selective bias and affinity of *Leptospira* towards EMJH as it is used for routine *Leptospira* maintenance in ^{m}Epilab. While EMJH was the best-performing media in this experiment, the field strain may not necessarily grow in EMJH. However, the EMJH used in ^{m}Epilab has been successfully used to isolate *L. borgpetersenii* serovars Hardjo, Ballum, and Balcanica from field samples from cattle, sheep, rodents, possums, and deer (Subharat *et al.* 2011; Fang *et al.* 2017; Vallée *et al.* 2017; Ryan 1978).

3.6 Conclusions

For future studies, the use of HAN media at 37C° and 5% CO_2 for *Leptospira* isolation from field samples needs to be investigated as new reports show HAN media efficiency for isolating *Leptospira borgpetersenii* from the urine of dairy cows and questioned the use of STAFF for this particular genomospecies.

3.7 References

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CHAPTER 4

Chapter 4 Epidemiology of *Leptospira borgpetersenii* strain Pacifica in New Zealand Primiparous Dairy Cows during 2020-2022

4.1 Abstract

Aims: This study aimed to evaluate *Leptospira borgpetersenii* strain (str.) Pacifica's shedding pattern and its persistence in high-risk str. Pacifica dairy herds in the 2020-2022 milking seasons.

Materials and Methods: Blood and urine samples were collected from primiparous dairy cows at three different time points: early and late in the milking season (N=16), followed by a one-time collection during the next season (N=1). The blood samples (N= 1364) were tested using the Microagglutination test (MAT) to detect antibodies against *Leptospira borgpetersenii* serovar Tarassovi as a proxy for str. Pacifica, and four others endemic serovars. DNA was extracted from the urine samples (N= 564) and typed using PCR targeting *glmU* genetic loci. The amplicons were compared to known sequences to determine the presence of str. Pacifica shedders. Descriptive analysis was conducted on the geometric mean titre (GMT), seroprevalence, and urinary shedding prevalence.

Results: The results of this study showed that in 2020, in sixteen high-risk herds, Tarassovi seroprevalence was 69% (95 CI: 24.6-81.9), and 63% (95% CI:31.6- 87.6) of herds had at least one animal shedding str. Pacifica. Comparison between Tarassovi seroprevalence and urinary shedding in the same animals, groups, and herds at the start and the end of the milking season showed that while the Tarassovi seroprevalence was very similar at 41.9% (95%CI: 37.3-46.6) and 48.7% (95%CI: 43.7-53.6), the Tarassovi geometric mean titre (GMT), however, was 13% lower at the start of the milking season at 316 (95%CI: 259.1-384.2) in comparison with the end of the season at 357 (95%CI:293.9-433.5).

Of 564 urine samples, 52 Pacifica shedders were confirmed by amplicon sequencing. Moreover, the estimated prevalence ratio (PR) was seven times higher at the start of

the season than at the end. The association between Tarassovi seropositivity and Pacifica shedding was statistically significant ($p \leq 0.05$) in all sampling stages.

Conclusions: This study's main finding provided further evidence of str. Pacifica host maintenance status in dairy cows by showing the continuity of str. Pacifica shedding in high-risk herds. Furthermore, we identified that in the same herds, there were more str. Pacifica shedding dairy cows at the beginning of the milking season compared to the end.

Clinical relevance: Veterinarians and farmers are updated on the epidemiology of str. Pacifica in dairy herds and on the serovar-host relationship between str. Pacifica and dairy cows in New Zealand.

Abbreviations MAT: microscopic agglutination test, GMT: geometric mean titre; PCR: polymerase chain reaction.

Keywords: *Leptospira borgpetersenii,* Pacifica, epidemiology, endemicity, dairy cows, New Zealand

4.2 Introduction

Leptospirosis is the most widespread spirochaetal zoonotic disease caused by pathogenic *Leptospira* from the *Leptospiracae* genus (Vincent *et al.* 2019). The international nomenclature recognises >71 species of *Leptospira* described in the past 20 years due to the advancement of genome sequencing technologies (Korba et al. 2021), and a convenient serological classification includes over 300 serovars *(Thibeaux et al. 2018)*. Serological MAT is considered a more serogroup-specific test than species-specific (Levett 2003). Conversely, genomic classification is the most precise method of typing *Leptospira* species. Nevertheless, while genomic and serological classifications still co-exist, they do not match well (Picardeau 2017).

Most mammalian species, including humans, are susceptible to leptospirosis, and a person can get infected after contact with an infected animal's urine or contaminated

environment (Haake and Levett 2015). Additionally, renal colonisation by *Leptospira* has been reported in several non-mammal species, such as birds and reptiles (Picardeau 2017). Specific serovars are adapted to specific hosts, often identified in the literature as "maintenance hosts", a species where the infection is endemic and is usually transferred from animal to animal by direct contact (Levett 2001). Maintenance hosts are essential links in the transmission of *Leptospira*. However, endemic serovars can also infect non-serovar-adapted or "accidental hosts".

In New Zealand, there are six pathogenic *Leptospira* serovars; bovines are considered maintenance hosts of serovars Hardjo, pigs of Pomona and Tarassovi (Marshall and Manktelow 2002), rats and mice of Copenhagen and Ballum (Moinet 2020) and possums of Balcanica (Hathaway *et al.* 1981). The occupational nature of leptospirosis in New Zealand has been long established, and people having day-to-day professional contact with animals are considered most at risk, with dairy beef and sheep farmers having the most at-risk occupations (Marshall and Manktelow 2002; Dreyfus *et al.* 2015; Benschop *et al.* 2021). In the 1970s, the incidence of human leptospirosis was the highest at 30/100,000 (Marshall and Manktelow 2002); by 1981, it had dropped to 3.0/100,000 (Thornley *et al.* 2002), and by 1995 it further declined to 1.4/100,000 (El-Tras *et al.* 2018). This decrease was mainly attributed to the implementation of vaccination programs against Pomona and Hardjo in dairy cattle and Pomona and Tarassovi in pigs in the early 1980s. However, a shift in the proportion of the notified serovars was observed, and the incidences of serovars not included in commercially available livestock vaccines, hereafter referred to as non-vaccine serovars, Ballum and Tarassovi, increased (Thornley *et al.* 2002; Moinet *et al.* 2017; Yupiana *et al.* 2019; Nisa *et al.* 2020; Wilkinson *et al.* 2023). Moreover, among cases notified from 1 January 1999 to 31 December 2016, the highest incidence of Tarassovi at 12.59/100,000 was reported amongst dairy farmers (Nisa *et al.* 2020). Thus, the rise in human Tarassovi cases is causing a concern.

The emergence of Tarassovi was noted in dairy cows (Yupiana *et al.* 2019). Serosurveys of apparently healthy cattle in New Zealand showed that over the past fifty years, Tarassovi seroprevalence increased from 6% (50/300) at MAT ≥ 50 (Ryan and Marshall 1976) to 18% (698/3,878) at MAT ≥ 48 (Wilson *et al.* 2021). More recently, a

serology and urine shedding study from 2015-2016 evaluated the status of 4,000 dairy cows. Cows were randomly selected from 200 farms and stratified by New Zealand's geographical location and herd size (Yupiana *et al.* 2019). The study found that on the animal level, 17% of the study animals showed evidence of past infection with Tarassovi, as defined by at least one sample reacting at MAT ≥ 48 for the study's purposes. Moreover, 68% (54/80) of urine samples from the cows that were shedding were sequence-typed using amplicon sequencing. Sequence data indicated the presence of *Leptospira* species *borgpetersenii*, referred to as *Leptospira borgpetersenii* str. Pacifica (Pacifica) (Wilkinson *et al.* 2021). Further analysis showed a statistically significant association ($p < 0.01$) between urinary shedding and seropositivity to Tarassovi, and it was suggested that MAT titre ≥ 48 might indicate active urinary shedding. Because of this two-fold association, str. Pacifica is thought to belong to the serogroup Tarassovi (Wilkinson *et al.* 2023). Therefore, when identified by serology only, Tarassovi infections in New Zealand animals and humans could at least partially be attributed to str. Pacifica. The isolation of str. Pacifica by culture is essential for its characterisation and understanding of the epidemiology and disease transmissibility.

Informed by the increased incidence of Tarassovi in humans and dairy cows, we hypothesise that str. Pacifica is endemic in New Zealand dairy cows and maintained within the herds. Thus, the specific aim of this study was to evaluate the shedding pattern and persistence of str. Pacifica in high-risk dairy herds in 2020-2022.

4.3 Methods

4.3.1 Identification of Farms and Sample Size Calculation

This study design was based on the findings of the 2016 cross-sectional study of 200 dairy herds (Yupiana et al. 2019). From these, in a subset of 35 herds, a herd was identified as high-risk if any of the 20 cattle urine samples were either positive to Tarassovi by serological test (MAT ≥48) and/or positive by qPCR with Ct values less than or equal to 40, using *gyrB* and *glmU* as a target gene for *Leptospira borgpetersenii* str. Pacifica DNA. The sample size was calculated to estimate shedding prevalence in

high-risk herds and is based on previous survey results (20% seropositive, of which 15% are urine-positive) and an assumption that 1 in 20 urine PCR-positive animals may generate an isolate in pure culture (Yupiana *et al.* 2019). Thus, 1,000 blood-sampled animals were identified as the minimum to obtain 1 isolate. The following equation was used to calculate sample size: n= (1,000 x 20% x 15% x 5%).

In 2022, an additional herd, not sampled previously, was added to this study. The motivation to include the additional herd was to utilise a HAN media (Hornsby *et al.* 2020) for field isolates, as successful isolation of Tarassovi in HAN media from the urine of dairy cows was reported, before this sampling (Hamond *et al*. 2022). This herd was categorised as high-risk as per the criteria above, because of Tarassovi MAT titres ≥48, and a two-fold increase in MAT titres between March 2022 and April 2022, as reported by the farm veterinarian. Moreover, this farm was associated with a human case of Tarassovi leptospirosis.

4.3.2 Ethical Considerations

The Massey University Animal Ethics Committee (MUAEC) granted study approval, Protocol 20/11 (and Amendment to Protocol 20/11).

4.3.3 Recruitment of Farms and Veterinary Clinics

Dairy herds identified as high-risk (n=35) were contacted via e-mail, post, and personal phone calls addressed to the farm owner/manager. From these, approximately two-thirds (n=15) of the farm owners were no longer farming, and four farmers did not want to participate in the study due to the staff shortage and upcoming farm sales. Thus, 16 high-risk dairy farms identified from the previous study were enrolled in this study. Next, farmers were asked to nominate their preferred veterinary practice for sample collection. Then, once all fifteen nominated veterinary practices agreed to participate in the study and a key veterinarian or veterinary technician was designated to the job, veterinarians contacted their clients to confirm sampling dates. Following that, sampling instructions and collection kits were sent out. An additional dairy farm was enrolled in the study via.

its veterinarian, who arranged the sampling times and dates with the owners and helped with animal sampling.

4.3.4 Study Design

Samples were collected at temporally distinct points, early and late in the milking season, followed by one-off sampling (Figure 4.1). A three-stage sampling strategy was developed for the early lactation sampling (July-Oct 2020) (Figure 4.1). During stage one, blood was taken from 1,364 primiparous dairy cows to identify animals seropositive for Tarassovi at MAT ≥ 48. At stage two of early lactation sampling, urine samples were collected from the MAT-positive (MAT ≥ 48) Tarassovi animals identified in stage one. During stage three, the collection of kidneys was organised from any animals sent to abattoirs from MAT and PCR-positive herds identified at stages one and two (Figure 4.1). The kidneys were cultured and tested by PCR. These cows were culled for various reasons, mainly reproductive and milking, but the exact reasons for culling were not available.

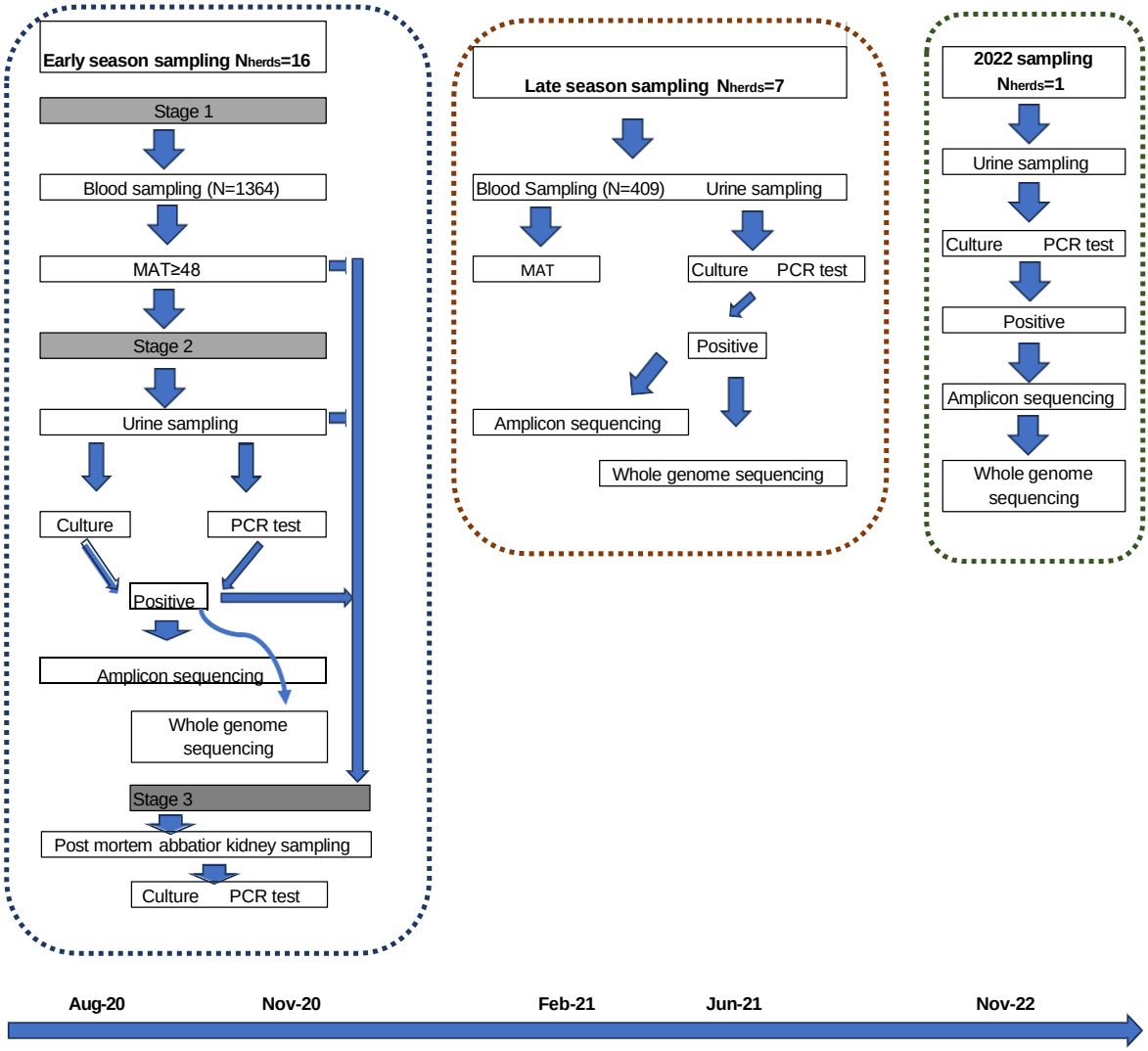

Figure 4.1 The flow diagram showing sampling stages, the number of samples collected, the testing flow, and the test types used for a study of *Leptospira borgpetersenii* str. Pacifica epidemiology in 17 New Zealand dairy herds (2020-2022).

In addition, farmers were asked if they would like to participate in late season (February-June 2021) testing at the end of a milking season, which involved blood and urine sample collection targeting dairy cows previously sampled earlier in the season. The difference between early and late lactation sampling was that blood and urine were collected simultaneously at the latter. Additionally, fewer farms participated in the late lactation sampling. Therefore, the staged approach for late-season sampling was unnecessary; however, the sequence of the testing steps was the same as at the early-season sampling (Figure 1). For the 2022 sampling, which was carried out on one farm only, urine samples were collected from primiparous cows with MAT >= 48 for Tarassovi (Fig. 4.1). Sera collected from these animals were tested by the farm`s veterinarian through a commercial veterinary laboratory prior to this study.

4.3.5 Sample Collection

Blood and urine samples were collected after milking in the yard or on the milking platform. A minimum of 10 ml of blood was collected into plain Vacutainter® (red top) by caudal vein venipuncture. Urine samples were collected in a 50 ml clear plastic, screw-top container, with urination induced by stimulating the ventral vulva. Before urine collection, each animal was cleaned by wiping off faecal material with disposable wet wipes. Animal details were recorded on the submission form and duplicated by cow number on blood and urine vials. Urine samples were immediately placed into a cool insulated box and were separated from ice packs by a layer of insulation material to prevent freezing. Upon completing on-farm sample collection, a disposable syringe was used to seed 100 µl of each urine sample aseptically into four types of media in duplicate that had been pre-made under laboratory conditions. During this process, urine samples were taken from the chilled environment for two minutes and placed back immediately after inoculation. Blood samples were kept at ambient temperature (~18-20ºC) during collection and transportation. Blood samples arrived at the laboratory within 24 hours of collection, and urine samples within 10 hours. Kidney sample collection by AsureQuality from animals sent to abattoirs from high-risk farms was overseen by the Ministry for Primary Industries (MPI) verification services and

dispatched to the *m*EpiLab within five days after collection. Kidney samples were kept chilled while waiting for dispatch.

4.3.6 Laboratory Procedures

4.3.6.1 Microscopic Agglutination Test (MAT)

All collected samples were processed at the *m*EpiLab, on the first day after collection. Blood samples were centrifuged at 3,000 *g* for 10 minutes, and sera were collected as supernatant. Thirty microliters of each serum were mixed with 150 µl standard saline into 96-well plates as a master plate to make 1/6 dilution for testing. The master plates were then stored at -20°C. The remaining sera were stored at -80°C. All serum samples were tested by MAT using the standard laboratory strains of Tarassovi, Ballum, Copenhageni, Pomona, and Hardjo cultures. Positive control antisera were sourced from the World Organisation for Animal Health (WOAH) Reference Laboratory for Leptospirosis. The MAT method was based on the method described by Faine (Faine 1982). From laboratory stock cultures, test cultures were prepared and used for MAT when the concentration of each strain of *Leptospira* reached ~1x10^8. Eight two-fold dilutions (inclusive of antigen) from 1/24 to 1/3072 of sera using standard saline as the diluent were prepared in 96-well flat-bottomed plates. Positive and negative controls were prepared using the same method, with standard antisera used for positive control and saline used for negative control. Plates were then incubated at 37°C for two hours and read under a dark field microscope (Olympus BH2, Tokyo, Japan); the endpoint titre was determined by agglutination of 50% leptospires at the lowest dilution. MAT results were reported to veterinarians, who then reported it to farmers as soon as possible over the phone and via e-mails, and a follow-up urine testing was arranged for the animals with Tarassovi MAT titres ≥48.

4.3.6.2 Bacterial Culture

Four different types of media prepared at the *m*EpiLab were used to culture urine samples. Specifically, the Ellinghausen-McCullough-Johnson Haris (EMJH) medium was made as described by (Ellinghausen Jr and McCullough 1965), modified by (Johnson and Harris 1967), and some EMHJ was also supplemented with 10% rabbit

serum (Faine 1994). *Leptospira* transport medium (LTM) was prepared as described by (Faine 1994) and updated by Guedes *et al.* (2020), and Hornsby, Alt, and Nally (HAN) media was prepared as described by Hornsby *et al.* (2020). All media and cultures, but HAN and cultures seeded in HAN media, were supplemented by STAFF (Chakraborty et al. 2011). It consists of sulfamethoxazole (S), trimethoprim (T), amphotericin (A), fosfomycin (F), and 5FU (F).

Immediately after on-farm collection, 100 µl of freshly collected urine samples were seeded in duplicate into three media types (EMHJ, alternatively EMJH 10% RS, LTM, and HAN) as mentioned above. The remaining urine samples collected on-farm were chilled and taken to ^{m}EpiLab, where the urine was centrifuged at 10,000 *g* for 30 minutes, the supernatant was discarded, and sediment was collected and divided for media inoculation and DNA extraction.

On-farm seeded media samples (sample A) were kept at room temperature. Upon delivery to the ^{m}EpiLab laboratory, 100 µl of sediment was seeded into three media types in duplicate (sample A) under laboratory conditions and incubated at 28°C. On the next day, after initial inoculation of on-farm and in-laboratory urine samples (sample A), two serial dilutions were made using sample A from each seeded tube, where 100 µl were transferred to another tube containing 9 ml of the same media (sample B), followed by another 100 µl to the last 9 ml of media (sample C). All EMJH and LTM seeded media were kept at 28°C. Seeded HAN media was kept at 38°C, and 5%CO_2 was on the shaking platform at 30 RPM. Seeded media was examined weekly for the first four weeks, fortnightly for another four weeks, and then monthly. It was declared negative and discarded if no *Leptospira* were detected under dark field microscopy after six months.

Kidney samples were dispatched to Massey University's ^{m}EpiLab within a maximum of 5 days after collection. Upon arrival at the ^{m}EpiLab laboratory, 10-20g of kidney sectioned from renal cortex to medulla was removed aseptically with a single-use sterile scalpel blade. Then, this section was doused with 70% ethyl alcohol, and the residual surface alcohol was removed by flaming with a Bunsen burner. It was then homogenised in 50-

100 ml sterile Phosphate Buffered Solution (PBS) using the Colworth Stomacher 400 (AJ Seward Ltd, London, UK). A 160 µl of a kidney slurry was used for DNA extraction. After processing (sample A), another 100 µl aliquot of this suspension was immediately seeded into four types of media. Then, 100 µl of sample A was subbed into the corresponding media labelled sample B and incubated at 28°C. Additionally, 2 ml of each kidney suspension was stored in the -80° C freezer.

4.3.6.3 DNA Extraction and PCR Testing

The DNA from urine (100 µl) and kidney (160 µl) samples were extracted using a QIAamp DNA mini kit®, per the manufacturer's instructions (Qiagen, Bio-Strategy Ltd, Auckland, New Zealand). After extraction, DNA samples were stored at -20°C. A conventional PCR assay was performed targeting the UDP-N-acetylglucosamine pyrophosphorylase (*glmU*) gene locus, the targeted amplicon length of which is 567 base pairs (bp) (Wilkinson *et al.* 2021). 5 x HOT FIREPol® Master Mix 15 mM Mg 1ml (Solis BioDyne OÜ, Tartu, Estonia) was used for DNA amplification. The 20 µl total reaction volume also contained 8.2 µl PCR-grade water (Invitrogen UltraPure™ Distilled Water, Thermo Fisher Scientific, Auckland, New Zealand), 5 µl of *glmU*_DW_forward primer F 5'-CCC GTA TGA AAA CGG ATC AGC C-'3' (*glmU*_DW), 5 µl *glmU*_DW reverse primer_R 5'-ATT CTC CCT GAG CGT TTT GAT TTC-'3' (*glmU*_DW) (Wilkinson *et al.* 2021) and 5µl of the DNA sample. Primer concentrations of PCRs were 400 nM. The thermal cycle conditions on the SensoQuest labcycler (Gottingen, Germany) consisted of an initial denaturation step at 95°C for 5 minutes, followed by five cycles of 95°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 1 minute and another 50 cycles of 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 7 minutes as described by Wilkinson *et al.* (2021).

4.3.6.4 PCR Purification and Amplicon Sequencing

DNA products were analysed by gel electrophoresis in a 1% agarose gel at 110 mA for 30 minutes. The gel was stained with a 1kb ladder (Invitrogen ®, Thermo Fisher Scientific), positive control of laboratory culture of Tarassovi validated by MAT and Whole Genome Sequencing (WGS), and PCR-grade water (Nuclease-free water,

Thermo Fisher Scientific, Auckland, New Zealand) was used as the negative control. Gels were read under the Molecular Imager® Gel Doc™ XR transilluminator, and gel images were saved and annotated. The positive gel bands closely matching 567bp size were excised from the gel and immersed overnight in a 50 µl elution buffer sourced from QIAamp® DNA mini kit (Qiagen). Eluted DNA product was submitted to the Massey Genome Services in sterile DNAse and RNAse free 0.2 mL SnapStrip® II PCR tubes (SSIbio, Lodi, CA, USA). Each tube contained 20 ng/µl and either 4 pmol/µl *glmU* primer DW_F or 4 pmol/µl glmU_DW_R primer. Massey Genome Services used a Sanger-based sequencing method, in which samples were prepared with BIgDye™ Sequencing Ready Reaction mix; then the XTerminator® system was used for cleanup after cycle PCR sequencing by separation using the ABI3730 DNA Analyzer. The resulting sequence data were aligned with control sequences for serovars endemic in New Zealand, including str. Pacifica using the bioinformatics software Geneious 10.2.6. Unknown sequences were checked against known sequences using the nucleotide Basic Local Alignment Search Tool (BLASTn) available at the National Center for Biotechnology Information (NCBI), part of the United States National Library of Medicine website, assessed in January 2022 (http://www.ncbi.nlm.nih.gov/BLAST). The sample was denoted as "confirmed positive" if its sequence matched str. Pacifica. Results from positive gel bands were denoted as "unconfirmed positive" if the sequence quality was low but identifiable as *Leptospira,* according to the NCBI database.

4.3.7 Statistical Analysis

Statistical analysis was performed using R version 4.1.0 (2021-05-18) 'Camp Pontanezen' (R Core Team, 2021). The percentage of PCR-positive results in the seropositive animal group was calculated by dividing the number of confirmed positive animals by the number of seropositive animals. Similarly, to calculate the number of PCR-positive animals in seronegative animals, the number of confirmed PCR animals from the seronegative group was divided by the total number of seronegative animals. This calculation was performed for each serovar for early and late lactation season. Geometric mean titre (GMT) was calculated for MAT-positive samples. Welch Two Sample t-test was used to compare the GMT between the start and end of the season

sampling. Pearson's Chi-square with Yates continuity correction was applied to compare the proportion of positive to PCR and MAT between herds and sampling times. The 95% confidence intervals for proportions were calculated using the Wald interval for a binomial proportion. Kappa statistics were used to assess the pairwise agreement between serovars. The interpretation for the kappa statistic is as follows:<0.2 slight agreement, 0.2-0.4 fair agreement, 0.4-0.6 moderate agreement, 0.8 almost perfect agreement (Viera and Garrett 2005). Urinary shedding prevalences were estimated by dividing the number of Pacifica-positive animals, as confirmed by amplicon sequencing, by the total number of urine-sampled animals. In addition, the prevalence ratio (PR) was calculated to compare shedding prevalence between early-season and late-season The confidence intervals for PR were calculated by the Clopper-Pearson method (Clopper and Pearson 1934). Analyses were performed using R with the packages epiR (Stevenson *et al.* 2018), tidyr (Wickham *et al.* 2019), and ggplot2 (Wickham 2011). Statistical significance was set at $p \leq 0.05$.

4.4 Results

4.4.1 Farms and Veterinary Clinics

From the total of seventeen high-risk farms, sixteen farms were enrolled in the early sampling at the start of the milking season; from these, six farms participated in the late lactation sampling round at the end of the milking season, and one additional farm participated in the later sampling (November 2022). Geographically, from 13/17 of the North Island farms, five were in the Northland region, three in Waikato, and one each in the Hawkes Bay, Bay of Plenty, Manawatu, and Taranaki regions (Figure 4.2). Three South Island farms were in Otago, and two were in Canterberry regions. Blood and urine samples were collected by sixteen veterinary practices local to farms; one of the practices had two participating farms as their clients. All farms had vaccination programmes in place, and on all participant farms, animals were vaccinated at least three months before sampling. However, on one of the farms, the last vaccination was recorded more than one year before this sampling, and at one other farm, not all animals were vaccinated. It is important to note that detailed vaccination records were

not collected in this instance.

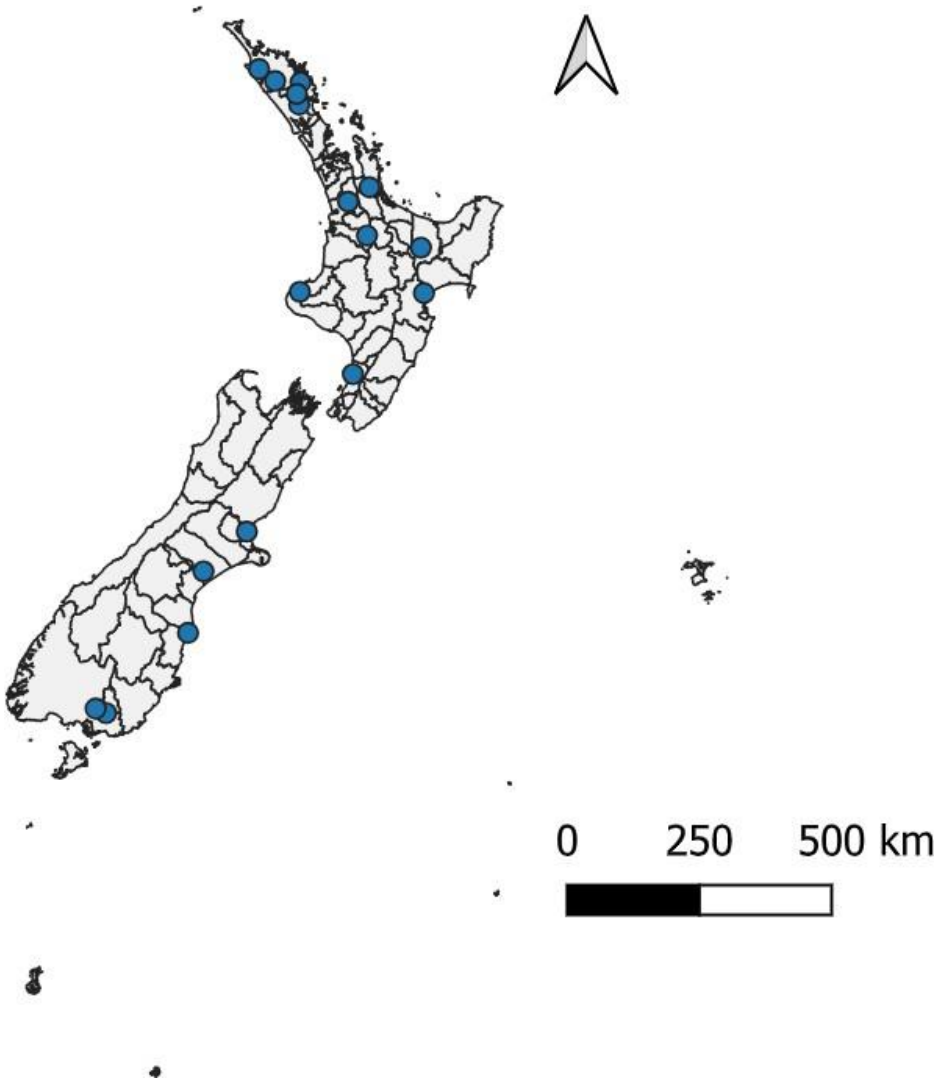

Figure 4.2 A map of New Zealand showing the geographical locations of participating farms denoted by blue dots (N=17).

4.4.2 MAT and urinary shedding at the early, late season and 2022 sampling.

On a herd level, from sixteen high-risk farms that participated in blood sampling, eleven farms, or 69% (95 CI: 25-82), were positive for Tarassovi, where at least one animal tested at MAT≥ 48 in the 2020-2021 milking season. Of seventeen farms that participated in the urine sampling, nine or 53% (95% CI:32-88) had at least one str.

Pacifica shedding animal, identified by conventional PCR test and DNA amplicon sequencing.

On the animal level, 1,424 dairy cows were sampled, and 1,324 serum samples were tested by MAT (n= 6,640, Figure 4.3) with five standard laboratory cultures used as antigens. From the total number of serum samples, 455 (34.3%;95% CI: 31.7 - 36.9) of cows were MAT positive (≥ 48) to Tarassovi (Table 4.1, Figure 4.3), 403 or 30.5% (95% CI: 28.0 - 33.1) to Pomona, 95 or 7.2% (95% CI: 5.9 - 8.79) to Hardjo, 79 or 5.9 % (95% CI: 4.6 - 7.3) to Copenhageni, and 31 or 2.3 % (95% CI: 1.43 - 3.1) cows were seropositive to Ballum. There were 59.8% (95% CI: 57.1 - 62.4) or 794 dairy cows positive for at least one serovar. Pairwise comparisons between serovars were evaluated as independent as their corresponding Kappa values were <0.1. Pairwise comparisons of serovar pairs such as Pomona and Hardjo were also independent at <0.1. As herds were vaccinated at least three months before sample collection, vaccination titres were low, and no Pomona PCR shedding was detected.

In total, 564 dairy cows had urine samples collected; 504 of these were sampled in early and late lactation of the 2020/2021 milking season. The remaining 60 samples were at the 2022 one-off sampling event (Table 4.1). The total number of urine PCR-positive samples identified as unconfirmed positive was 82 or 14.5 % (95% CI: 11.8% – 18.2%). Of these, 52 or 9.2% (95% CI: 7.2 – 12.52) shedders of the str. Pacifica were confirmed by amplicon sequencing using the PCR assay targeting the *glmU* locus (confirmed positive). The estimated PR was seven times higher at the start of lactation at 27.3 (95% CI: 19.8-36.3) than at the end and 3.9 (95%CI: 2.3-6.5), respectively.

Of the 62 kidney samples, none were PCR-positive. None of the 2,858 EMJH and LTM media samples seeded with urine were positive, although one sample was positive by dark field microscopy observation in freshly collected urine. However, this positive sample did not result in a positive culture.

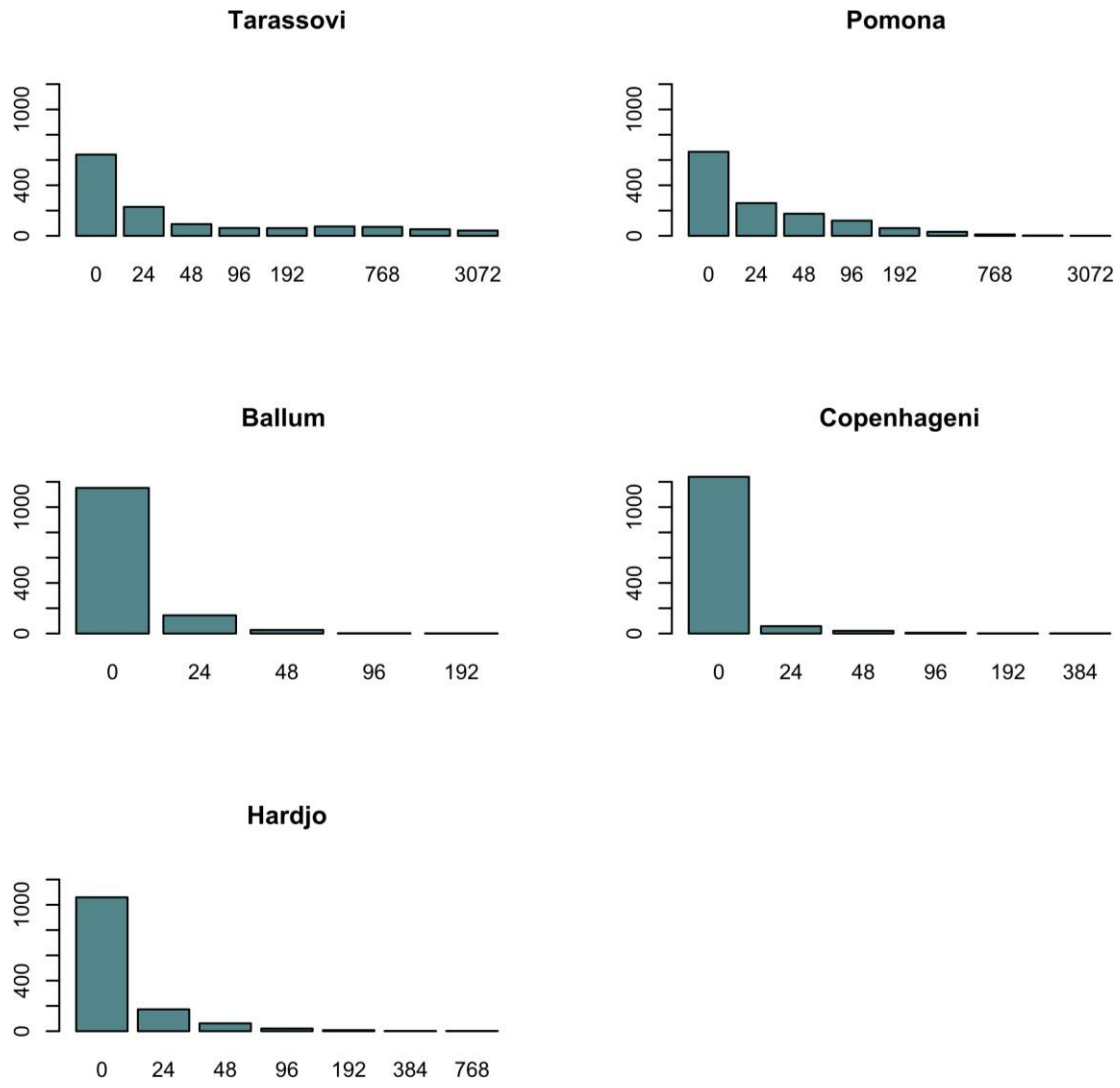

Figure 4.3 Bar graphs showing the distribution of MAT titres for five different serovars. MAT titres are shown on the x-axis, and the number of samples is shown on the y-axis. Samples were collected from 1,324 primiparous dairy cows in 16 herds in 2020-2021 to study *Leptospira borgpetersenii* str. Pacifica epidemiology in New Zealand dairy herds.

Table 4.1 Comparison of test results for the early and late season 2020-2021 and one-off sampling in 2022, samples collected for a study of *Leptospira borgpetersenii* str. Pacifica epidemiology in primiparous New Zealand dairy cows. The table shows the number of cows tested the percentage of MAT positive (titre≥48) (95% CI) to Tarassovi, Ballum, Hardjo, Copenhageni, Pomona, and overall geometric mean titre (GMT) (95% CI) of positive samples as well as the number of urines tested cows, the number and percentage of PCR positive samples, and shedding prevalence.

Sampling Time	**Early Season**				**Late Season**				**One-off sampling**
Number of Farms	16				6				1
Total Serum Samples (N)	915				409				0
MAT Positive	Seroprevalence MAT ≥ 48	95% CI	GMT	95% CI	Seroprevalence MAT ≥ 48	95% CI	GMT	95% CI	Not available
Tarassovi	27.9% (256/915)	25.1-31.0	261	222.2-307.6	48.7% (199/409)	43.7-53.6	357	293.9-433.5	
Ballum	1.5% (14/915)	0.9-2.6	56	44.2-70.2	4.2% (17/409)	2.3-6.1	50	45.9-54.5	
Hardjo	7.3% (67/915)	5.8-9.3	73	62.0-86.9	6.8% (28/409)	4.3-9.0	61	52.9-71.4	
Copenhageni	8.5% (78/915)	6.8-10.6	63	52.0-76.8	0.2% (1/409)	0.0-1.5	48	NA*	
Pomona	34.8% (318/915)	31.7-38.0	98	51.7-76.8	20.9% (85/409)	15.5-2.3	90	77.1-104.9	
Overall			92				86		Not available

Sampling Time	**Early Season**		**Late Season**		**One-off sampling**	
Total Urine Samples (N)	121		383		60	
PCR Positive	Shedding prevalence	95% CI	Shedding prevalence	95% CI	Shedding prevalence	95% CI
PCR [1]Unconfirmed Positive	37.2% (45/121)	28.7-46.5	7.6% (29/383)	5.2-10.8	13.3% (8/60)	12.3-15.9
[2]PCR str. Pacifica Confirmed Positive	27.3% (33/121)	19.8-36.3	3.9% (15/383)	2.3-6.5	6.66% (4/60)	4.6-9.4
[2,3]PCR Hardjo Confirmed positive	none	none	1.04 (4/383)	0.4-3.5	none	

[1]PCR Unconfirmed Positive- sample where the presence of the leptospiral DNA was confirmed by conventional PCR test; [2]PCR Confirmed positive sample where the amplicon sequencing confirmed the presence of leptospiral DNA; [3] *Leptospira borgpetersenii* serovar Harjo shedders were found in a single herd.

4.4.3 Test Results from the Subset of Six Farms that Participated in Early and Late Lactation Sampling

The subset of the same six farms was used to compare seroprevalence, shedding, and PR between the early (August to November 2020) and late (February to May 2021) seasons (Table 4.2). Where possible, the same animals were targeted at the start and end of the sampling. From these six farms, 449 cows were sampled in the early season and 409 in the late season (Table 4.2). The prevalence of Tarassovi in both sampling steps was very similar at 41.9% (95%CI: 37.3-46.6) and 48.7% (95%CI: 43.7-53.6); however, the prevalence of Copenhageni and Pomona at early season was almost twice the prevalence in the same group of animals sampled at late season (Table 4.2). All sampled animals were vaccinated against Pomona and Hardjo at least three months prior to the first sampling. Moreover, Ballum prevalence was higher at late lactation at 4.2 % (95%CI: 2.5-6.7) than in the early season at 1.11% (95%CI: 0.5-3.0).

Tarassovi GMT was the highest among all serovars (Table 4.2), and it was lower but not statistically different in the early season at 316 (95%CI: 259.1-384.2) compared to the late season at 357 (95%CI: 293.9-433.5). While GMT for all other serovars in the early and late seasons was also similar, the distribution of the MAT titres differed for all serovars at the start and the end of the milking season (Table 4.2).

Table 4.2. Comparison of MAT and GMT for six high-risk farms during the early and late season sampling of 2020-2021. Samples were collected to investigate the epidemiology of *Leptospira borgpetersenii* strain Pacifica in primiparous New Zealand dairy cows.

Serovar / Sampling Step	**Seroprevalence MAT ≥ 48 %**	**95% [2]CI**	**[1]GMT**	**95% [2]CI**	**[3]MAT Titre (number of animals)**								
					0	**24**	**48**	**96**	**192**	**384**	**768**	**1536**	**3072**
Tarassovi													
Early Season	41.9 (188/449)	37.3-46.6	316	259.1-384.2	178	83	35	32	17	36	34	17	17
Late Season	48.7 (199/409)	43.7-53.6	357	293.86-433.5	157	53	34	24	33	24	29	32	23
Ballum													
Early Season	1.11 (5/449)	0.5-3.0	48	NA	352	28	5						
Late Season	4.2 (17/409)	2.5-6.7	50	45.9-54.5	338	60	16	1					
Hardjo													
Early Season	4.9 (22/449)	3.3-5.2	53	47.4-58.8	363	64	19	3					
Late Season	6.9 (28/409)	4.7-9.9	61	52.9-71.4	314	67	19	8	1				
Copenhageni													
Early Season	5.6(25/449)	3.7-8.2	63	50.9-78.8	379	45	18	5	1	1			

Serovar / Sampling Step	**Seroprevalence MAT ≥ 48 %**	**95% [2]CI**	**[1]GMT**	**95% [2]CI**	**[3]MAT Titre (number of animals)**								
					0	**24**	**48**	**96**	**192**	**384**	**768**	**1536**	**3072**
Late Season	0.2(1/409)	0.0-1.6	48	NA	406	2	1						
Pomona													
Early Season	30.1(135/449)	25.9-34.6	85	75.6-95.3	218	96	65	42	16	11	1		
Late Season	20.885/409)	17.02-25.11	90	77.1-104.9	245	79	38	26	14	5	2		

[1] GMT =geometric mean titre; [2] CI= confidence interval;[3] MAT=microscopic agglutination test

The association between seropositivity of MAT ≥ 48 and urine shedding, as identified by PCR, was statistically significant for Tarassovi for both the early season (p=0.005) and late season (p=0.003) and for Hardjo in the late season (p=0.04236). In contrast, the association was not statistically significant for the rest of the serovars. When the percentage of PCR-positive animals was compared in seropositive and seronegative animals in early and late lactation, results showed that most were seropositive to Tarassovi and Pomona, then Hardjo (Table 4.3). All sampled animals were vaccinated against Hardjo and Pomona at least three months before sampling. On the other hand, the lowest number of PCR-positive results was found in animals seronegative to Tarassovi and Hardjo (Table 4.3). Moreover, when PCR-positive results in Tarassovi seropositive and seronegative were attributed to the infecting serovar, as determined by amplicon sequencing of PCR-positive products, the percentage of Pacifica shedders in seronegative animals was less than 1% in both early and late season (Table 4.4).

Table 4.3 A The association of PCR unconfirmed positive results, as determined by a PCR test and MAT serology, for six high-risk farms that participated in early season (n=449) and late season (n=409) sampling. Samples were collected to study the epidemiology of *Leptospira borgpetersenii* strain Pacifica in primiparous New Zealand dairy cows.

Serovar/Sampling	**% [1]PCR positive in seropositive animals**	**95% [2]CI**	**%[1]PCR positive in seronegative animals**	**95% [2]CI**
Tarassovi				
Early Sampling	19.2% (36/188)	13.9-25.7	0.8% (2/261)	0.8-3.0
Late Sampling	10.1% (20/199)	6.40-15.30	2.9% (6/210)	1.2-6.4
Ballum				
Early Sampling	20.0% (1/5)	10.5-70.12	9.5% (36/380)	6.8-13.0
Late Sampling	5.-% (1/17)	3.1-30.8	6.3% (25/398)	4.2-9.3
Hardjo				
Early Sampling	0% (0/22)	0	8.7% (37/427)	6.3-11.9
Late Sampling	11.1% (3/27)	2.9-30.3	6.0% (23/381)	3.94-9.05
Copenhageni				
Early Sampling	4.0% (1/25)	0.2-22.3	8.5% (36/424)	6.1-11.7
Late Sampling	0% (0/1)	0	6.37% (26/408)	4.3-10.2
Pomona				
Early Sampling	11.9-% (16/135)	7.1-18.8	6.7% (21/314)	4.3-10.2
Late Sampling	7.1% (6/85)	2.9-15.3	6.2% (20/324)	3.9-9.5

[1] PCR= polymerase chain reaction;[2] CI= confidence interval

Table 4.4 The association between *Leptospira borgpetersenii* strain Pacifica PCR and Tarassovi seropositive results. These were identified by PCR and amplicon sequences, as well as MAT serology, for six high-risk farms that participated in early season (n=449) and late season (n=409) sampling. Samples were collected to study the epidemiology of *Leptospira borgpetersenii* strain Pacifica in primiparous New Zealand dairy cows.

Serovar/Sampling str. Pacifica	**% str. Pacifica [1]PCR positive in Tarassovi seropositive animals**	**95%[2]CI**	**% str. Pacifica [1]PCR positive in Tarassovi seronegative animals**	**95%[2]CI**
Early Sampling (n=121)	14.4% (27/188)	9.8-20.4	0.4% (1/261)	0.03-2.8
Late Sampling (n=383)	4.0% (8/199)	1.0-8.1	1.0% (2/210)	0.03-3.7

[1] PCR= polymerase chain reaction;[2] CI= confidence interval

4.4.4 One-off 2022 Sampling Results

The one-off urine sampling of 60 primiparous dairy cows showed that at the time of sampling, 13% (8/60) (95% CI:6.3- 25.1) of animals were shedding *Leptospira spp*. From these, 7% (4/60) were shedding str. Pacifica, as confirmed by amplicon sequences (Table 4.1). Additionally, 12% (7/60) (95% CI:5.2- 23.2) of samples seeded into the HAN media were identified as culture-positive, and 3% (2/60) (95% CI:0.6-12.5) were both culture and PCR-positive, confirmed by the amplicon sequencing. None of the positive by-culture samples in HAN media resulted in viable str. Pacifica cultures. None of the samples seeded into EMJH media were positive.

4.5 Discussion

The specific aim of this study was to evaluate the shedding pattern and persistence of *Leptospira borgpetersenii* str. Pacifica in primiparous dairy cows from high-risk dairy herds in 2020-2022.

The results of the sampling and testing confirmed that str. Pacifica is still present in

approximately half, 69% (95 CI: 24.6-81.-9) of the high-risk New Zealand dairy herds five years after str. Pacifica's initial detection in the same herds was in 2015/2016. Test results showed that the seropositivity to Tarassovi and str. Pacifica shedding in the same number of herds (N=6) was higher at the start of New Zealand's milking season (September and October) than at the end (April, May, and June), and the overall urinary shedding of str. Pacifica was statistically significantly associated with Tarassovi seropositivity by MAT (≥48), in line with what has been reported previously (Yupiana *et al*. 2019). The estimated PR of urinary shedding was seven times higher at the start of the season than at the end. While some live Pacifica cultures were initially isolated in HAN media, further propagation failed, and no viable cultures were obtained from urine or kidney samples, indicating that str. Pacifica growth requirements are fastidious and differ from other common pathogenic *Leptospira* strains.

A comparison of Tarassovi GMT between early and late season sampling showed that Tarassovi GMT at the start of the season was approximately 13% lower than at the end of the season (Table 4.2). More animals with lower MAT titres at the start of the milking season contributed to lower GMT. On the other hand, towards the end of the season, there were fewer Tarassovi seropositive animals with higher MAT titres, contributing to the higher GMT (Table 4.2). Low titres and more reactors detected in the early season may indicate that early season testing picked up rising titres and may indicate very recent exposure to Tarassovi, as it is generally accepted that raising titres generally indicate the beginning of infection (Durfee and Allen 1980; Ellis 2015). On the other hand, the higher GMT titres at the end of the season likely indicate the post-peak of the immune response in the sampled group of primiparous dairy cows (Guedes *et al.* 2021).

One of the possible *Leptospira borgpetersenii* str. Pacifica transmission pathways to the primiparous dairy cows could be after their re-introduction to the main milking herd via contact with the older animals. Therefore, the primiparous dairy cows could be subjected to a high level of exposure over a short time, which could lead to concurrent seroconversion and urinary shedding of many animals. In this study, participating primiparous cows were grazed separately from the main milking herd before calving, similar to many other New Zealand milking herds, and these animals typically enter the main herds only prior to calving (Yupiana *et al.* 2019). Therefore, naive new entrants

may be even more susceptible to infections due to the stress of first calving and milking, which weakens immune systems (Fraga *et al.* 2011). The immune response and symptoms associated with *Leptospira* infection depend on the immunological status of the host (Levett 2001), and the antibody production starts 3 to 10 days post-infection infection and peaks from two weeks to a few months post-infection (Faine 1994). Therefore, the findings of this study suggest that the antibody response to natural Tarassovi or str. Pacifica infection in the first calvers may have peaked between the first and second samplings, mid-miking season, or around five months after infection.

The overall shedding pattern was assessed by evaluating PCR test results and comparing sequences of the amplicons of the PCR samples to the known sequences of *Leptospira borgpetersenii* str. Pacifica. Results showed that 63% (95% CI:31.61- 87.63) of herds had at least one animal shedding str. Pacifica. Moreover, 34% (95% CI: 31.72 - 36.89) or 455 cows and 11 or 69% (95 CI: 24.56-81.86) of herds were seropositive at MAT≥ 48 to Tarassovi. Thus, we report the urinary shedding of str. Pacifica in 63 % of high-risk herds five years after the initial study in 2016. As the average annual replacement rate in New Zealand dairy herds is estimated at 20% (Lopez-Villalobos and Holmes 2010), it means that approximately 80%-100% of dairy cows from each herd sampled in this study were replaced from 2016 to 2021; therefore, str. Pacifica urinary shedding in this herds suggests that str. Pacifica is maintained within and well-established in the dairy cow population.

Moreover, the PCR results from early and late season sampling further support the hypothesis of infection entering the herd at the start of the season. Although the group sampled at the end of the season did not match the first one exactly, it included 86% of cows already sampled at the start of the season. Therefore, the results show that the number of str. Pacifica shedders dropped approximately by a third in an average of 5 months in the high-risk seropositive three-year-old dairy cows' group (Table 4). Specifically, a comparison between early and late milking season PCR results from the same high-risk farms and animals showed a difference in the estimated prevalence ratios. In this study, from 564 analysed urine samples, 52 shedders of the Pacifica strain were confirmed by amplicon sequencing using the PCR assay targeting the *glm*U locus.

(Wilkinson *et al.* 2021). However, at the start of the season, the estimated PR was seven times higher than at the end at 27.3 (95% CI: 19.8-36.3) and 3.9 (95%CI: 2.3-6.5),

The following factors may have contributed to this difference in prevalence ratio. Firstly, the intermittent nature of *Leptospira* shedding. It is generally accepted that infected animals may not continuously shed the live bacteria (Faine 1994), and intermittent shedding can sometimes last for years (Levett 2001; Monti *et al*. 2023). A study investigating the intermittent shedding of different *Leptospira* strains (Copenhageni L1-130, Canicola LO-4 and Pomona Fromm) in cattle and sheep by PCR reported that 43.3% of experimentally infected cows were shedding intermittently (Rocha et al. 2017). Similarly, an intermittent shedding pattern of *Leptospira borgpetersenii* was reported in a dairy cow kept at Iowa, USA research facilities (Hamond et al. 2022).

Urinary shedding of *Leptospira* starts after renal colonisation of the host by bacteria. It may begin as early as eight days from the disease onset in the acute stage (Levett 2001). Thus, more urine PCR-positive animals in our study at the start of the season indicated more shedding animals than in the late season. Moreover, natural infection, as opposed to experimental infection, may result in prolonged shedding periods (Faine 1994). For example, a study reported various intermittent shedding patterns in six dairy herds in Chile; specifically, the mean elimination of 259 days, ranging from 207 to 311 days was reported. These animals were shedding *L. borgpetersenii* serovar Ballum (34.5%), *L. interrogans* serovar Hardjo (type Hardjo-Prajitno) (20.7%), *L. interrogans* serovar Autumnalis (17.2%), *L. interrogans* serovar Pomona (13.8%), most animals (81.5%) presented MAT at 100, and for the rest titers ranged between 200 and 400 (Monti *et al.* 2023). Therefore, the detection of shedders at the end of the season sampling may be potentially attributed to re-infection within herds and a longer shedding time due to the natural infection.

Furthermore, an epidemiological study of notified cases of *Leptospira* in the New Zealand population 1999-2017 reported that dairy farmers had the highest incidence of Tarassovi (12.59/100,000) and Tarassovi incidences peaked in summer (Nov-Feb)

(Nisa *et al.* 2020). Thus, the summer peak of Tarassovi infections in farmers could most likely be attributed to the greater number of dairy cows shedding Pacifica, as identified at the start of the season in this study. Therefore, Tarassovi leptospirosis in dairy farmers could hypothetically result from occupational exposure to str. Pacifica has long been considered an occupational disease (Marshall and Manktelow 2002).

Current study results showed a statistically significant association between urinary shedding of str. Pacifica and Tarassovi seropositivity at MAT≥48 in all sampling stages ($p<0.05$). Thus, a Tarassovi-positive MAT titre of ≥48 most likely indicates urinary shedding of str. Pacifica. These findings are similar to the reports by other researchers who also demonstrated the ability of MAT to predict shedding (Lilenbaum *et al.* 2009; Philip *et al.* 2020; Guedes *et al.* 2021). Moreover, a strong association between shedding or PCR-positive results and serology for Tarassovi where 6.5% (95% CI: 18.9–35.9) of herds and 2.4% (95% CI:1.8–3.1) of individual cows were confirmed urinary shedders of *Leptospira* spp. (Yupiana *et al.* 2019). While a positive PCR indicates a current infection and acute phase of the disease, MAT titres may indicate past infection or vaccination, and a single high MAT titre >400 or fourfold increase between acute and convalescent titre may also indicate current infection (Lau *et al.* 2018). Although these two tests are different and best applied in the different stages of the disease, they give a sufficient indication of leptospiral infection (Klement-Frutos *et al.* 2020). Therefore, comparing results obtained by two methods is not uncommon, and most researchers report that the two tests are in good agreement (Yupiana *et al.* 2019); hence, the MAT may indicate the PCR result. For example, Fang *et al.* (2014), in the paper investigating inter-laboratory agreement between results of different tests on different specimens, reported that MAT titres may provide a limited prediction of shedding in urine or renal carriage in cattle as determined by PCR testing.

Interestingly, the prevalence of Pomona detected in the early milking season sampling at 30.1% (95% CI: 25.9-34.6) was most likely due to the recent vaccination of dairy cattle, as most Pomona titres were low positive (Table 4.2), and Pomona GMT was nearly four times lower than Tarassovi, and there were no Pomona positive PCR results (Table 4.1). Therefore, Pomona's prevalence at the start of the season was most likely.

due to vaccination. Pomona and Hardjo antigens are included in all commercially available New Zealand bovine leptospiral vaccines, and all participating herds were vaccinated at least three months before the first sampling, but no additional details on vaccination are available.

The unexpected finding of this study was the detection of five animals shedding Hardjo at the late season sampling. These cows were in the same herd and were vaccinated with a vaccine containing antigens to Hardjo and Pomona 11 months before sampling. Their corresponding serological MAT titres to Hardjo ranged from 48 to 96. In line with this finding, several other studies report infections in dairy herds with different *Leptospira* serovars and shedding of vaccine-containing serovar in the vaccinated animals (Bolin 2003; Lilenbaum and Martins 2014; Yupiana *et al.* 2021). While 99% of New Zealand dairy herds are vaccinated against *Leptospira borgpetersenii* serovar Hardjo, only approximately half of the farmers follow the best practice vaccination guidelines, and 61% of farmers are unsure of the vaccination status of the stock they purchased (Yupiana *et al.* 2021). Therefore, detection of the Hardjo shedders does not necessarily mean the lack of protection by the vaccine but instead highlights the importance of timely vaccination and the possibility of other strains of *Leptospira*, i.e. str. Pacifica infection in the high-risk herds vaccinated against other serovars.

Although str. Pacifica in bovines has been detected by PCR testing and amplicon sequencing in New Zealand and overseas (Yupiana *et al.* 2019; Wilkinson *et al.* 2021; Wilkinson *et al.* 2023), but there are no reports of successful isolation of this strain. Successful isolation of str. Pacifica is important for preventing animal and human infections. It will lead to a better understanding of str. Pacifica's epidemiology and growth requirements. It may enable further researchers to culture Pacifica, characterise it by WGS, and advance diagnostic methods and vaccine development to prevent Pacifica leptospirosis.

The current study's limitations included using different sampling strategies instead of one, which resulted in different numbers of blood and urine samples collected. Moreover, sampling only one class of animal, primiparous dairy cows, may have.

resulted in biased results. Additionally, a small number of herds (n=6) participated in the late-season 2021-2022 sampling. The reason for the small number of herds enrolled in the late season was due to COVID-19 travel and work restrictions.

It is important to note that this study investigated str. Pacifica shedding in 17 high-risk herds. The seroprevalence and shedding rates reported here represent those seen in high-risk herds; however, their locality may show "hot spots" of str. Pacifica strain in New Zealand. Nevertheless, given the statistically significant association between str. Pacifica shedding and Tarassovi seroprevalence, and considering that Tarassovi seroprevalence is estimated at 75% in the national dairy herd (Yupiana et al. 2019), the findings of this thesis could be extrapolated to the national herd level, suggesting that dairy cows may have become a maintenance host for str. Pacifica.

Moreover, a factor or a combination of unknown factors contributed to the failure to maintain the growth of the Pacifica strain despite successful initial isolation in HAN media at 38°C and 5 CO_2. Future studies may consider the intermittent nature and seasonality of strain Pacifica shedding and the locality of high-risk herds for its successful isolation.

4.6 Conclusion

This study provided evidence of str. Pacifica's endemicity in New Zealand dairy herds, thus adding weight to the evidence that dairy cows are maintenance hosts of str. Pacifica. Although Pacifica's initial isolation in HAN media was successful, culture could not be maintained and was subsequently lost. Thus, further str. Pacifica maintenance experiments are needed, as the isolation of str. Pacifica will be necessary for a better understanding of its epidemiology and transmission, which may help prevent human leptospirosis, particularly in dairy farmers.

4.7 References

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CHAPTER 5

Chapter 5 Investigation of the Association Between *Leptospira borgpetersenii* strain Pacifica and Reproductive Performance of Dairy Cows in New Zealand

5.1 Abstract

Aims: To investigate the association between *Leptospira borgpetersenii* strain Pacifica (str. Pacifica) and the reproductive performance of dairy cows in New Zealand herds during the 2015/2016 milking season.

Methods: Reproduction animal and herd records collected from 44 New Zealand dairy herds during the 2015/2016 and 2016/2017 milking seasons were matched with *Leptospira* PCR and serology test results from 20 animals from each herd. Data was collected from herds classified as either high or low risk for str. Pacifica. At the animal level, a shared frailty survival model was used to examine the calving to conception interval, and a generalised mixed effect model evaluated pregnancy from the first mating. In both models, independent variables included str. Pacifica positivity, breed, age, and herd were random effects. Both final animal-level models included breed as a variable of interest and were adjusted for the clustering effect of herds. A multivariable linear regression model was fitted to the data at the herd level to estimate the relationship between the average first service to conception rate and the prevalence of str. Pacifica positivity adjusting for herd size and average age.

Results: In the final dataset, there were 131 str. Pacifica positive and 551 negative animal records from 44 herds, of which 27 were str. Pacifica positive. Results of the statistical analysis of the association between str. Pacifica positivity and reproduction of dairy herds showed that str. Pacifica did delay the time from calving to conception (HR = 0.84; 95% CI 0.74-0.96), although there was no effect on the pregnancy rate. However, the generalised mixed effect model at the animal level and the multivariable linear regression model at the herd level did not reveal an association between str. Pacifica positivity and pregnancy to the first mating. Similarly, no association was detected at the herd level between the first service to conception rate and str. Pacifica positivity.

Conclusion: The results of these analyses indicated that positivity to str. Pacifica is associated with an increase in the time from mating to pregnancy, resulting in later pregnancies, although there was no effect on the pregnancy rate. These findings suggest that str. Pacifica developed adaptation mechanisms allowing for its maintenance within the New Zealand dairy cow population.

5.2 Introduction

Leptospirosis is a zoonotic infectious disease caused by pathogenic spirochetes of the genus *Leptospira*. *Leptospira* is a diverse bacterial genus with more than 300 serovars (Picardeau *et al.* 2014) and seventy-one genomospecies (Korba *et al.* 2021), which are classified into two distinct clades: saprophytic (S1 and S2) and pathogenic (P1 and P2) (Vincent *et al.* 2019).

Leptospira colonises the kidneys of the infected host and is excreted with urine into the environment, where it can survive for months under favourable conditions (Barragan *et al.* 2011). All mammals are susceptible to leptospirosis, while amphibians, birds and reptiles can also be infected (Picardeau 2017). Commonly, particular *Leptospira* species and serovars from pathogenic clades (P1 and P2) have preferred maintenance host species. Within maintenance populations, *Leptospira* does not cause severe disease, ensuring the host's long-term survival (Haake and Levett 2014). Mammalian species recognised as long-term maintenance hosts demonstrate prolonged renal carriage of the bacteria; for example, bovines are considered maintenance hosts of *L. borgpetersenii* serovar Hardjo (Hardjo) (Putz and Nally 2020).

Livestock are currently considered the primary source of human leptospirosis in New Zealand (Thornley *et al.* 2002; Yupiana *et al.* 2019; Nisa *et al.* 2020; Benschop *et al.* 2021). There was an increase in notified human cases of leptospirosis attributed to Tarassovi from 1999 to 2017 in New Zealand, particularly noticeable among dairy farmers, with a reported incidence of 12.59/100,000 (Nisa *et al.* 2020). The increased detection of cases attributed to Tarassovi in humans and the significant proportion of animals shedding str. Pacifica and seropositive to Tarassovi since 2021 is likely due to the emergence of *Leptospira borgpetersenii* strain Pacifica, which serologically reacts to

Tarassovi antigens. However, it is possible that some of the cases serologically attributed to Tarassovi before 2021 (Ryan and Marshall 1976; Nisa *et al.* 2020; Wilson *et al.* 2021) could also be attributed to str. Pacifica, as, at the time, serological tests were the preferred testing method, and a molecular test method capable of distinguishing Tarassovi and Pacifica was only described and published in 2021 (Wilkinson *et al.* 2021).

Historically, Tarassovi infection in New Zealand cattle was considered clinically unimportant, and cattle were not recognised as maintenance hosts (Ryan and Marshall 1976). In a 2016 study, dairy farmers completed a questionnaire investigating the presence of clinical signs in Tarassovi-positive herds shedding str. Pacifica, identified by both serology and amplicon sequencing (Yupiana *et al.* 2019; Wilkinson *et al.* 2021). The study noted no clinical manifestations of Tarassovi-Pacifica leptospirosis in dairy cows. Therefore, the importance of str. Pacifica infection and its effects on dairy cattle remain to be determined.

The clinical importance of other *Leptospira* in cattle, such as *Leptospira interrogans* serovar Pomona and *Leptospira borgpetersenii* serovar Hardjo, was proven by earlier researchers (Reichel *et al.* 2018). The most significant reported impacts of Pomona leptospirosis in cattle were abortion, stillbirth, premature birth (Te Punga and Bishop 1953; Carter *et al.* 1982), and reproductive failure (Mori *et al.* 2017). Additionally, mastitis and infertility were also reported, with the latter two syndromes being mostly reported overseas (Prescott *et al.* 1988; Langoni *et al.* 1999). Similarly, *L. interrogans* sv. Hardjo type prajitno and *L. borgpetersenii* sv. Hardjo type bovis were primarily associated with cattle reproductive failures, manifested by decreased conception rates and increased calving to conception interval (Ellis *et al.* 1985; Miller *et al.* 1991; Guitian *et al.* 2001; Rajeev 2022). Susceptibility of cattle to other serovars and species, namely, *Leptospira kirschneri* serovar Grippotyphosa and *Leptospira noguchii,* was also noted (Miller *et al.* 1991; Martins *et al.* 2015; Nally *et al.* 2018).

More recently, the *L*. *borgpetersenii* serovar Tarassovi was isolated from the urine of a dairy cow in Iowa, USA (Hamond *et al.* 2022). This was the first reported isolation of

Tarassovi from bovine species in the USA. In this case, the herd's poor reproductive history, specifically failure to breed and maintain pregnancy, prompted a veterinary investigation and led to the successful isolation of *L*. *borgpetersenii* serovar Tarassovi. However, the individual cow from which the isolate was obtained did not display clinical signs of leptospirosis during the observation period, as reported by the study`s authors.

Regardless of the infecting species, there are reports that *Leptospira spp.* have been isolated from aborted foetuses, uteri, follicular fluid, and oviducts (Bielanski *et al.* 1998; Monte *et al.* 2015; Loureiro and Lilenbaum 2020). These studies suggested that *Leptospira* causes endometrial inflammation, which can lead to a change in the intra-uterine environment, affecting conception or early pregnancy and resulting in reproductive failure (Loureiro and Lilenbaum 2020). However, PCR results from urine and vaginal fluid samples were poorly correlated (Loureiro *et al.* 2017; Pinna *et al.* 2018). Meanwhile, reproductive issues are well recognised as the effect of leptospiral infection in cattle, but the actual cellular mechanism of infection is still largely unknown.

The question of whether str. Pacifica impacts the reproduction of dairy cows remains. Moreover, it is unknown to what extent str. Pacifica may impact the reproduction of animals under seasonal conditions of the New Zealand pastoral farming system, where young animals are often grazed separately before their first calving. Hence, the main aim of this study was to investigate the effects of *Leptospira borgpetersenii* strain Pacifica on the reproductive performance of dairy cows.

5.3 Methods

5.3.1 Data Sources

Three data sources were used for this analysis. The primary dataset was drawn from a seroprevalence and shedding study (Yupiana *et al.* 2019), for which the national database of New Zealand dairy herds was stratified by region and herd size and then randomly selected. Blood and urine samples were collected from 20 in-milk dairy cows randomly selected from each of the 200 participating farms from 5 January 2016 to 26 April 2016. Sera were tested with microscopic agglutination test (MAT), as described by

Faine (1982) against the following leptospiral antigens: *Leptospira borgpetersenii* serovars Hardjo, Ballum and Tarassovi and *Leptospira interrogans* serovars Pomona and Copenhageni. DNA was extracted from urine samples and tested by qPCR, using *gyrB* as a target gene for pathogenic *Leptospira* DNA, as described earlier (Subharat *et al.* 2011; Fang *et al.* 2014). As a result, 4,000 individual animal data records, including unique animal IDs and herd supplier numbers, were available for this analysis and included by MAT and PCR test results.

The second data source was reproduction data extracted from the Dairy Industry Good Animal Database (DIGAD) (Government of New Zealand 2014). These data are supplied to DIGAD by private companies that provide herd test services to New Zealand dairy farms. Data availability allowed extraction from DIGAD for only seventy-seven farms of the 200 farms in the primary dataset and included 34,652 individual animal records.

For this study, we defined two "types" of herds:

(1) high risk, where at least one of the 20 cattle sampled had a positive serology test (MAT ≥ 48) for Tarassovi and/or tested PCR positive for str. Pacifica at Ct ≤ 40 using *glmU* as a target gene for *Leptospira* DNA

(2) low risk, where no PCR positives were detected, and serology tests to Tarassovi were negligible (MAT < 48).

Fields extracted comprised unique animal IDs, herd participant codes, supplier numbers, animals' date of birth, dates when animals entered and left the herd, breed codes, and animal fate. The extract also included reproduction records such as mating date, mating type, bull identification, calving date, number of calves, calf fate, drying off date, and drying off description. Additionally, lactation information, such as the number of lactations, parturition date, fat and protein percentages, milk volume and weight per milking, and somatic cell count (SCC), was also extracted. These extracted data contained records from 1 June 2015 to 31 May 2016.

The third data source was a dataset from the 2016-2017 milking season provided by the Livestock Improvement Corporation (LIC), which holds approximately 90% of herd-test data from individual New Zealand farmers who herd-test their animals (DairyNZ 2018). These data were required for cross-checking with the 2015-2016 DIGAD data to determine the conception dates of pregnant cows. For example, 2016/2017 calving records were matched with the last mating date of 2015/2016 mating records, as well as to animal age, parity, and other reproductive-related data and dates. Signed permissions were collected from each farm owner, allowing for data access for research purposes, and upon presenting these, LIC supplied data files. Data contained data fields for 62 herds and 31,752 individual animal records from 1 June 2016 to 31 May 2017. These included herds' test records, such as test dates, milk composition, volume, somatic cell count (SCC), mating dates, types, parturition dates, the number, fate, and sex of offspring, animal lactation number and lengths, live weights, and animal health records. In addition, individual animal data such as unique ID assigned by LIC, farm ID, date of birth, breed code, dates when the animal entered and exited the herd, unique herd ID such as participant code and supplier numbers, as well as an internally assigned unique herd ID, and reason for leaving the herd, were also included in the extract.

Three data sets were merged using unique individual animal IDs, birth dates, and farm IDs, with all duplicated values removed after merging. All data used in this analysis were collected during the 2015/2016 and 2016/2017 milking seasons. All data manipulations were done using R-Studio, version 4.2.1 (R CoreTeam 2021).

5.3.2 Animal-Level Data Categorisation

5.3.2.1 Reproduction Events Variables

For this analysis, one of the created binary variables was variable PREG, where animals were assigned 'pregnant' status and recorded as 1 when they had a calving event recorded in the subsequent 2016/2017 milking season (Table 5.1), and if the animal was not pregnant, its status was recorded as 0. Similarly, the binary variable PREGBY1ST was created when the animal had PREG status with only one mating.

event, and subsequent calving events were recorded in the 2016/2017 milking season, and it was coded 1. When the binary variable PREGBY1ST was zero, it meant that the cow was not pregnant after the first insemination, indicating a silent fertilisation failure, early embryonic death, poor heat detection, or subfertility, which may otherwise not be evident. Any of these reproductive issues could potentially be associated with genital leptospirosis (Aymee *et al*. 2021).

Animals with mating and calving dates recorded for the 2015/2016 season were accordingly assigned MATED and CALVED binary statuses. These statuses were also used to cross-check the animal's pregnancy status in 2015/2016 using 2016/2017 calving records. Other created variables (Table 5.1) included calving to calving interval (CI), which represented the time difference between subsequent calvings and is widely accepted as the measurement of cow's fertility.

Several dates indicative of animals' reproductive performance were calculated (Table 5.1). Variables representing the first and last mating dates were determined by comparing matings and recorded as binary indicators. For analysis, the first mating date was one, and the last was zero. Identifying the last and first mating dates was important for verification of the length of calving to conception interval, which is the reproductive performance indicator. The conception date was calculated by subtracting an average gestation length of 283 days from the 2017 calving date and matching it with the nearest corresponding recorded mating date. The conception date was also used to cross-check which mating date resulted in pregnancy.

5.3.2.2 Str. Pacifica Status Variables

For this study, an animal was deemed str. Pacifica positive (PACPOS =1) if its test results from 2016 (Yupiana *et al.* 2019) were either positive to Tarassovi by serological test (MAT ≥48) and/or positive by qPCR with Ct values less than or equal to 40, using *gyrB* and *glmU* as a target gene for *Leptospira borgpetersenii* str. Pacifica DNA. Otherwise, the animal was deemed str. Pacifica negative (PACPOS=0). PACPOS was the binary variable of interest in the animal-level analysis.

5.3.2.3 Dairy Cows' Demographic Variables

The age of the animals was calculated by subtracting their date of birth from the approximated mid-milking season date of 1 October 2015 and converted to the number of years. Then, all animals were divided into three age groups (AGECAT): one to three years, four to six years, and greater than six years. Breed code (BREED) was available in the extracted data, but for analysis, it was categorised into three categories: Ayrshire (AYS), Friesian (FRI), and Jersey (JER). Crossbreds were denoted under their main breed category, whichever constituted the bigger proportion of breed composition (Table 5.1).

Table 5.1 The description of the animal level variables used for the animal level analysis from the dataset containing herd test records of 2015/2016/2017 milking seasons from 44 New Zealand dairy herds.

Variable	Description
PACPOS	str. Pacifica status
0	negative when MAT <48 and/or qPCR>40
1	positive when MAT ≥48 and/or qPCR≤40
AGECAT	age of the animal
1	1-3 years
2	4-6 years
3	>6 years
BREED	breed of the animal
AYS	Ayrshire
FRI	Friesian
JER	Jersey
NM	number of matings per animal
LG	lagdate number of days between consecutive matings
MI	LG are categorised as mating intervals
1	LG>18 or<23 or their multiples
2	LG=0
3	LG<17 or >23 or multiples
PREG	pregnancy status:

Variable	Description
0	If no calving was recorded in the following season and the animal had a mating record
1	if calving was recorded in the following season
PREGBY1ST t	Pregnant from the first mating: status was assigned if the animal was pregnant from the first mating
CALVED	Calving status:
0	If the animal did not have a calving record in the 2016/2017 season
1	If the animal had a calving record in the 2016/2017 season
MATED	Status of the
0	If the animal did not have a mating record in the 2015/2016 season
1	if the animal had a mating record in the 2015/2016 season
CTCI	Calving to conception interval
CI	Calving to calving interval

5.3.2.4 Created Variables

The following variables were created to categorise and order each animal's mating dates and multiple mating records (Table 5.1).

i. The number of matings (NM) was created to order the matings from first to last. NM showed each animal's recorded matings in the 2015/16 season.

ii. Lagdate (LG) was created to measure intervals in days between two consecutive matings.

iii. The mating Interval (MI) was created to categorise LG and convert the time variable into three categories. Mating intervals were calculated to describe

reproductive performance, as regular mating may indicate that the animal had no reproductive issues.

- MI was one (regular) if LG was 21+/-3 days or either 42+/-5 or 63+/-7 days.
- MI was two if the animal had only one mating recorded or when LG was zero and was excluded from the analysis.
- MI was three (signifying irregular oestrus) if LG was less than 17 days, greater than 23 days, or their multiples.

5.3.3 Herd-Level Data Categorisation

5.3.3.1 Reproduction Events Variables

The first service to conception rate was a continuous outcome variable on the herd level. It was calculated by dividing the number of cows that became pregnant after the first mating by the overall number of cows in the herd and was coded 1STCR (Table 5.2). Similarly, the average calving to conception rate was calculated for each herd using CTCI, which was defined earlier and coded as AVECTCI.

5.3.3.2 Pacifica Status Variable

The Pacifica's status of the herd (PACPOSH) was recorded as positive if there was one PACPOS animal. If there were no PACPOS animals, the herd was assigned a negative status.

5.3.3.3 Herd Demographic Variables

The average age in the herd AVEAGE was calculated for each herd. Also, the herd size was coded as SIZE and split to obtain three somewhat equal groups into three categories: 1 if the herd was less than 399 cows, 2 if it was more than 400 but less than 550, and 3 if it had 551 or more cows (Table 5.2).

Table 5.2 A summary of the herd-level variables used for the herd-level analysis from the dataset containing herd test records from 44 New Zealand dairy herds' 2015/2016/2017 milking seasons.

Variable	Description
PACPOSH	Str. Pacifica positivity in the herd:
0	If there were no PACPOS animals, the herd was negative
1	if there was at least one PACPOS animal, the herd was positive
1STCR	An average rate of the first service to conception per herd
AVECTCI	An average calving to conception interval per herd
AVEAGE	The average age of the animals in the herd
SIZE	Herd size:
1	<399 cows
2	400-550 cows
3	>551 cows

5.3.4 Statistical Analysis

5.3.4.1 Data Preparation and Descriptive Analyses

First, on the animal level, repeated mating dates for each animal were removed, and only one measurement corresponding to the last recorded mating date was retained. Similarly, repeated values and measurements were removed from the herd-level dataset, and only one unique set of measurements per herd was retained. Next, on animal and herd levels, count tables describing categorical variables and continuous variables were assessed by calculating standard deviations, means, and interquartile ranges. Contingency tables and percentages were calculated to assess possible

collinearity amongst variables of interest. The Goodman-Kruskal asymmetric test (Somers 1962) was applied to compute forward and backward associations between all pairwise combinations of categorical variables in the data sets.

5.3.4.2 Animal-Level Analyses

The survival time was expressed in days from the start of the calving to the conception, or CTCI (Table 5.1). The data was left-censored, and the end of the follow-up was set at 200 days, or by events such as pregnancy, death, culling, or non-conception. Therefore, the survival time was the time between calving and the end of follow-up. The status variable used was the PREG variable defined earlier. The Kaplan-Meier survival curves were plotted using CTCI as a time variable stratified by str. Pacifica positivity. The median calving to conception for cows that conceived was determined by the Kaplan-Meier model of the survival analysis method. In addition, the association between each explanatory variable thought to influence CTCI was tested using the log-rank test.

A preliminary Cox proportional hazard model was fitted to estimate the hazard of an animal at risk of getting pregnant over time. The calving to conception interval was used as a time variable, and PREG was a status variable. All data were left censored, and the end of the follow-up was set at 200 days, or by an event such as pregnancy, death, culling, or non-conception. The independent variables were PACPOS, AGE, and BREED. A backward elimination technique was used to build a multivariable model, where the variables with a p-value ≥ 0.2 were excluded. The estimates for survival function, hazard ratios, PACPOS, AGE, and BREED, and 95% confidence intervals were included in the final model. Next, a multivariate shared frailty survival model was fitted to account for animal data clustering within the herds. PACPOS and BREED were independent variables, and HERD was added as a clustering variable. The baseline hazard function was calculated using the Breslow estimator (Lin 2007).

Bivariate screening of variables was performed for preliminary logistic regression models before stepwise forward and backward elimination methods. The outcome variable was PREGBY1ST, and the variables of interest were NM, MI, PACPOS, AGECAT, and BREED. Variables with statistically significant p-values (≤0.2) identified

by the Likelihood Ratio (LRT) test were retained for the preliminary and final logistic models.

In a preliminary logistic regression model, the PREGBY1ST was the outcome binary variable, and PACPOS, AGECAT, and BREED were included as independent variables.

Interactions were tested by adding biologically plausible interactions into the preliminary final logistic regression models. The statistical significance of *p-value* ≤0.05 for an interaction multiplicative term was the threshold for retention.

Confounding variables were identified by a change of the coefficient of any other variable of more than 20% in the presence of a confounder and their association with both dependent and independent variables, assessed by $\chi 2$ tests, as recommended by (Dohoo *et al.* 2003). When any predictor was found to be a confounder, it was retained in the model regardless of its statistical significance. The generalised binomial random and fixed effect (GLMER) models were used for the multivariate analyses.

The stepwise forward and backward elimination approach described above was applied to GLMER models where the HERD variable was added as a random effect to account for clustering. The predictors in GLMER models were retained in the model when 90% probability intervals of their corresponding regression coefficients did not overlap. Models were tested by evaluating their convergence, divergence, and *p*-values and comparing the models' Akaike information criterion (AIC) values. Thus, in the final GLMER model, PREGBY1ST was the binary outcome variable, and PACPOS, AGECAT, and BREED were independent variables. HERD was added as a clustering variable.

5.3.4.3 Herd-Level Analyses

Bivariate screening of the effects of the continuous outcome variable 1StCR and variables of interest PACPOSH, AVECTCI, AVEAGE, and SIZE was performed for preliminary linear regression models. Variables with statistically significant *p*-values (≤0.2) identified by the $\chi 2$ test were retained for the preliminary linear models. Interactions were tested by adding biologically plausible interactions into the preliminary

final linear regression models. Interactions and confounding variables were identified as described in the animal-level methods section.

Next, the stepwise forward and backward elimination approach was applied to the intermediate linear regression model. Variables with statistically significant *p*-values (≤0.2) identified by the Likelihood Ratio (LRT) test were retained. Thus, on the herd-level data in the final linear regression model, 1StCR was the continuous outcome variable, and PACPOSH, AVERAGE, SIZE, and AVECTCI were independent variables. ANOVA values of bivariate, intermediate, and final models were compared, the smallest value indicated the best fit of the model. Statistical significance of *p*-value ≤ 0.05 for an interaction multiplicative term was the threshold for retention.

All statistical analyses were carried out using R Version 4.1.2 (R Core Team, 2021). Various packages were used, namely 'epiR' (Stevenson *et al.* 2018) ', car' (Fox and Weisberg 2018), 'ggplot2' (Wickham 2011), 'lme4' (Bates *et al.* 2009), 'survminer' (Kassambara *et al.* 2017), frailtyEM (Balan and Putter 2019) and gee (Højsgaard *et al.* 2006). Statistical significance was set at a *p*-value ≤ 0.05.

5.4 Results

5.4.1 Animal-Level

5.4.1.1 Survival Analysis

The dataset included 682 unique animal records from 44 dairy herds in New Zealand collected in the 2015/2016 milking season. The median calving to conception interval was 78 (95%CI: 65-87) days for str. Pacifica negative cows and 86 (95%CI: 75-91) days for str. Pacifica-positive cows.

The Kaplan-Meier curve for survival time and pregnancy is shown in Figure 1. It shows that for str. Pacifica-positive animals took longer to get pregnant when compared to Pacifica-negative cows. Positivity to str. Pacifica was statistically significantly associated (*p*=0.015) with the calving to conception interval (Figure 1).

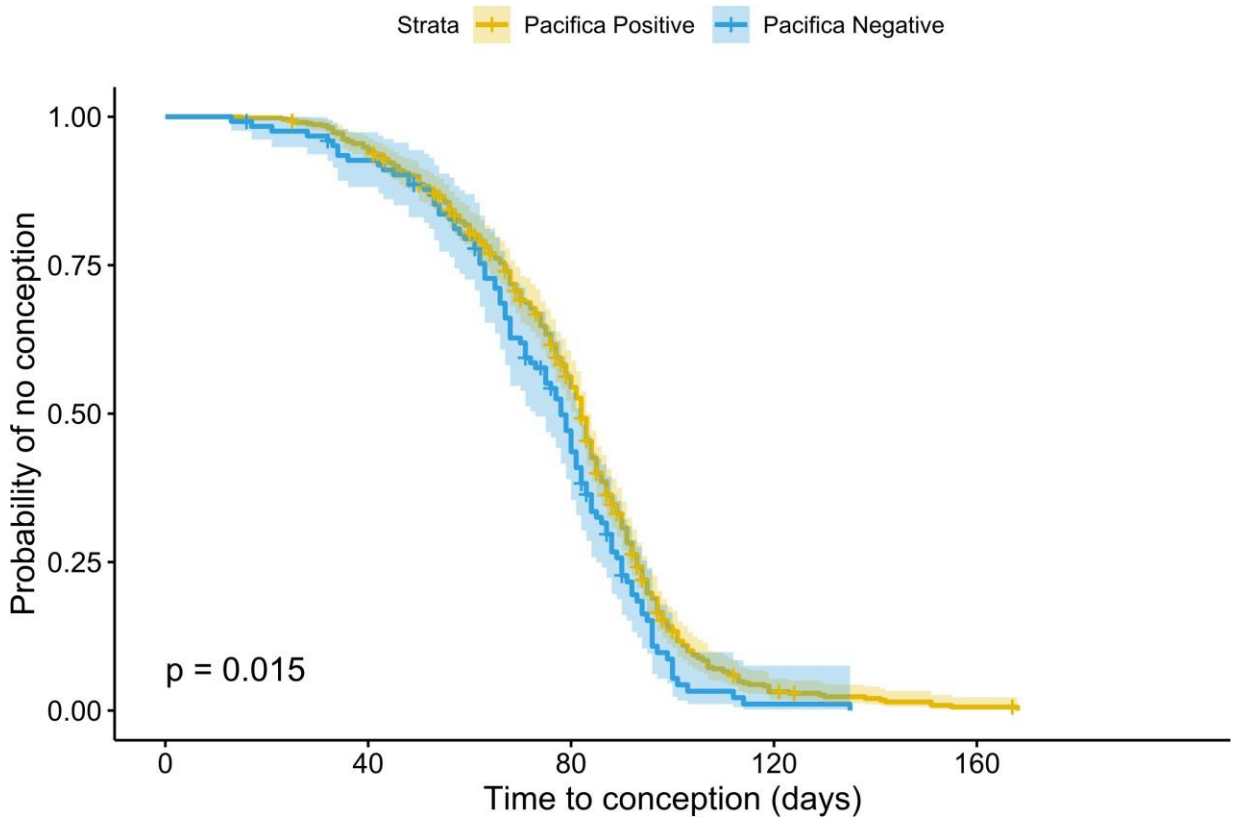

Figure 5.1 The plot of Kaplan-Meier survival functions (y-axis) for calving to conception interval (x-axis) stratified by their str. Pacifica status. Data was collected in the 2015/2016 milking season and included 682 unique animal records from 44 dairy herds in New Zealand

The estimates of the survival function using a penalised likelihood on the hazard function and their standard errors were obtained for all levels of PACPOS and BREED; these independent variables were included in the final shared frailty survival model (Table 5.3). The results show that PACPOS was statistically significantly (*p*-value=0.01) associated with pregnancy. Both the Commenges-Andersen test for heterogeneity and the one-sided likelihood ratio test deem the random effect highly significant (*p*-value <0.001), therefore confirming the model`s validity. This is also suggested by the confidence interval for the frailty variance, which does not contain zero (95% CI 0.05 - 0.20) Table 5.3.

Table 5.3 The results from the shared frailty survival model using a penalised likelihood on the hazard function of pregnancy, with calving to conception interval designated as the time variable for selected New Zealand dairy herds were adjusted for different categories of breed, age, positivity to str. Pacifica and for the clustering effect of the herd. Data was derived from 2015-2016 herd test records.

Variable	**[1]Coeff**	**[2]SE**	**[3]HR**	**95% [4]CI**	***p*-value**
[5]PACPOS					
0	Ref				
1	-0.18	0.07	0.84	0.74 - 0.96	0.01
Breed					
[6]AYR	Ref				
[7]FRI	-0.20	0.11	0.98	0.87 - 1.10	0.07
[8]JER	-0.41	0.84	1.51	0.67 - 2.40	0.62

Fit Summary:

Commenges-Andersen test for heterogeneity: *p*-value 1.17e-06

no-frailty Log-likelihood: -3269.367

Log-likelihood: -3256.827

[9]LRT: 1/2 * pchisq (25.1), p-value 2.75e-07

Frailty summary:

	Coeff	95% CI
Variance	0.01	0.05 - 0.20
Kendall's tau	0.05	0.02 - 0.09
Median concordance	0.05	0.02 - 0.09

[1]Coeff= coefficient; [2]SE = standard error; [3]HR = Hazard ratios in the shared frailty model; [4]CI= confidence intervals based on the likelihood function.[5]PACPOS = positivity to str. Pacifica; [6]BREED[AYR] = animals of the Ayrshire breed; [7]BREED [FRI]= animals of the Friesian breed; [8]BREED[JER] = animals of the Jersey breed; [9]LRT: 1/2 * pchisq one-sided likelihood ratio test;

5.4.1.2 Descriptive, Bivariate, And Multivariable Analysis

After data cleaning, 682 animal records were retained from a total of 31,694 records. Among the 682 animals, 131 were str. Pacifica-positive and 551 were str. Pacifica-negative (Table 5.4). These animals were from 44 herds, 17 of which were str. Pacifica-negative.

Descriptive data for categorical variables stratified by the animal's str. Pacifica status is captured in Table 5.4.

Table 5.4 A descriptive table presenting animal-level categorical variables stratified by str. Pacifica positive status of the animals based on data measured in the 2015-2016 milking season at the time of the first service from 682 unique animal records from 44 dairy herds in New Zealand. *p*-values presented in this table are obtained from χ2 tests.

	str. Pacifica Positive			
Characteristic	**N = 131[1]**	**%**	**95% CI[2] (%)**	***p*-value**
[3]PREGBY1ST				<0.01
0	54	41	33 - 50	
1	77	59	50 - 67	
[4]AGECAT				<0.01
1 (Under 3 years)	39	30	22 - 38	
2 (three to six years)	63	48	39 - 57	
3 (More than six years)	29	22	16 - 30	
[5]BREED				0.2
AYR	5	3.8	0.59 - 7.1	
FRI	68	52	45 - 62	

Characteristic	str. Pacifica Positive			
	N = 131[1]	%	95% CI[2] (%)	*p*-value
JER	58	44	36 - 53	
[6]MI				0.35
0	110	84	76 - 90	
1	10	7.6	3.9 - 14	
2	11	8.4	4.5 - 15	
[7]PREG				<0.01
0	15	11	6.8 - 18	
1	116	89	82 - 93	
[8]NM				0.29
1	103	79	70 - 85	
2	19	15	9.7- 23	
3	7	3	2.4-11	
4	2	1.5	0.3 – 5.9	

[1] n/N (%) number (percent);[2] CI= Confidence Interval; [3]PREGBY1ST= pregnancy to 1st mating, [4] AGECAT= animal age categorised into categories; [5]BREED[AYR]: animals of Ayrshire breed, BREED [FRI]: animals of Friesian, BREED[JER]: animals of Jersey breed;[6]MI=mating interval;[7]Preg =pregnant; [8]NM= number of matings recorded for animal

The bivariate relationships between pregnancy at the first mating as an outcome variable and independent variables are presented in Table 5.4. After variables with *p*-values <0.2 were added and removed stepwise from the final logistic regression model and their statistical relation to an outcome and to identify confounding variables, animal's str. Pacifica positive status *(p*-value = 0.74) and breed were identified as confounders (*p*-value =0.36) because of the change of the coefficient of any other

variable of more than 20% in the presence of a confounder as outlined in the methods sections. Thus, these were retained in the model.

Next, the GLMER model was constructed, where pregnancy to the first mating (PREGBY1ST) was an outcome variable, positivity to str. Pacifica (PACPOS), age (AGECAT), and breed (BREED) were independent variables, and the herd was included as a random effect variable. The results of the final GLMER model show that pregnancy at the first mating and cows' age were significantly associated (Table 5.5), as demonstrated by confidence intervals derived from the GLMER model, which did not cross zero. When exponentiated to OR, coefficients showed that cows of 6 years and older were less likely to get pregnant from the first mating (adj. OR 0.55, 95 CI: 0.35, 0.87) compared to cows in the younger group (under three years of age) when adjusted for the random effect of the herd. Both positivity to Pacifica (PACPOS) and breed (BREED) were not statistically significantly associated with pregnancy to the first mating, as shown by 95% confidence intervals (Table 5.5).

Table 5.5 The adjusted odds ratios derived from the GLMER model show the relationship between the outcome variable, the pregnancy to the first mating, and age and breed categories. Animal level data was collected in the 2015/2016 milking season and included 682 unique animal records from 44 dairy herds in New Zealand.

Parameter	**[1]OR**	**95% [2]CI**	
[3]PACPOS			
0	Ref		
1	1.05	0.69- 1.59	
[4]AGECAT			
1 (< 3 years)	Ref		
2 (3 -6 years)	0.93	0.65- 1.32	
3 (>6 years)	0.55	0.35- 0.87	
[5]BREED			
AYR	Ref		
FRI	1.27	0.36- 4.51	
JER	0.96	0.27- 3.43	
Random Effects			
Parameter SD (Intercept: HERD)	Coefficient		0.73

[1]OR= odds ratios;[2] CI= confidence interval,[3]PACPOS= positivity to str. Pacifica, [4]AGECAT: age category, [5]BREED[AYR]: animals of Ayrshire breed, BREED [FRI]: animals of Friesian, BREED[JER]: animals of Jersey breed

5.4.2 Herd-Level Descriptive, Bivariate, and Multivariable Analysis

At the herd level, raw data comprised 31,694 animal records for 77 herds. After data cleaning, 44 individual herd-level records were retained. There were 27 Pacifica positive herds and 17 Pacifica negative herds.

ANOVA test results showed that the final linear regression (model 3) performed better than intermediate models (model 1, model 2) (Table 6); however, none of the ANOVA p-values were statistically significant. In the final linear model, the outcome variable was the average values of first service to conception rate (1StCR), and the independent variables were average age (AVEAGE), str. Pacifica herd positivity (PACPOSH), average calving to conception interval (AVECTCI), and the herd size (SIZE). None of the included variables were statistically significantly associated with the calving to conception rate in this model (Table 6).

Table 5.6 The table compares the herd level intermediate (Model1 and Model2) and final linear multivariate (Model3) models where age and breed were independent variables and pregnancy to the first mating was the dependent variable. Data was collected in the 2015/2016.

Characteristic	**Model 1**			**Model 2**			**Model 3**		
	[1]Coeff	[2]Std.Error	*p*-value	[1]Coeff	[2]Std.Error	*p*-value	[1]Coeff	[2]Std.Error	*p*-value
[3]PACPOSH	0.57	0.036	0.787	0.012	0.036	0.7077	0.022	0.038	0.559
[4]AVECTCI				0.002	0.012	0.2392	0.002	0.002	0.198
[5]SIZE									
1 <399 cows		Ref					—	—	—
2 400-550 cows							0.01	0.04	0.767
3 >511 cows							-0.07	0.04	0.089
ANOVA F-Value	0.4795			0.2552			0.2392		

[1] Coeff = coefficient of the linear regression model, [2] Std.Error = Standard Error, [3]PASPOSH= Herd-level str. Pacifica positivity; [4]AVECTCI= the average age of the animals in herds, [5]SIZE=herd size

5.5 Discussion

In this study animal and herd-level analyses were performed to estimate the association between str. Pacifica positivity and dairy cows' reproductive performance.

On the animal level, the shared frailty model, when adjusted for effects of age and breed and random clustering effect of the herd, showed a statistically significant association (*p*-value= 0.01) between time to conception and positivity to str. Pacifica (HR=0.84; 95%CI: 0.74 - 0.96). Specifically, the estimates indicated that str. Pacifica-positive animals had 16% longer calving to conception interval than Pacifica-negative animals.

On the other hand, the final GLMER animal level model results showed that the association between pregnancy to the first mating and str. Pacifica positivity was insignificant (adj. OR 1.05, 95% CI: 0.69- 1.59) when age and breed were added to a generalised binomial model as fixed effects and herd as a random effect (Table 5.5). Similarly, the herd level results of the linear model (Table 5.6) showed no statistically significant associations between str. Pacifica positivity and the rate of pregnancy to the first mating, herd size, animals` average age, or calving to conception interval.

Therefore, the results of this study showed that str. Pacifica positivity is associated with a cow's time to pregnancy, as shown by the outputs of the shared frailty survival model, in which the calving to conception interval was included as a time variable, although there was no effect on the pregnancy rate. The difference in the results could be explained by the potential difference in the data structure, as the survival analysis data was left-censored, and survival analysis may be less biased by a 'healthy-worker effect'.

Calving to conception interval is often used to indicate the economic impact of herd reproductive performance. Increased calving to conception interval decreases milk yield compared to labour and feed costs (Cabrera 2014) and increases breeding costs and culling (González-Recio *et al.* 2004). Although there are no recent economic estimates available of the increased calving to conception costs for New Zealand dairy herds, in

the US, for every additional day of the calving to conception interval, average milk production decreases by 2.40 kg, resulting in a reduced number of calves, and accounted for an economic loss of 0.25 to 0.71 USD (Louca and Legates 1968). A more recent study from Korea estimated that an additional US$622.40 per cow was spent on animals that failed to get in-calf at the first mating, and these estimates included the cost of replacement heifers, treatment, semen, nutrition, calf price, and labour among the significant costs (Kim and Jeong 2019). Therefore, str. Pacifica positivity may potentially result in increased farming costs for Pacifica-positive New Zealand dairy herds, as it negatively affects the calving to conception interval, as shown by this analysis.

Calving to conception intervals differ in different milking systems; for example, in Ethiopia, the median calving to the conception interval is 154 days (Temesgen *et al.* 2022), and its length is very similar in other low-intensity milking system countries (Vargas *et al.* 1998; Punyapornwithaya and Teepatimakorn 2004). However, in the more intensive farming systems such as New Zealand, the recommended calving to conception interval is one of the shortest amongst high-intensity systems. It is reported to be 80-100 days (Ma *et al.* 2020). Within the New Zealand pastoral farming and breeding systems, where the calving interval averages 365 days, the cow should be mated in 12 weeks to allow farmers to meet market requirements (McNaughton *et al.* 2007). This is because, in such a system, the highest peak of milk production coincides with the highest grass growth rates in spring and summer, and in winter, the grass growth is not high enough to support milk production. Therefore, dairy cows are purposely selected to breed in a shorter period to align the highest production periods with the highest feed supply. Selection by the ability to breed in a short period is one of the attributes of high-performance dairy systems, where low-performing animals are culled after the first or the second lactation from the herd, and high-performing ones are retained (Craig *et al.* 2018).

Positivity to str. Pacifica may not be presented with acute leptospirosis symptoms and thus may not be associated with the overall ability of the cows to get in calf. It was reported that farmers participating in the seroprevalence study in their responses collected via questionnaire did not observe any leptospirosis-associated syndromes in

their dairy herds (Yupiana *et al.* 2019). Similarly, the absence of symptoms in a *L. borgpetersenii*-shedding animal was reported in the study where *L.borgpetersenii* serovar Tarassovi was isolated from an intermittently shedding dairy cow with persistent renal carriage (Hamond *et al.* 2022). However, "reproductive problems" were reported from the herd where this cow came from. In both studies mentioned above, cows were shedding str. Pacifica or were seropositive to Tarassovi, as confirmed by PCR results, also had elevated titres to Tarassovi.

The shedding of Pacifica, seroprevalence to Tarassovi, and the absence of clinical symptoms indicate that dairy cows may have adapted immune response to *L. borgpetersenii,* and cows have likely become the maintenance hosts of str. Pacifica in New Zealand. Literature suggests that no acute or mild symptoms or organ damage are observed in maintenance hosts (Faine 1994; Putz and Nally 2020; Monti *et al.* 2023), and this is typical for *Leptospira*'s non-symptomatic maintenance hosts (Chirathaworn *et al.* 2016). Experimental leptospiral infection in susceptible animal models shows that the immune cells produce an early inflammatory response to the first infection. Next, *Leptospira* is cleared out early from the blood with a response to early IL-10 and TNF- α production, and after this, infected animals' immune system reaches homeostasis, as described by Cagliero *et al.* (2018) in the paper investigating the inflammatory response to a *Leptospira* infection in susceptible animal models, where in response to infection the immune system reaches homeostasis earlier, the inflammatory stage shortens, and the immune system becomes adapted, defining host`s susceptibility. There are also reports that *Leptospira* adapts to particular hosts by switching on or off specific genes (Xue *et al.* 2010; Barbosa and Isaac 2020). It is unclear how the adaptation mechanism is inherited in *Leptospira,* but adaptation resulted in a mutually beneficial relationship between bacteria and host.

In the case of str. Pacifica, both the dairy cows and the strain may have had time to adapt to each other to reach homeostasis. Str. Pacifica may have been present in New Zealand since 1968 when the seroprevalence to *Leptospira* Tarassovi in New Zealand dairy cows was first reported at 4.8 % (Ryan and Marshall 1976). It is challenging to determine the timeline of str. Pacifica's arrival with accuracy. This is because the

method of differentiating between str. Pacifica and Tarassovi was only available in 2021 (Wilkinson *et al*., 2021). However, the retroactive molecular testing has detected str. Pacifica in cattle samples taken in 2007 (Wilkinson *et al*., 2023). Prior to this, serological methods could identify only Tarassovi antibodies, whose production is strongly correlated with str. Pacifica infection, i.e. the presence of str. Pacifica in dairy cows was confirmed by PCR retrospectively in 2019, and urine shedding was strongly associated with seroprevalence to Tarassovi (Yupiana *et al*., 2019). Although molecular identification did not accompany earlier seroprevalence studies, it is possible that seroprevalence to Tarassovi then was also Pacifica infection. Hence, between 1968 and 2021, while str. Pacifica became widespread in New Zealand dairy herds; dairy cows may have become its maintenance hosts and adapted to it, ensuring str. Pacifica survival.

Factors contributing to Pacifica's emergence could be due to an animal turnaround in herds. In 2014, 20% of the average herd was replaced annually in New Zealand (Edwards *et al.* 2014). Therefore, on average, 20% of replacement young animals are added to the herd, potentially increasing the proportion of the potentially naïve cow population, and consequently increasing re-infection rates. However, this may not be true when young and older cows are grazed together before entering the milking herd. Moreover, the intensification of dairying in recent years as shown by Stats NZ (2023), where the average number of cows in the national dairy herd increased from just over 3 million in 1968 to 6.2 million in 2018, may have contributed to this increase. Additionally, the practice of effluent spreading on paddocks may have contributed to Pacifica`s emergence, as *Leptospira* is spread from infected animal's urine and can survive in favourable damp and cool conditions (Haake and Levett 2014).

Another factor that could play a role in str. Pacifica`s presence in the national dairy herd is the widespread vaccination of dairy cows with vaccines containing *Leptospira* serovar Hardjo and *L. interrogans* serovar Pomona, which caused a significant reduction in leptospiral infection by these serovars and possibly leaving a "niche" for what was atypical at the time - infections with *L. borgpetersenii* serovar Tarassovi, which was

confirmed by PCR testing in 2019 as str. Pacifica in mainly younger animals (Yupiana *et al.* 2019).

In the final shared frailty survival model, age was not statistically significantly associated with time to pregnancy (*p-value* = 0.2). However, for the GLMER model on the animal level, age was statistically significantly associated with pregnancy, as defined by their CI ((95% CI, 0.35-0.87) Table 5.5). When individual animal data were adjusted for the random effect of the herd, the results showed that the cows of the age group older than six years were half as likely (adj. OR=0.55, 95% CI 0.35- 0.87) to get pregnant from the first mating when compared to the young (up to 3 years of age) group. These findings are consistent with other published reports, which show that pregnancy rates usually decline with age, regardless of the animal's health status. For example, (Gottschall *et al.* 2018), in a study of 868 cows from 3 to 11 years old (British breeds and crossbreeds) on a farm located in Brazil, shows that the pregnancy rate declined from 90.3% (6 years) to 77.1% (11 years). Another report (Gwazdauskas *et al.* 1975) mentioned that conception rates declined with age: heifers, 47.6%; young cows, 42.7%; older cows, 31.9%. As for the pregnancy to the first mating (Tillard *et al.* 2008), the study of risk factors affecting conception risk in cows on Reunion Island reported a similar trend where younger cows have a greater pregnancy to the first mating conception rates. Because the age of animals was not associated with time to pregnancy in survival analysis and the statistically significant association detected by the GLMER model on the animals' level showed reproduction trends in str. Pacifica-positive cows were similar to apparently healthy cows; these findings may signal the adaptation and maintenance of str. Pacifica to dairy cows, resulting in the survival of both bacteria and hosts.

There were two main limitations in this study. Firstly, the data used in this analysis was routinely collected and used primarily to evaluate cow production traits. Therefore, while specific reproduction categories were present, data were collected only a few times a year for some herds, and it was not available, nor was it collected only once per season for other herds. Thus, after data cleaning, the size of the dataset was reduced first from ~ 32,000 to 23,286 records and then to 682 records at the animal level. Additionally, records' accuracy may have been compromised in some cases due to recording errors.

Secondly, the use of the MAT as an identifier of recent infection to *L. borgpetersenii* serovar Tarassovi may have been unreliable, as animal seropositivity is subject to individual immunity response. Seroconversion may happen sooner in some animals or later in others, and titre may drop in one animal more rapidly than others. Moreover, the silent shedders that do not display MAT titres may not be identified (Zuerner 2014). Additionally, despite the MAT being a reference test recommended by the World Health Organization, the International Committee on Systematic Bacteriology, and the Subcommittee on the Taxonomy of *Leptospira*, it is considered serogroup rather than serovar specific, although in New Zealand, only 6 endemic serovars are reported, and from these six only two can cross-react. Therefore, these limitations may slightly influence the results.

5.6 References

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CHAPTER 6

Chapter 6 Exploring the Association Between *Leptospira borgpetersenii* Strain Pacifica and Milk Production of New Zealand Dairy Cows

6.1 Abstract

Aims: To investigate the association between *Leptospira borgpetersenii* strain (str.) Pacifica and milk production of dairy cows in New Zealand herds during the 2015-2016 milking season.

Methods: The dataset was created by matching individual animals` str. Pacifica PCR and serology test results from the 2016 study with the milk production data from the 2015/2016 milking season obtained from the Dairy Industry Good Animal Database (DIGAD). After data cleaning, the resulting dataset had 1443 repeated individual animal records from 43 herds. Positivity to str. Pacifica was defined as one or both of the following: results of Microscopic Agglutination Test (MAT) ≥48 to *Leptospira borgpetersenii* serovar Tarassovi antigen, as a proxy for str. Pacifica and, positive real-time PCR (qPCR) test result, where the amplicon sequencing of *gryB* and then *glmU* genes positively identified a sequence specific to str. Pacifica. To investigate the association of positivity to str. Pacifica with the milk production of dairy cows at the animal level, linear mixed effect models were fitted and adjusted for fixed effects of age, breed, and the random effects of the herd and animal. The outcome was milk production, defined as solids-corrected milk; values were standardised to 4% fat and 3.2% true protein per kilogram for each animal. At the herd level, a linear multivariable model was fitted to the data where 305-day milk production was an outcome variable and prevalence to str. Pacifica, average age, and herd size were independent variables.

Results: Statistical analyses showed no association between str. Pacifica positive status and dairy cows' milk production at both animal and herd levels

6.2 Introduction

Leptospirosis is a zoonotic disease caused by pathogenic spirochetes of the *Leptospira spp*., distributed worldwide and endemic in many countries. The *Leptospira* genus currently includes approximately seventy-one genomospecies (Korba *et al.* 2021) and more than 300 serovars worldwide (Thibeaux *et al.* 2018). Pathogenic *Leptospira* are able to establish chronic carriage in the kidney tubules of a large spectrum of wild and domestic mammals; moreover, amphibians, birds, and reptiles can also be infected (Picardeau *et al.* 2008; Vincent *et al.* 2019). Specific serovars are adapted to specific hosts, often identified in the literature as "maintenance hosts", a mammalian species in which the infection is endemic and is usually transferred from animal to animal by direct contact (Levett 2001). Maintenance hosts are essential links in the transmission of *Leptospira*. However, endemic serovars can also infect non-serovar-adapted or "accidental hosts". Accidental hosts are typically described as susceptible mammals that may develop a clinical disease of varying severity after being short-term renal carriers (Zuerner 2015).

Humans are considered accidental hosts of *Leptospira*, and the occupational nature of leptospirosis has been long-established (Bharti *et al.* 2003). People having day-to-day contact with animals are considered most at risk, with dairy, beef, and sheep farmers having the most at-risk occupations (Marshall and Manktelow 2002; Dreyfus *et al.* 2015; Benschop *et al.* 2021; Barragan *et al.* 2017).

There are many reports of the associations between *Leptospira* and dairy cows` milk production, and these effects are often associated with host maintenance status (Sykes *et al.* 2022). On the one hand, accidental leptospirosis in cows is often reported as an outbreak with acute disease symptoms, which may manifest as red water or haemoglobinuria, raised body temperature, mastitis, or a sudden drop in milk production, often described as a "flaccid udder" (Bautista *et al.* 2022). On the other hand, leptospirosis in cows, which are considered maintenance hosts of particular serovars, is often associated with subclinical mastitis and a decline in milk production (Alonso-Andicoberry *et al.* 2001; Bolin and Alt 2001; Ibrahim *et al.* 2022). Thus, the

severity and the impact of leptospirosis in dairy cows vary and depend on whether they are the maintenance or the accidental hosts of the infecting serovar (Putz and Nally 2020). The relationships between serovars and their hosts are complex. In New Zealand, dairy cows are considered maintenance hosts for *Leptospira borgpetersenii* serovar Hardjo and *Leptospira interrogans* serovar Pomona (Marshall and Manktelow 2002).

In New Zealand, the dairy industry generates 23% of export value (Yang *et al*. 2020), and dairy farming is considered one of the main contributors to the national GDP. To increase the dairy industry's profits, dairy cows are selectively bred to increase milk production (Bachman and Schairer 2003; McCarthy *et al.* 2013). For example, intensive genetic selection of the traits associated with high production in dairy cows in New Zealand resulted in an increase in the production of milk solids per animal by 290%, from 137 kilograms (kg) per cow in 1975–1976 to 397 kg per cow in 2020–2021 (Oltenacu and Broom 2010; Dairy NZ 2021). Milk is a profitable commodity and milk harvesting is the primary aim of dairy farming. The dairy industry's principal aim is to minimise losses and support milk production by controlling infectious diseases such as leptospirosis and reducing economic losses in dairy farming (Ellis 1984; Adler and de la Peña Moctezuma 2010).

Many countries successfully control leptospirosis in domestic and production animals by vaccinating against serovars that are endemic to particular countries or regions within countries (Bharti *et al.* 2003). These vaccines are serovar-specific and require annual boosters. Vaccination has been proven successful in reducing transmission between species and lowering the incidence of clinical cases in animals and humans (Thornley *et al.* 2002). For example, at the time of the study, in New Zealand, vaccines containing antigens to *Leptospira* serovars Pomona, Hardjo and Copenhageni are commercially available for use in cattle. The emergence of leptospirosis attributed to serovars that are not included in vaccines at the time (non-vaccine serovars) has also been reported (Moinet *et al.* 2017; Yupiana *et al.* 2019; Wilkinson *et al.* 2021; Wilson *et al.* 2021; Wilkinson *et al.* 2023). In particular, from 1968 to 2014, Tarassovi seroprevalence in New Zealand cattle increased from 4.3% (13/300) at MAT cut-off titre of ≥50 (Ryan and Marshall 1976) to 18% (698/3,878) at a cut-off of ≥48 (Wilson *et al.* 2021).

A serology and urine shedding study from 2015-2016 evaluated the status of 4,000 dairy cows. Cows were randomly selected from 200 farms and stratified by their geographical location and herd size (Yupiana *et al.* 2019). The study found that on the animal level, 17% of the study animals showed evidence of past infection with Tarassovi, as defined by at least one sample reacting at MAT ≥ 48. Further analysis showed a statistically significant association *(p-valu*e < 0.05) between urinary shedding and seropositivity to Tarassovi, and it was suggested that MAT titre ≥ 48 may indicate active urinary shedding. Moreover, urine samples from the cows that were shedding were typed using amplicon sequencing. Sequence data indicated the presence of a novel *Leptospira* species *borgpetersenii* in 68% (54/80) of samples (Collins-Emerson *et al*. 2021), referred to as *Leptospira borgpetersenii* str. Pacifica (Wilkinson *et al.* 2021). Because of this association, str. Pacifica is thought to belong to the serogroup Tarassovi (Wilkinson *et al.* 2023). Therefore, when identified by serology, Tarassovi infections in New Zealand animals and humans could at least partially be attributed to str. Pacifica. It is unknown when str. Pacifica emerged in New Zealand, as mostly serological methods were used in laboratory diagnostics. However, the retrospective molecular testing showed that str. Pacifica was present in New Zealand livestock as early as 2007 (Wilkinson *et al.* 2023). As a result of str. Pacifica detection by the work listed above, a vaccine containing Tarassovi antigen became available for dairy cows in early 2024 (Virbac 2024). However, its uptake is yet unknown.

Historically, Tarassovi leptospirosis in cattle was considered insignificant in New Zealand because no apparent clinical signs were associated with Tarassovi seroprevalence in dairy cows (Ryan and Marshall, 1976). There are also no reports on the specific effects of *Leptospira borgpetersenii* strain Pacifica on the milk production of dairy cows. It is important to investigate a possible relationship between str. Pacifica positivity and dairy cow milk production to determine if any preventable losses could be addressed to ensure milk production continuity, keeping in mind that 76% of dairy herds in New Zealand are seropositive to Tarassovi (Yupiana *et al.* 2019)

Thus, the main aim of this work was to investigate via analysis of the combined herd test data and animal test results, whether positivity to str. Pacifica is associated with the

milk production of dairy cows, defined as solids-corrected milk and 305 days milk production during the 2015-2016 milking season.

6.3 Methods

6.3.1 Data Sources

Two data sets were used for this analysis. The first dataset was collected during a study investigating serological *Leptospira* prevalence and urinary shedding in New Zealand dairy farms in the 2015-2016 milking season (Yupiana *et al.* 2019). In that study, 200 dairy farms in New Zealand were randomly selected, and the farms were stratified by the number of herds and the farm's geographical location by researchers. Researchers collected urine and blood samples from 20 random animals from each participating farm (Yupiana *et al.* 2019). At the time, whole blood samples were coagulated and spun on arrival at the laboratory. Separated sera samples were tested against leptospiral antigens: *Leptospira borgpetersenii* serovars Hardjo, Ballum, and Tarassovi and *Leptospira interrogans* serovars Pomona and Copenhageni by microscopic agglutination test (MAT) as described by (Faine 1982).

Additionally, DNA was extracted from urine samples and tested by qPCR, using *gyrB* as a target gene for *Leptospira* DNA (Slack *et al.* 2009), as described by Subharat *et al.* (2011), Fang *et al.* (2014) followed by molecular testing using *glmU* as a target gene (Wilkinson *et al.*2021). MAT and PCR test results were recorded in a Microsoft Access database. As a result, 4,000 individual animal data recordings, including unique animal and herd IDs, complemented by MAT and PCR test results, became available.

For the current study, we selected two "types" of herds:

(1) high risk, where at least one of the 20 cattle sampled were one or both of the following: had a positive serology test (MAT ≥ 48) for Tarassovi, as a proxy for str. Pacifica, and tested PCR positive for str. Pacifica at cycle threshold (Ct) ≤40 using *glm*U as a target gene for *Leptospira* DNA.

(2) low-risk herds where no PCR positives were detected, and serology tests to Tarassovi were negative (MAT ≤ 24)

The second dataset was the animal herd test data for 77 herds, extracted from the DIGAD, a subsidiary of DairyNZ (access number AP 125). It included 34,652 individual animal records. The DIGAD holds animal production and reproduction data from several private companies that provide herd test services to New Zealand's dairy farmers.

Data fields extracted for this study included unique animal ID, herd participant code and supplier number, animal's date of birth, herd relationship start and end dates (when the animal entered and was removed from herd records), breed code, and animal fate. The following records were also extracted: calving date, number of calves, calf fate, drying off date, days in milk, and drying off description. Additionally, lactation information was extracted, such as the number of lactations, fat and protein percentages, somatic cell count (SCC), and the dates of the herd tests. Extracted data contained records from 1/06/2015 to 31/05/2016. This period represents a typical austral spring milking season (Dairy NZ, 2021).

6.3.1.1 Animal Level Data Categorisation

For this study, two data sets were merged by individual animal IDs, and the duplicate rows were removed; data were cleaned and, as a result, two subsets of the raw data were retained for further analysis. The subset on the animal level included 1443 repeated animal records from 43 herds. In New Zealand, the production of dairy cows is measured several times per milking season via a herd test. The recommended number of tests per season is four times throughout lactation stages: 61-120, 121-180, 181-240, and 241-300 days of lactation (Dairy NZ 2021). In the dataset used in this analysis, milk production was measured from one to eight times per year, but 2/3 of animals with production data recorded had three herd tests per milking season. Therefore, the individual animal-level data had repeated production measurements per animal.

Production Variables

Fat and protein-corrected or solids-corrected milk (SCM) was standardised to 4% fat and 3.2% true protein per kilogram using the following equation from the International

Dairy Federation (IDF) (2015): (kg/test) = milk production(kg/test) × (fat%/4 + protein%/3.2) and this variable was the outcome variable in this analysis (Table 6.1)Morning and afternoon test results were combined for each animal`s herd test to get a daily production herd test result. Average values for individual cows per herd test for protein (%), fat (%), and weight (kg) were calculated.

Leptospira Test Results Variables

On the animal level, the binary variable PACPOS was created by combining individual cow test results that were one or both of the following: MAT positive, PCR (≤ 40 Ct) positive, and both MAT and PCR (≤ 40 Ct) positive results. MAT positive status was a titre ≥48 to *Leptospira borgpetersenii* serovar Tarassovi antigen. If positive, PACPOS was recorded as one (1); if negative, it was recorded as zero (0) (Table 6.1).

Demographic Variables

The age of the animals was calculated by approximating their date of birth from the approximate mid-milking season date of the 1st of October 2015 and converted to the number of years. Then, all animals were divided into three age groups (AGECAT): under three years of age, three to six years, and greater than six years. Breed code (BREED) was available in the extracted data and was categorised into three categories: Ayrshire, Friesian, and Jersey. Crossbreeds were assigned their primary breed category. Days in milk (DIM) for each animal were already available from the original dataset. Each animal in the data set had a unique ID; this variable was denoted as the ANIMAL ID variable. Additionally, every herd included in the analysis was coded under the HERD variable, where each herd was assigned a unique numerical ID.

Table 6.1. A description of the animal level variables used for the animal level analysis from the dataset containing herd test records of 2015/2016 milking seasons from 43 New Zealand dairy herds.

Variable	Description
PACPOS	str. Pacifica status
0	negative when MAT <48 and/or qPCR>40
1	positive when MAT ≥48 and/or qPCR≤40
AGECAT	age of the animal
1	1-3 years
2	4-6 years
3	>6 years
BREED	breed of the animal
AYS	Ayrshire
FRI	Friesian
JER	Jersey
SCM	solids-corrected milk was standardised to 4% fat and 3.2% true protein per kilogram
ANIMAL ID	unique animal identifier
HERD	unique herd identifier
DIM	Days in milk, showing for how many days the animal was milked

6.3.1.2 Herd-Level Data Categorisation

Herd-level data included records from 43 herds, 33 of which had str. Pacifica prevalence greater than zero.

Production Variables

Each herd`s average 305-day milk production (HM305) was adjusted for days in milk (DIM) variable calculated as described by Cole and VanRaden (2006).

Leptospira Test Results Variables

On the herd level, the prevalence rate (PREV) was calculated using the PACPOS variable, where the number of positive animals (PACPOS=1) was divided by the number of tested animals (N=20) in each herd (Table 6.2).

Demographic Variables

The average age in the herd, AVEAGE, was calculated for each herd. Also, the herd size was coded as SIZE and split to obtain three somewhat equal groups into three categories: 1 if the herd had less than 399 cows, 2 if it had more than 400 but less than 550, and 3 if it had 551 or more cows (Table 6.2).

Table 6.2 A summary of the herd-level variables used for the herd-level analysis from the dataset containing herd test records from 43 New Zealand dairy herds' 2015/2016 milking seasons.

Variable	Description
PREV	Prevalence of str. Pacifica positivity in the herd: the number of positive animals (PACPOS=1) was divided by the number of tested animals (N=20)
AVEAGE	The average age of the animals in the herd
SIZE	Herd size:
1	<399 cows
2	400-550 cows
3	>551 cows
HM305	Herd`s average 305-day milk production

6.3.2 Statistical Analyses

All data manipulations were done using R-Studio version 4.2.1, the R Foundation for Statistical Computing (R Core Team 2021). Analyses were performed using R with the packages 'epiR' (Stevenson *et al.* 2018), 'tidyr' (Wickham and Henry 2018), 'lme4' (Bates *et al.* 2009), and 'ggplot2' (Wickham 2011). Statistical significance was set at a *p*-value ≤ 0.05.

6.3.2.1 Animal Level

Descriptive Analyses

Continuous variables were described by their standard deviation, means, and interquartile ranges. Categorical variables were described by counts and two by two tables. Next, the chi-squared ($\chi 2$) tests were performed to establish the associations

between the variables of interest, including the outcome variable SCM and the independent variables PACPOS, BREED, and AGECAT. The Goodman-Kruskal asymmetric test (Somers 1962) was applied to compute associations between all pairwise combinations of categorical variables in the data sets to measure the correlation between variables.

Bivariate Analyses and Preliminary Models

On the animal level in each dataset, the bivariate relationship between the SCM, age, breed of animals, and their positivity to str. Pacifica was evaluated using bivariate linear models. Then, the stepwise screening method was applied, where variables of interest were added and then removed from the model. Variables with *p-values* (≤0.2) identified by the Likelihood Ratio (LRT) test were retained for the preliminary and final linear mixed-effect models.

Confounding variables were identified by a change of the coefficient of any other variable of more than 20% in the presence of a confounder and their association with both dependent and independent variables, assessed by $\chi 2$ tests, as recommended by (Dohoo *et al.* 2003). When any variable was found to be a confounder, it was retained in the model regardless of its statistical significance.

Interactions were tested by adding biologically plausible interactions into the preliminary final linear regression model. The statistical significance of *p-value* ≤ 0.05 of an interaction multiplicative term was a threshold for retention.

Final Models

The generalised linear mixed effect models were used for the multivariate analyses on the animal level. Mixed effects models containing fixed and random effects are considered the gold standard for addressing are justified when group factors are correlated (Dohoo et al. 2003). Therefore, their use in this analysis to account for data clustering on the animal and herd levels is justified. The stepwise forward and backward elimination approach was applied to construct mixed models, and the HERD and ANIMAL ID variables were added as random effect variables to account for multiple

data records on the animal level and for clustering effect on the herd level. The predictors in mixed effect models were retained in the model when 95% probability intervals of their corresponding regression coefficients did not overlap zero. Models were tested by evaluating their convergence, divergence, and *p*-values and comparing the models 'Akaike information criterion (AIC) values. Thus, in the final mixed effect model, SCM was the outcome variable, PACPOS, AGECAT, and BREED were independent variables, and HERD and ANIMAL ID were clustering variables. The model's AIC values were compared to the final and intermediate models, and the model with the lowest AIC was chosen.

6.3.2.2 Herd Level

Bivariate Analyses and Preliminary Models

Bivariate screening of the effects of the continuous outcome variable HM305 on PREV, AVEAGE, and SIZE was performed via constructing bivariate linear regression models. Variables with statistically significant *p*-values (≤0.2) identified by the $\chi 2$ test were retained in the preliminary linear model. Interactions were tested by adding biologically plausible interactions into the preliminary linear regression models.

Multivariate Analyses

Next, the stepwise forward and backward elimination approach was applied to the preliminary linear regression model. Variables with statistically significant *p-values* (≤ 0.2) identified by the Likelihood Ratio (LRT) test were retained. Thus, on the herd-level data in the final linear regression model, HM305 was the continuous outcome variable, and PREV, AVEAGE, and SIZE were independent variables. Statistical significance of *p*-value ≤ 0.05 for an interaction multiplicative term was the threshold for retention. Interactions and confounding variables were identified as described in the animal-level methods section.

6.4 Results

6.4.1 Animal-Level Results

6.4.1.1 Descriptive Analysis Results

On the animal level, there were 1443 repeated animal records. The number of animals participating in the Herd Test 1 included 526 individual animal records, 405 in Herd Test 2, 322 in Herd Test 3, 178 in Herd Test 4, and 12 in Herd Test 5. Thus, the number of in-milk animals participating in herd testing between each subsequent herd test, apart from herd test 5, was reduced by approximately 20% within one milking season. The distribution of the age categories between different herd tests remained very similar (Table 6.3).

Table 6.3 Animal level data showing the number and percentage of animals in each corresponding category. Data for this analysis were collected over the 2015/2016 milking season from 43 New Zealand dairy farms.

Characteristic	**[1]N**	**%**	**95%[2] CI**
[3]PACPOS			
0	404	77	74-80
1	122	23	20-26
[4]AGECAT			
1	198	38	35-41
2	208	40	37-43
3	120	22	20-25
BREED			
[5]AYR	15	3	2-4
[6]FRI	322	61	58-64
[7]JER	189	36	33-39

[1]N= the number of animals in each category; [2] CI=confidence interval; [3]PACPOS= positivity to str. Pacifica; [4]AGECAT= age category; [5] BREED[AYR]=animals of Ayrshire breed,[6]BREED [FRI]= animals of Friesian breed, [7]BREED[JER]=animals of Jersey breed

There were 122 str. Pacifica positive and 404 str. Pacifica negative animals. The average values of fat, protein, somatic cell count, solid-corrected milk, and age for all herd tests were very similar in str. Pacifica positive and negative groups (Table 6.4).

Table 6.4 Descriptive animal-level analysis of continuous variables from the 2015/2016 milking season. Data were collected from 43 farms, stratified by positivity to str. Pacifica, as identified by MAT≥48 to Tarassovi and Ct ≤40 for PCR.

Characteristic	Pacifica Negative [1]N = 404	95% [2]CI	Pacifica Positive [1]N = 122	95% [2]CI
[3]PROT (%)	3.79	3.6-3.9	3.77	3.6-3.9
[4]FAT (%)	4.78	4.5-4.9	4.64	4.5-4.8
[5]SCM (kg/test)	21.03	19-22	23.23	21-25
[6]DIM (days)	278	275-281	275	273-278

[1] N=Number of animals [2] CI = Confidence Interval; [3]PROT=protein milk content, measured in kilograms; [4]FAT= protein milk content, measured in kilograms; [5]SCM=solids corrected milk measured in kg/test;

Multivariate Analyses Results

In the mixed effects models fitted to the dataset, PACPOS was not significantly associated (*p-value* >0.05) with SCM when adjusted for the effects of BREED and the random effects of ANIMAL ID and HERD.

In this analysis, AGECAT was statistically significantly associated (95% CI; 2.12-3.45 and 1.46- 3.15) with solid-corrected milk when adjusted for the effects of PACPOS and BREED and the random effects of ANIMAL ID and HERD (Table 6.5), and both categories of older cows produced more than 2 kg of solids-corrected milk than younger cows (0-3 years) (Table 6.5).

Table 6.5 Results of the mixed-effects linear model fitted to the animal level of the herd test data from 43 New Zealand dairy herds from the 2015/2016 milking season. Analysis in each subset included ANIMAL ID and HERD as fixed-effect variables.

Predictors	**Estimates**	**[1]SE**	**95% [2]CI**
[3]PACPOS			
0	Reference		
1	-0.18	0.39	-0.94-0.59
[4]AGECAT			
1	Reference		
2	2.78	0.34	2.12-3.45
3	2.31	0.43	1.46-3.15
BREED			
[5]AYR	Reference		
[6]FRI	-0.08	1.77	-3.53-3.38
[7]JER	-1.44	0.77	-4.91- 2.02

Groups	Name	Variance	Standard Deviation
Animal ID (Intercept)		8.774e-07	0.0009367
Herd (Intercept)		2.875e-07	0.0005362
Residual		5.133e-06	0.0022656

Number of observations: 1443; groups: Animal ID, 526; HERD, 43

[1]SE= standard error;[2]CI = Confidence Interval; [3]PACPOS= positivity to str. Pacifica; [4]AGECAT= age category; [5] BREEDS [AYR]=animals of Ayrshire breed,[6]BREED [FRI]= animals of Friesian breed, [7]BREED[JER]=animals of Jersey breed

6.4.2 Herd Level Results

On the herd level, the prevalence rate of str. Pacifica was greater than zero in 36/43 herds. The table below shows bivariate relationships between HM305 and independent variables, specifically, AGEAVE, PREV, and SIZE (Table 6.6).

Table 6.6. Bivariate associations between herd's average 305-day milk production and age, str. Pacifica prevalence and herd size were drawn from herd-level herd test data from the 2015/2016 milking season.

Characteristic	**Estimates**	**95% CI[1]**	***p-value***
[2]PREV	0.97	0.54-1.23	0.11
[3]AGEAVE	1.5	1.2- 1.7	<0.02
[4]SIZE			
1 (<399)	—	—	—
2 (400-550)	-0.57	-0.65- 1.7	0.736
3 (>550)	1.16	0.89- 1.5	0.468

[1]CI = Confidence Interval; [2]PREV= str. Pacifica prevalence; [3]AGEAVE= age category; [4]SIZE= size of the herd

In the multivariable linear model, HM305 was an outcome, and PREV and AGEAVE were independent variables. As shown by the *p-values* in Table 6.7, none of the independent variables was statistically significantly associated with an outcome.

Table 6.7. Estimates from the multivariable linear model drawn from herd-level, herd test data from the 2015/2016 milking season, where the herd`s average actual 305-day milk production was an outcome, and average age per herd was included as an independent variable.

Predictors	**Estimates**	**95% [1]CI**	***p-value***
(Intercept)			
[2]PREV	0.36	-5.54- 6.26	0.90
[3]AGEAVE	0.94	-0.27-2.15	0.13

[1] CI = Confidence Interval; [2]PREV=Pacifica prevalence; [3]AGEAVE= age category.

6.5 Discussion

This analysis aimed to investigate the association of str. Pacifica and the milk production of dairy cows in New Zealand dairy herds.

The results showed that positivity to *Leptospira borgpetersenii* strain Pacifica in high-prevalence herds, as defined in this work, was not associated with dairy cows' milk production on both animal and herd levels.

Early researchers evaluated the possible effect of seroprevalence to Tarassovi on the production of dairy cows and concluded that there was no apparent drop in milk production or clinical signs associated with Tarassovi leptospirosis (Ryan and Marshall 1976; Milner *et al.* 1980). However, whether Tarassovi infections reported before 2021 could be attributed to str. Pacifica is unknown because the molecular test method that differentiates Tarassovi and str. Pacifica was only published in 2021 (Wilkinson *et al.* 2021). However, since then the DNA of str. Pacifica has been retrospectively detected in cattle and deer samples dating back to as early as 2007 (Wilkinson *et al.* 2023). The first report of str. Pacifica shedding in New Zealand dairy cows seropositive to Tarassovi was published by Yupiana *et al.* (2019), who investigated the seroprevalence to *Leptospira* in dairy herds. As a part of this investigation, farmers and the farms' usual

veterinarians were asked to report any clinical signs or production effects that could be attributed to leptospirosis (Yupiana *et al.* 2019). None of the responders reported any production-associated effects attributable to leptospirosis. Although there are no published reports on the prevalence or detection of *Leptospira* strain Pacifica in overseas herds, researchers in the USA have reported the isolation of *Leptospira borgpetersenii* serovar Tarassovi from a dairy cow. Additionally, in the herd to which this cow belonged, there were no reported effects on milk production associated with leptospiral infection (Hamond et al. 2022). Therefore, the lack of production effects attributed to str. Pacifica or Tarassovi found in earlier work supports the findings of the current analysis, where no association was detected between str. Pacifica and milk production.

There could be a few reasons why no association was detected. Firstly, the lack of effects could be due to the absence of an association between milk production and positivity to str. Pacifica in this analysis. The absence of production effects or clinical manifestations in animals seropositive to Tarassovi and shedding str. Pacifica in this study, suggests that dairy cows serve as maintenance hosts of str. Pacifica. Literature reports that it is typical that no acute symptoms or organ damage are observed in infected maintenance host animals (Faine *et al.* 1999; Putz and Nally 2020; Monti *et al.* 2023). The mechanism of immune adaptation of animals to *Leptospira*, resulting in this beneficial bacterium-host relationship, is not fully understood. However, experiments with susceptible animals show that the inflammatory stage of the immune response shortens over time determining the host`s susceptibility to *Leptospira spp* (Cagliero *et al.* 2018). Moreover, recent research showed that *Leptospira* can also adapt to particular hosts or environments by switching on and off specific genes (Barbosa and Isaac 2020).

Additionally, as shown in Chapter 4 of this thesis, str. Pacifica was still circulating in the same New Zealand dairy herds 6 years after initial detection in 2015/2016. Also, it likely took some time for dairy cows to develop immune adaption mechanisms that would allow them to become str. Pacifica maintenance hosts since str. Pacifica emergence, and similarly, str. Pacifica's adaptation to dairy cows may also have been happening

over time. However, it is unknown when str. Pacifica emerged, and it could be argued that the early Tarassovi antibody detections in dairy cows could also be partially attributed to str. Pacifica, as discussed in the previous paragraph. Thus, no production effects in Pacifica-shedding herds point to the well-developed adaptation of both bacteria and dairy cows, therefore adding further evidence towards the maintenance-host relationship of dairy cows and str. Pacifica.

Secondly, the probable reason why no effect of str. Pacifica positivity on milk production was detected could be attributed to the low frequency of herd test measurements, as most animals were herd tested two or three times per season. Suppose it assumed that the effect of str. Pacifica on dairy cows` production is similar to Hardjo's effects because they belong to the same species (*Leptospira borgpetersenii).* Hardjo's effects on milk production include a “milk drop syndrome”, mastitis, and the “flaccid udder syndrome” which could last for one or two weeks (Alonso-Andicoberry *et al.* 2001; Bolin and Alt 2001; Ibrahim *et al.* 2022). In that case, the hypothetical effect of the "milk drop syndrome" due to str. Pacifica may also last for one or two weeks in individual animals and may therefore be undetected when animals that are typically herd tested three to four times a year.

Additionally, supposing the possible "milk drop syndrome" is accompanied by mastitis due to str. Pacifica infection occurring during the herd test, then this animal would be excluded from the analysis because farmers in New Zealand commonly exclude animals with mastitis from the herd test. Therefore, hypothetical str. Pacifica-induced mastitis or the milk drop may be undetected due to hypothetical str. Pacifica-infected animals are not being herd tested.

Thirdly, as shown in Chapter 4 of this thesis in the str. Pacifica shedding herds, an active infection peaked at the start of the milking season or approximately at the time of the first herd test. Milk production in New Zealand dairy herds also peaks at the time of the first herd test, as shown by Edwards (2019) in their paper on the analysis of milking characteristics in New Zealand dairy cows. Specifically, the author showed that the milk yield was the highest in the first 60 days of milking at an average of 12.07 kg/milking of

milk compared to 11.02, 9.76, 8.92, and 7.45 kg/ milking at 61-120, 121-180, 181-240 and 241-300 days, accordingly, based on the data collected at four herd tests from two farms. Therefore, a potential "milk drop syndrome" may not be detected due to a cumulative effect of the milk output increase at this lactation stage, as the increased milk production coincides with the time of active infections in the herd.

Lastly, it is possible that the emergence of str. Pacifica, confirmed in 2015 by molecular methods in cows seropositive to Tarassovi (Yupiana *et al.* 2019), could have been happening or accompanied simultaneously by intensive genetic selection for milk production traits targeted by New Zealand breeding companies over the past fifty years (Harris and Clark 1996). Due to selective breeding, milk production per dairy cow increased by 290% from 137 kilograms per cow in 1975/1976 to 397 kg per cow in 2021 (Dairy NZ 2021). Therefore, selective genetic breeding for increased milk output may also have been coincidentally helping to select NZ dairy cows based on their adaptiveness to str. Pacifica, thus minimising its effect on milk production. The possibility of the use of genetic selection to reduce the prevalence of endemic infection diseases was recently reported in a study that used stochastic simulation models for population and herd heritability models (Hulst *et al.* 2021). For example, the selection study observed a 15% reduction in clinical mastitis prevalence after 5 generations of single trait selection, compared to only 8% predicted by estimated breeding values (Halloran *et al.* 1992).

In this analysis, age was significantly associated with standardised milk production on the animal level in high-risk *Leptospira borgpetersenii* str. Pacifica herds, when adjusted for effects of str. Pacifica, breed, and random effects of animal and herd (Tables 4). There was a difference in standardised solid-corrected milk output between age groups on the individual animal level. For example, solid-corrected milk output in both older age groups was greater than in the younger group of cows under three years by at least 2 kg per test when adjusted for the random effects of animal and herd and fixed effects of str. Pacifica and breed. Therefore, these results show that the youngest animal group cows in the high-risk str. Pacifica herds had lower milk output than the older age groups. In general, healthy older cows produce more milk than younger cows, as reported by

Edwards (2019), where the average milk yield produced by the heifers was 7.23 kg/milking, compared with older animals at 8.00 kg/milking. Additionally, Dairy NZ (2021) reported that six-year-old cows were the highest-producing age group in 2019/2020, and five-year-old cows were the highest-producing age group in the 2018/2019 season. These results indicate that in this analysis, the milk production trend in different age groups was similar to that of healthy New Zealand dairy herds.

Similarly, in this analysis, the breed of the animal was also statistically significantly associated with standardised milk production; Friesians were producing the most milk, followed by Jerseys and then Ayshires (Table 5). In the published literature and scientific research, there is no agreement on which breed of dairy cows produces more milk. For example, an Australian study shows that Holstein and Friesian crossbreds produced 22 kg more than Jerseys and 12 kilograms more than Ayrshires per season (Madgwick and Goddard 1989). On the contrary, in a paper where the efficiency of energy conversion in primiparous Jersey and Friesian cows were compared, Mackle *et al.* (1996) commented that healthy Jersey cows produced more SCM than Friesians under the same conditions. Therefore, while different studies show that milk production varies between breeds, most agree that, in general, the breed of the cow is associated with milk production (Meyer *et al.* 1994; Oltenacu and Algers 2005; Walsh *et al.* 2008; Lu *et al.* 2020). Therefore, a significant association between milk production and breed detected by this analysis is what is commonly found in healthy herds.

Thus, the absence of str. Pacifica's effect on cow production, combined with a statistically significant association of age and breed with production commonly detected in healthy herds, may indicate that dairy cows in New Zealand could already be well adjusted to str. Pacifica's effects on their production. However, it is important to note that str. Pacifica shedders can infect people such as dairy farmers, milkers, and farm workers who are in regular contact with str. Pacifica-shedding animals. Str. Pacifica poses a public health concern, as previously stated by Yupiana *et al.* (2019). Additionally, amplicon sequencing of the *glmU* gene positively identified a sequence specific to str. Pacifica in the specimen collected from a notified case of leptospirosis in a dairy farmer (S. Nisa, *personal communication*, March 25, 2024). Therefore, the

prevention of str. Pacifica transmission via vaccination or the use of appropriate personal protective gear should be prioritised.

One of the limitations of this study includes possible data collection errors and biases. The DIGAD data relies on the accuracy of the farmers' record-keeping and identifying individual animals. Record keeping is a tedious task, and it is common for farmers to have some unresolved data entry and animal and calf identification errors in their herd records. An additional source of data errors could come from the identification of milk samples during herd tests; sometimes, during herd tests, pottles of milk samples are mislabelled, and the test results of one animal could be assigned to another. However, it is important to note that in this analysis, all efforts were made to rectify mistakes by comparing herd records from consecutive seasons. Other possible errors include the mislabelling of blood and urine samples during collection and possible labelling errors during sample processing in the laboratory. Such errors could lead to test results being incorrectly matched to animals, which could have influenced the results and introduced non-differential bias.

Moreover, the production effect might be too small to be detected with the power of the current study, as the power of the study and sample size calculations were obtained from non-str. Pacifica-specific scientific literature. Thus, this study may help to identify if str. Pacifica sample size needed to be increased to detect production effects in future research.

One more source of potential errors in this study was the use of the MAT as one of the identifiers of positivity to str. Pacifica. Although MAT is considered a "gold standard" test for identifying serovars, and it is also a reference test recommended by the World Health Organization and the International Committee on Systematic Bacteriology, MAT is considered serogroup-specific rather than serovar-specific (Levett 2001). However, where all present serovars are known to belong to different serogroups, serovars can be identified with high confidence. Additionally, reading the MAT involves a subjective interpretation of the degree of agglutination. Therefore, interpretation bias may be introduced into test results, which may have slightly altered reported MAT values.

6.6 Conclusion

This study analysed the association between *Leptospira borgpetersenii* strain Pacifica on dairy cows' production as measured by their standardised solids-corrected milk output and the herd`s average actual 305-day milk production. Although this analysis found no statistically significant association in the putative highly adapted maintenance host population of New Zealand dairy cows. A possible reason for this could be that New Zealand dairy cows adapted to str. Pacifica and became maintenance hosts of this strain and that str. Pacifica developed adaptation mechanisms allowing for its maintenance within New Zealand dairy cows. Future studies, including targeted data collection, improved sample size at the start of the milking season and further analysis may contribute to a better understanding of str. Pacifica's effects on milk production.

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CHAPTER 7

Chapter 7 General Discussion

7.1 Introduction

Leptospirosis remains a major global zoonosis, and in New Zealand, it is considered the most important zoonotic occupational disease. A 2016 study found that on the animal level, 17% of the study animals showed evidence of past infection with Tarassovi, as defined by a sample reacting at MAT ≥ 48. Additionally, 96% of urine samples were PCR-positive for *Leptospira*, with 68% (54/80) containing a novel allele in the *glmU* gene, referred to as *Leptospira borgpetersenii* strain Pacifica (Yupiana *et al*. 2019; Collins-Emerson *et al*. 2021; Wilkinson *et al.* 2021). This novel strain is genetically different from the Tarassovi reference str. Perepelitsin, but it is thought to belong to the *Leptospira borgpetersenii* serogroup Tarassovi because of the strong association between seropositivity to serovar Tarassovi and the shedding of Pacifica (Yupiana *et al.* 2019). It was shown that str. Pacifica is commonly shed by dairy cows into their environment, potentially presenting a risk of infection for dairy workers (Wilkinson *et al.* 2021). There was a reported incidence of 12.59/100,000 in notified human cases of leptospirosis attributed to Tarassovi from 1999 to 2017 in New Zealand dairy farmers (Nisa *et al.* 2020). Thus, due to the recent detection of str. Pacifica, its epidemiology, morphology, maintenance in the host population, and associations with the host's health, as well as the transmission mechanism, were not understood well.

This thesis starts with a comprehensive literature review focusing on serovar Tarassovi (Chapter 1). This is followed by analytical sections with the following aims. Firstly, to evaluate different media types for their suitability to support the isolation and growth of str. Pacifica culture from the urine of dairy cows under laboratory conditions (using laboratory-adapted strains of *L. borgpetersenii* as its proxy) (Chapter 3). Secondly, to investigate the epidemiology of str. Pacifica in dairy herds (Chapter 4) by comparing serology and shedding at the start and the end of the milking season in herds previously identified as str. Pacifica positive. Assessment of a possible association between str. Pacifica and reproduction (Chapter 5), and milk production of dairy cows (Chapter 6),

using linear, logistic, mixed models containing fixed and random effects, and shared frailty survival statistical models, formed the final two aims.

This thesis was formatted and presented as independent chapters suitable for peer-reviewed journal publications and each chapter already includes an extensive discussion of the relevant findings. Thus, this chapter is a brief discussion of the major findings of the thesis. They are summarised under the headings 'Isolation of str. Pacifica', 'Maintenance hosts status of str. Pacifica of dairy cows', and 'Public Health importance of str. Pacifica'. This chapter then reviews experimental designs and methodologies and suggests areas for future research.

7.2 Main Findings

7.2.1 Isolation of Strain Pacifica

Chapter 3 of this thesis showed that str. Pacifica is a fastidious bacterium that requires modified growing conditions, different from the conditions sufficient for the maintenance of the laboratory-adapted strains. It also requires media different from the media used for the isolation of other *Leptospira spp*. strains from deer, sheep, cattle, and wildlife by ^{m}Epilab researchers (Subharat *et al.* 2011; Fang *et al*. 2014; Vallée *et al*. 2015; Moinet *et al.* 2023). In this thesis, we showed that str. Pacifica was successfully isolated from the urine of dairy cows, but it failed to grow. We also confirmed that the HAN media at 37°C and 5% CO_2 is suitable for str. Pacifica's initial isolation. The possible reasons for losing the culture after successful isolation are discussed in Chapters 3 and 4. The isolation of str. Pacifica is essential to genetically describe the strain, allowing differentiation from known strains and to precisely identify the causative agent of infection. Obtaining an isolate will allow for the development of a strain-specific vaccine and facilitate future challenge studies.

An essential prerequisite to the isolation of *Leptospira* species is identifying suitable conditions and a medium that will support the growth of bacteria and provide the essential nutrients. Overall, *Leptospira spp*. are known to be fastidious and slow growing (Rahelinirina *et al.* 2019), and *Leptospira borgpetersenii* are reported to have

preferred medium and growth conditions (Haake and Levett 2015). There are no reported successful isolations of *Leptospira borgpetersenii* serovar Tarassovi from New Zealand cattle, despite widely reported cattle seropositivity in New Zealand at MAT ≥ 48 (Ryan and Marshall 1976; Yupiana *et al.* 2019; Wilson *et al.* 2021). This thesis provided further evidence towards understanding the epidemiology, and maintenance within the host population (Chapter 4). Therefore, these findings could provide the guidelines for future research and its possible directions are discussed under the heading ‘suggestions for future research.

7.2.2 Str. Pacifica Maintenance Host Status of Dairy Cows

In this work (Chapter 4), we identified shedding patterns of str. Pacifica in 16 dairy herds identified as str. Pacifica positive in a 2016 study (Yupiana *et al.* 2019; Wilkinson *et al.* 2021) and from one additional herd in a follow-up 2022 sampling. We collected urine samples from 564 primiparous dairy cows, from those, 54 str. Pacifica shedders were confirmed by a PCR assay targeting the *glmU* locus. The results of this study also showed that in 2020, in sixteen high-risk herds, 63% (95%; CI:31.6- 87.6) of herds had at least one animal shedding str. Pacifica six years after initial detection in the same herds. Moreover, the estimated prevalence ratio (PR) of 7 indicated that the prevalence of shedders was seven times as high at the beginning of the season as at the end.

Results of the statistical analysis of the association between str. Pacifica positivity and reproduction (Chapter 5) of dairy herds showed that str. Pacifica did delay the time from calving to conception (HR = 0.84; 95% CI 0.74-0.96), although there was no effect on the pregnancy rate. At the herd level, no statistically significant associations were detected between str. Pacifica and reproductive performance (Chapter 5) or milk production of dairy cows (Chapter 6).

The lack of an association could be explained by a host adaptation of str. Pacifica to dairy cows. As discussed by other researchers, maintenance hosts do not display clinical signs or adverse reactions when infected by their host adapted *Leptospira* (Faine 1994; Putz and Nally 2020; Hamond *et al.* 2022b; Monti *et al.* 2023). To summarise, we propose that New Zealand dairy cows may be recognised as a

maintenance host population of str. Pacifica in New Zealand.

7.2.3 Public Health Importance of Strain Pacifica

In New Zealand, the occupational nature of leptospirosis has been long-established, and dairy, beef, and sheep farmers who have day-to-day contact with animals are most at risk (Dreyfus *et al.* 2015; Nisa *et al.* 2020; Benschop *et al.* 2021). To prevent human infections and protect animals, New Zealand started implementing livestock vaccination programs in the early 1980s, and this was associated with a drop in the incidence of reported human cases of leptospirosis. Since then, a shift in the proportion of the notified serovars in humans was observed, and the incidence of serovars not included in commercially available vaccines for cattle, hereafter referred to as non-vaccine serovars, Ballum and Tarassovi, increased (Thornley *et al.* 2002; Moinet *et al.* 2023; Yupiana *et al.* 2019; Nisa *et al.* 2020; Wilkinson *et al.* 2023). Moreover, among cases notified from 1 January 1999 to 31 December 2017 the highest incidence of Tarassovi at 12.59/100,000 was reported amongst dairy farmers (Nisa *et al.* 2020). Thus, the rise in Tarassovi cases among dairy farmers is causing concern, especially because str. Pacifica is detected within the population of New Zealand dairy cows (Yupiana *et al.* 2019; Wilson *et al.* 2021; Wilkinson *et al.* 2023) and there appears to be a transmission between dairy cows and farmers, as amplicon sequencing of the *glmU* gene positively identified a sequence specific to str. Pacifica in the specimen collected from a notified case of leptospirosis in a dairy farmer (S. Nisa, *personal communication*, March 25, 2024).

At the time of the aforementioned studies, serovars Hardjo, Pomona, and Copenhageni were included in commercially available vaccines for cattle, and Pomona and Tarassovi were included in pig vaccines. From these, only Pomona was causing severe disease in pigs and cows, and Tarassovi was regarded to cause milder disease, but occasionally, abortions and infertility in sows were also reported (Davos 1977). Similarly, vaccines against Hardjo in cattle are also used despite Hardjo not causing acute clinical disease in cattle (Hathaway 1981). The main aim of including clinically non-important serovars in livestock vaccines was to prevent human leptospirosis, reduce transmission, as well as

to prevent subclinical disease in animals. The uptake of vaccination in dairy and pork industries was estimated to be 99%, but only an estimated 10% of beef herds and sheep flocks are currently being vaccinated against *Leptospira* (Sanhueza *et al.* 2017; Lawrence *et al.* 2018; Yupiana *et al.* 2021).

As described above, over 50% of str. Pacifica positive herds were still positive six years after initial detection and results showed that str. Pacifica appears to have little or no clinical or (re-) production effects in dairy cows. In general, clinical signs of leptospirosis in cattle may include a drop in milk production, mastitis, reproductive failure, abortion, stillbirth, and premature birth (Te Punga and Bishop 1953; Ellis *et al.* 1985; Miller *et al.* 1991; Guitian *et al.* 2001; Martins *et al.* 2015; Nally *et al.* 2018; Rajeev 2022). Therefore, str. Pacifica may not be causing acute leptospirosis in shedding dairy cows, and while protecting animals remains an important goal, the protection of people who have contact with str. Pacifica-positive animals especially dairy workers, is increasingly important now that str. Pacifica has become well-established in New Zealand dairy herds.

The multiple research publications, presentations and workshops delivered by the *Leptospira* Research Group at Massey University (Yupiana *et al.* 2019; Nisa *et al.* 2020; Benschop *et al.* 2021; Wilkinson *et al.* 2021; Yupiana *et al.* 2021; Wilkinson *et al.* 2023), as well as this thesis's findings, provided ample scientific evidence for introduction of a new commercial vaccine containing the Tarassovi antigen. Consequently, a commercial vaccine became available for use in dairy cows in December 2023 (Virbac 2024). Cross-protection between serogroups is commonly used in leptospirosis vaccines (Sonrier *et al.* 2000), and this vaccine was designed to protect against Tarassovi-associated leptospirosis and potentially provide immunity against str. Pacifica. Str. Pacifica is thought to belong to serogroup Tarassovi because seropositivity to Tarassovi was statistically significantly associated with the shedding of str. Pacifica in dairy cows, as shown by Yupiana *et al.* (2019) and Chapter 4 of this thesis. The vaccine uptake is unknown, but if farmers use it, we may see a drop in the number of notified human Tarassovi leptospirosis cases. A programme of vaccination of dairy herds was proven to break the transmission cycle and prevent infection in both humans and infected animals in New Zealand (Thornley *et al.* 2002; Marshall and Manketlow 2002) and overseas

(Levett 2001; Azevedo *et al.* 2023). Str. Pacifica shedding patterns and Tarassovi seroprevalence in dairy herds reported in this study may help inform the strategies for reducing the transmission of str. Pacifica between animals and humans, such as using personal protection equipment or updating the vaccination strategy.

7.3 Chosen Methodology and Impact on Conclusions

7.3.1 Media Types and Preparation

The media evaluation study (Chapter 3) involved a stepwise assessment of different media by seeding laboratory-adapted cultures of *Leptospira borgpetersenii* serovars Hardjo and Tarassovi to selected media. Then, the growth rates of *Leptospira* were evaluated by estimating the number of bacteria per ml of medium over time. The selection of the best medium was based on several criteria, the main one being the ability of the medium to support the growth of the selected cultures and suppress contamination. Then, the urine of PCR-positive dairy cows was seeded into selected media, which was observed for up to six months. Two inoculates were initially positive, but none resulted in an established str. Pacifica culture.

While the stepwise media selection approach provided a robust structure to this media experiment, the main weakness of this design was the use of laboratory-adapted strains or ”wild“ strains already cultivated in the laboratory as they had a strong affinity towards particular media types and growth conditions commonly used in the laboratory. Therefore, the selected best media was not necessarily suitable for the "wild" str. Pacifica. Thus, using laboratory strain-adapted culture media was initially unsuccessful when trying to isolate str. Pacifica from the urine of dairy cows.

The 2022 follow-up isolation attempt (Chapter 4) was influenced by the successful isolation of *Leptospira borgpetersenii* serovar Tarassovi from the urine of dairy cows in the USA (Hamond *et al.* 2022a). In their study, authors isolated and cultivated *Leptospira* Tarassovi at 37°C and 5% CO_2 in HAN media (Hornsby *et al.* 2020) without the addition of a cocktail of antibiotics. Thus, for the 2022 follow-up isolation attempt,

freshly collected urine from 64 dairy cows was seeded into HAN medium alongside the traditionally used EMJH supplemented by 5`flurouracil. Seeded samples were kept under 37°C and 5% CO_2. As a result, str. Pacifica's successful isolation in HAN media was confirmed by PCR test, but then, HAN medium failed to maintain the growth of str. Pacifica and the cultures were lost.

Thus, the findings of the media experiment (Chapter 3), such as the use of unsuitable media, growth conditions, or the addition of antibiotics, helped to exclude unsuitable media, supplements, and growth conditions from further use and helped to refine methods and resulted in the successful initial isolation of str. Pacifica (Chapter 4). Although we did not obtain str. Pacifica culture this time, these experiments will help to shape the strategy for future attempts. The possible future approach is discussed under the heading "suggestions for future research".

7.3.2 Sampling Design

The sampling design of the study is described in detail in Chapter 4 of this thesis, thus it`s only briefly summarised further below.

- The sampling design was based on results from previous research conducted in 2016.
- There were two stages of sampling: the start of the milking season (July-Oct 2020) and the end of the season (February-June 2021).
- Blood samples were collected only at the start of the season to inform urine sampling at the start and the end of the season
- Additional farm was sampled at the start of the 2022/2023 milking season, it was added due to the history of seropositivity to Tarassovi. Only urine samples were collected from the additional farm.
- Blood samples were tested by MAT against the Tarassovi antigen.
- Bacterial DNA was extracted from urine samples tested using PCR assay and followed by amplicon sequencing of the *glmU* gene.
- Urine samples used as inoculum for media inoculation to isolate str. Pacifica.

This study design allowed for the identification of shedding animals at the different stages of lactation and the evaluation of seropositivity levels in the same animals during

one milking season. The sampling design had advantages and disadvantages. On the one hand, identifying seropositive animals first, then targeting them for urine sampling, helped focus on animals that were the most likely shedders. On the other hand, the low-titre shedders might have been missed. Additionally, the duration of shedding and possibly low amounts of shed bacteria by selected animals may have resulted in missing some shedders. It is generally accepted that infected animals may not continuously shed the live bacteria (Faine 1994), and intermittent shedding may indicate that a period of shedding can sometimes last for a short time (Levett 2001; Monti *et al.* 2023). Therefore, this study`s design did not fully account for the different shedding patterns of *Leptospira*. However, we did get 52 /564 urine PCR-positive animals for the study (Chapter 4). For example, a study of *Leptospira* shedding patterns in southern Chile reported five distinctive shedding patterns, non-persistent, short persistent, high persistent, intermittent, and multiple patterns *(*Monti *et al.* 2023). Str. Pacifica shedding patterns can be explored more by future studies. This work helped to determine the best potential sampling time for future studies, which is the start of the milking season when the most shedding animals were detected in the herd.

7.3.3 Laboratory Tests

Two types of laboratory tests were used to determine the exposure and shedding status of cattle in this study. The first type, serological microscopic agglutination test (MAT), was used to identify the presence of specific *Leptospira* antibodies in the animal's blood. MAT is considered a "gold standard" test and remains one of the most accessible and widely applied tests for identifying *Leptospira* serovars as well as diagnosing leptospirosis in clinically ill humans and animals or as a proxy for exposure in "healthy" ones, MAT is generally serogroup rather than serovar-specific (Levett 2003). However, where a limited number of serovars are known to be present, serovars can be identified by MAT with a high degree of certainty (Levett 2003). In New Zealand, where two pairs of serovars can cross-react (Hardjo and Balcanica, Tarassovi and Pacifica), identification of infecting serovars by MAT has limitations, but in our study, MAT was fit to determine Tarassovi seroprevalence in high-risk herds. The MAT was the only serological test available for serovar identification in animals. In this study, the

specificity of the MAT was estimated to be high, as 99% of sera samples were negative to Copenhageni and 98% of samples were negative to Ballum, these results suggest that these are the minimum estimates for this test. The positive results likely represented true positives, therefore confirming the high specificity of the MAT.

The second type of laboratory test was the molecular PCR test. The results from two types of PCR tests were used in this thesis. One was a qPCR method, followed by amplicon sequencing targeting the *gyrB* locus, as described by Slack *et al.* (2009). It was used to determine *Leptospira* urinary shedding by dairy cows in a previous study during the 2015-2016 milking seasons. This test method was unable to differentiate str. Pacifica from other serovars. Another PCR test method was a conventional PCR assay, followed by amplicon sequencing targeting the *glmU* locus. This PCR assay was described by Wilkinson *et al.* (2021) and Wilkinson *et al*. (2023) and it allowed the differentiation str. Pacifica from Tarassovi. Thus, the use of the PCR assay targeting the *glmU* locus allowed for accurate str. Pacifica differentiation, which could not have been achieved via MAT or PCR assay targeting the *gyrB* locus.

For the purposes of this study, it was accepted that a positive MAT result (≥48) indicated past infection, and in contrast, the PCR positive result identified the presence of leptospiral DNA at the time of sampling (Haake and Levett 2015). However, it is important to note that in some cases, particularly in public health, a single MAT titre >400 or a fourfold increase between acute and convalescent titre may also indicate current infection (Lau et al. 2018; Faine 1994). In this thesis, animals were deemed positive if they were one or both of the following: positive to Tarassovi by serological test (MAT ≥48) or positive by qPCR with Ct values less than or equal to 40, using *glmU* as a target gene for *Leptospira borgpetersenii* str. Pacifica DNA. Thus, the use of combining indicators of a past and the present infection as an independent variable in regression analysis may have broadened the definition of animal`s positivity and may have slightly reduced the sensitivity of the analysis.

7.3.4 Statistical Methods

In this thesis, a variety of statistical methods were employed to analyse the data, these included descriptive analyses, bivariate models, and multivariate models. The multivariate models were logistic and linear regression models, Kaplan-Meier models, shared frailty survival models, and mixed models incorporating random and fixed effects. The choice of statistical models was tailored to best suit the data's characteristics.

The investigation into the associations between str. Pacifica and milk production and reproduction in dairy cows utilised a combination of historical MAT and PCR test results along with routinely collected herd test data, typically gathered three to four times a year. This herd test dataset comprised generic production and reproduction measurements, which provided a basis for analysis, although it had notable limitations. Enhancing data granularity by including specific health and production variables, such as disease reports, or recorded abortions would have been beneficial. Additionally, the data likely contained recording errors, such as mislabelling of test tubes, inaccurate or missing cow numbers or dates, and erroneous mating or calving records of this thesis, thus potentially the measurement error was introduced to this analysis, as discussed in Chapter 5.

The data were clustered at the herd level in the reproduction dataset and both the herd and animal levels in the milk production dataset due to multiple records of the herd tests or matings for the same animal. Consequently, an appropriate analytical approach was required to account for this clustering. The use of mixed-effects and frailty models, which incorporated random effects for herds and animals, was deemed appropriate. These models facilitated the examination of the variables of interest while accounting for variability within and across individual data points, such as multiple mating or herd test dates. They also managed missing data and unbalanced designs effectively, which could have arisen from missing records or a left-skewed distribution of data due to the majority of herd tests occurring at the start of the milking season.

Overall, the application of these advanced statistical methods enabled the restriction of

dependency between outcome and independent variables and minimised bias from cluster effects. This approach led to obtaining robust estimates, thereby enhancing the reliability of the findings.

7.4 Suggestions for Future Research

The knowledge gained through this research could provide a background for future work, which may lead to the development of farm management practices that reduce the risk of infection with strain Pacifica in ruminants and consequently protect humans against leptospirosis.

7.4.1 Str. Pacifica Isolation

Media experiments and follow-up inoculation work using the urine of dairy cows helped to identify media and growth conditions most suitable for the initial isolation of str. Pacifica. However, HAN media at 37°C and 5%CO_2 not supplemented by the cocktail of antibiotics failed to maintain the growth of str. Pacifica. This finding suggests that after initial isolation, str. Pacifica may change its growth requirement as it is no longer in an animal host, and further modification of media and growth conditions may be needed as many strains also become laboratory-adapted and initial isolation and are successfully maintained under laboratory conditions. Examples include *Leptospira* strains isolated by [m]Epilab researchers from various animal hosts (Subharat *et al.* 2011; Fang *et al*. 2014; Vallée *et al*. 2015; Moinet *et al.* 2023).

Some authors propose a practical approach to improving the maintenance of *Leptospira* isolates. This approach involves seeding initially isolated cultures into different media, often under different conditions (Chideroli *et al.* 2017; Guedes *et al.* 2022; Hamond *et al.* 2022a). On the other hand, some researchers have developed a new media composition that can provide the necessary conditions for the growth and further maintenance of these isolates (Ellinghausen Jr and McCullough 1965; Hornsby *et al*. 2020). Both strategies can be used for future isolation attempts of str. Pacifica, however, the first strategy may be more efficient, as in the absence of the isolate, it may

be challenging to empirically determine the exact type of media and growth conditions supporting the isolation and maintenance of bacteria.

Another option to consider is obtaining dairy cows that shed str. Pacifica and using a continuous sampling strategy to compensate for possible intermittent shedding or different amounts of shed bacteria (Monti *et al*. 2023). By increasing the frequency of sampling and using different combinations of media and growth conditions, the chances of isolating str. Pacifica can be enhanced.

A combination of multiple sampling strategies with the use of different media types and their combination was successfully employed for isolation from the urine of dairy cows and long-term maintenance isolate of *Leptospira borgpetersenii* serovar Tarassovi (Hamond *et al.* 2022a). Another option to consider is to use a susceptible animal and inoculate it with bodily fluids, such as blood or urine from an animal that is shedding str. Pacifica. Although this method is not commonly used for the isolation of *Leptospira* nowadays, it was one of the most effective techniques before the introduction of selective media (Noguchi 1917), but is still often used in research, specifically in *Leptospira* virulence studies if approved by the animal ethics (Haake 2006; Matsui *et al.* 2015; Wunder Jr *et al.* 2016). Other factors contributing to difficulties in the isolation of str. Pacifica from dairy cows may include higher frequencies and large urine volume excreted by dairy cows, possibly contributing to a low number of live bacteria collected during sampling. All these factors and considerations are discussed in chapters 3 and 4 and should be considered when planning a future str. Pacifica isolation study.

7.4.2 Bovine Genital Leptospirosis Investigation

One of the topics that has not been explored in this thesis is whether str. Pacifica could be isolated from the reproductive system of dairy cows, and if it could cause bovine genital leptospirosis (BGL). It has been suggested that the presence of *Leptospira* in the reproductive system is a manifestation of a syndromic disease named Bovine genital leptospirosis (Di Azevedo *et al.* 2020; Loureiro and Lilenbaum 2020; Aymée *et al.* 2021; Aymée *et al.* 2022; dos Santos Pereira *et al.* 2022; Aymée *et al.* 2023; Barnabé *et al.* 2023; Aymée *et al.* 2024). Bovine genital leptospirosis is characterised by silent

reproductive disorders, and the most common manifestation of BGL in cows is early embryonic death, which occurs in a period of up to 28 days after fertilisation (Loureiro and Lilenbaum 2020). Less frequently, BGL manifested as late embryonic death or late-term abortion. These may result in repeated mating, irregular mating intervals, or increased time to conception (Aymée *et al.* 2024), and an increased time to conception is one of the effects of str. Pacifica on the reproduction of dairy cows, as reported in chapter 5 of this study. Therefore, a possible association of BGL and str. Pacifica needs to be investigated.

The presence of leptospiral DNA in the reproductive system of cows has been reported by a number of studies. For example, in a study exploring leptospiral vaginal carriage among slaughtered cows, leptospiral DNA was detected in 50.4% (128/254) of vaginal fluid and 11% (28/254) urine samples of abattoir cows; authors concluded that these findings are suggestive of vaginal carrier status of culled animals and suggested that venereal transmission of leptospirosis may occur (Loureiro *et al.* 2017)Interestingly, authors reported that PCR results from urine and vaginal fluid samples were poorly correlated (Loureiro *et al.* 2017; Pinna *et al.* 2018) but did not offer an explanation why this can be happening.

As noted in chapter five of this thesis, none of the participating farmers who had str. Pacifica-positive cows in their herds reported adverse reproduction effects. The results of this study showed that str. Pacifica increased the time to conception in str. Pacifica-positive cows. Increased time to conception in the absence of other acute clinical or subclinical signs may be indicative of early embryonic death or BGL in New Zealand dairy cows. In the current study, 62 kidney samples collected from random cows originated from high-risk str. Pacifica herds were PCR-negative (chapter 4). However, no samples from the reproductive system were collected at the time due to logistical constraints.

Future studies will need to investigate the possibility of isolation of the str. Pacifica from the reproductive system of dairy cows, as str. Pacifica may have an affinity to the reproductive system of dairy cows. The first stage of such investigation could identify infected cows by obtaining urine, kidneys, and samples from the different organs of

reproductive systems at the abattoir from culled animals. This sampling strategy will help to identify the presence of str. Pacifica`s DNA in both urinary and reproductive systems. Sampling can focus on cows culled at the peak of shedding at the beginning of the milking season. The second stage could include sampling live animals and testing the presence or absence of str. Pacifica DNA in vaginal fluid and urine. The results of such investigation will indicate whether str. Pacifica has an affinity to the reproductive system of a living cow and may help to determine shedding levels.

7.4.3 Further Tarassovi vaccine and diagnostic tests research

A new vaccine for dairy cattle, which includes the Tarassovi antigen along with Hardjo, Copenhageni, and Pomona antigens (Virbac 2024), became commercially available in late 2023. This vaccine provides protection against *Leptospira* of the serogroup Tarassovi. Given that str. Pacifica is believed to belong to the Tarassovi serogroup (chapter 4), the vaccine could potentially offer protection against str. Pacifica as well. However, further research on str. Pacifica, including clinical trials and challenge studies, is dependent on obtaining a viable culture of str. Pacifica. An isolate is also needed for full genome sequencing, as only partial fragments of the *glmU* gene of str. Pacifica has been sequenced to date (Wilkinson et al. 2021)

The findings of this thesis provide a foundation for future evaluations of the economic impact of vaccination against str. Pacifica in dairy herds. Within New Zealand`s pastoral farming system, cows must be pregnant within the critical six to twelve-week period. Achieving pregnancy within this timeframe allows for aligning the peak of the grass growth with peak milk production and to maximise the profitability of the dairy farm. The results of this study indicate that str. Pacifica was found to delay the time from calving to conception HR = 0.84; 95% CI: 0.74-0.96). Consequently, additional inseminations, pregnancy diagnoses, and veterinary visits may be required to ensure str. Pacifica-positive cows are pregnant within the 12-week period. The costs for these additional measures can be significant, starting at 150 NZD per hour for a veterinary technician callout, with veterinarian`s charges ranging from 250-300 NZD per callout and 3.00 NZD

per cow for pregnancy diagnosis (when testing at least 15 cows per visit). These additional costs can be assessed using appropriate economic models and analyses, such as those discussed in a publication by Sanhueza et al. (2020). It could also be evaluated whether the vaccination against str. Pacifica, costing approximately 2.85 NZD per dose (J Watts, *personal communication*, May 2, 2024), is economically beneficial for dairy farmers.

In conclusion, the research findings of this thesis indicate that str. Pacifica can be isolated from the urine of dairy cows using HAN media at 37°C and 5% CO_2, although it cannot be maintained in this media. The study also demonstrated that individual dairy herds remain infected for at least six years from the initial detection of str. Pacifica, suggesting that the bacterium is being maintained within New Zealand's dairy cow population.

It is important to note that this study investigated str. Pacifica shedding in 17 high-risk herds. The seroprevalence and shedding rates reported here represent those seen in high-risk herds; however, their locality may show "hot spots" of str. Pacifica strain in New Zealand. Nevertheless, given the statistically significant association between str. Pacifica shedding and Tarassovi seroprevalence, and considering that Tarassovi seroprevalence is estimated at 75% in the national dairy herd (Yupiana et al. 2019), the findings of this thesis could be extrapolated to the national herd level, suggesting that dairy cows may have become a maintenance host for str. Pacifica. Furthermore, the absence of clinical signs is shedding animals, and the lack of production effects are also adding weight to this argument. However, str. Pacifica was found to delay the time from calving to conception (HR = 0.84; 95% CI 0.74-0.96), although it did not affect the overall pregnancy rate. Consequently, continued research on str. Pacifica is recommended based on the findings reported in this thesis.

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Appendix

Appendix Table1. List of chemicals used for media preparation.

Reagent name	**Made by**	**Lot/ batch number**	**Catalogue/Product number**
5-fluorouracil	Sigma	MKBX3795V	F6627-56
Ammonium chloride	Sigma	55H107015	A9434
Amphotericin	Sigma	0000096326	A2942
BD Diffco *Leptospira* Enrichment EMJH	Diffco TM	8011968	279510
BSA "Bovolep"	Bovogen	328	261
Calcium chloride	Sigma	57H1053	M0250
Copper Sulphate	Sigma	67H3607	C6283
Cyanocobalamin (vitamin B12)	Sigma	MKCB771	V2876
Diffco Lepto medium	BD	279410	707670
Disodium phosphate	JT Baker	J05147	FN141.96
Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12	Sigma	51448 C	Sigma51448 C
Ferrous sulphate	BDH	63177501500	10112
Fosfomycin	Sigma	BCBS2720V	P5396-59
Glycerol	Fisher Chemical	1922421	10650117

Reagent name	**Made by**	**Lot/ batch number**	**Catalogue/Product number**
Magnesium chloride	Merk	57H1053	M0250
Monopotassium phosphate	BDH	A398525	10034B
Newborn Calf Serum from tissue culture	Sigma	1659947	MFCD00163621
Noble agar	Sigma	A5431	SLBV7797
Phenol red	BDH	20090	0497980
Rabbit serum	Sigma	BP-E21,	R4505
Sodium acetate	Sigma	78H0127	P5396-56
Sodium chloride	Pancrae	00000205090	121659.1210
Sodium pyruvate	Merk	46000002074	K34077019509
Sulfamethoxazole	Sigma	BCBS2720V	S7507109
Thiamine chloride (vitamin B1)	Sigma		T1270
Trimethoprim	Sigma	097M4049V	92131
Tween 80	Sigma	MKBT8700V	P6224
Zinc sulphate	Sigma	3811302	Z-4750

Appendix Table 2. *Leptospira* EMJH media components

Ingredients	**Volume/L**
Basal solution	
Disodium phosphate	1g
Monopotassium phosphate	0.3g
Sodium chloride	1g
Ammonium chloride stock solution	1ml (25g/100ml water)
Thiamine stock solution	1ml (0.5g/100ml water)
Glycerol stock solution	1 ml (10ml/100ml water)
Sterile distilled water	800ml
BSA stock solution	
Calcium chloride stock solution	1ml (1 g/100 ml water),
Magnesium chloride stock solution	1ml (1 g /100 ml water)
Zinc sulphate stock solution	1ml (0.4 g /100 ml water)
Copper sulphate stock solution	0.1ml (0.3 g/100 ml water)
Ferrous sulphate stock solution	10ml (0.5g/100 ml water)
Vitamin B12 stock solution	2ml (0.02 g/100 ml water)
Tween 80 stock solution	12.5 ml (10 ml/100 ml water)
Enrichment	
Rabbit serum	10ml
Newborn Calf Serum	10ml

Appendix

Ingredients	**Volume/L**
BSA	10 g
BD Diffco *Leptospira* Enrichment EMJH	100ml
Selective supplement	
Sulfamethoxazole	40 µg/ml
Trimethoprim	20 µg/ml
Amphotericin B	5 µg/ml
Fosfomycin	400 µg/ml
5-fluorouracil	100 µg/ml

Appendix Table 3. *Leptospira* LTM media components

Ingredients	**Volume/L**
Basal medium	0.5g
Meat peptone	0.3g
Beef extract	0.3g
Monopotassium phosphate	0.3g
Disodium phosphate	1.0g
Sodium Chloride	1.0g
Ammonium Chloride	0.25g
Phenol red	0.03g
Agar	1.5g

Ingredients	**Volume/L**
Ultrapure water	900ml
Selective supplement	
Sulfamethoxazole	40 µg/ml
Trimethoprim	20 µg/ml
Amphotericin B	5 µg/ml
Fosfomycin	400 µg/ml
5-fluorouracil	100 µg/ml

Appendix Table 4. *Leptospira* HAN media components

Ingredients	**Volume/L**
BSA	10g
Calcium chloride	1 ml (3.0 g/100 ml water)
Magnesium chloride	1 ml (3.0 g/100 ml water)
Zinc sulphate	1 ml (0.4 g/100 ml water)
Ferrous sulphate stock solution	10 ml (0.5g/100 ml water)
Tween 80	12.5 ml (10 ml/100 ml water)
Vitamin B12	1 ml (0.02 g/100 ml water)
Glycerol	1 ml (10% solution in water)
Ammonium chloride	1 ml (25% solution in water)
Disodium phosphate	1.0 g

Ingredients	**Volume/L**
Monopotassium phosphate	0.3 g
Sodium chloride	1.0 g
Sodium pyruvate	0.1g
Ultrapure water	900ml
Enrichment	
Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12	200ml
Selective supplement	
Sulfamethoxazole	40 mg/ml
Trimethoprim	20 mg/ml
Amphotericin B	5 mg/ml
Fosfomycin	400 mg/ml
5-fluorouracil	100 mg/ml