Final report

Improving fertility in northern cattle through host and pathogen molecular diagnosis

Project code: P.PSH.0799

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Abstract

Vibriosis or Bovine Genital Campylobacteriosis is a widespread venereal disease of cattle which is carried by asymptomatic bulls causing infertility or abortion in heifers and cows. It can be prevented by effective VibroVax® vaccination of maiden heifers and bulls, maintaining a young bull herd (culling old bulls), and by mating *Campylobacter fetus* subspecies *venerealis* negative bulls. Issues with BGC are thus mostly due to poor vaccination compliance and the retainment of old bulls in the herd. Currently there are no tests available to detect *C. fetus venerealis* (to differentiate from *C. fetus* subsp. *fetus* which is less likely to cause abortion) in clinical samples, and the available IgA ELISA cannot confirm a current infection. Through undertaking vaccination challenge trials in heifers and bulls, and through sampling field herds, this project has developed a 'tool kit' for managing BGC. The trials demonstrated that *C. fetus venerealis* can be detected in vaccinated and unvaccinated animals and that qPCR positivity is transient. Trials confirmed that young bulls clear the pathogen compared to old bulls. A newly developed TaqMan SNP qPCR assay was less sensitive than targeted long read genome sequencing in detecting low levels of *C. fetus venerealis* in clinical samples. Poor *C. fetus venerealis* detection in heifers rendered the ability to identify EBVs for BGC resistance not possible in this research. Serum biomarkers were identified which can confirm BGC infection and/or vaccination status. If an entity could be identified to deliver the genomic sequencing screening as a service, the tools developed here will ultimately enable red meat producers to manage vibriosis/BGC and other reproductive diseases.

Executive summary

Background

Campylobacter fetus venerealis is known as the venereal disease agent of bovine genital campylobacteriosis (BGC or Vibriosis) causing infertility and abortion, while a related subspecies *C. fetus fetus* only causes sporadic abortions. Currently there is only a herd IgA ELISA diagnostic test for vibriosis/BGC offered by Veterinary diagnostic laboratories in Australia, which retains long term positivity following initial exposure to the disease. This test cannot be used to confirm the presence of the causative pathogen *C. fetus venerealis*. Diagnostic assays need to discriminate between the two subspecies which has proven to be problematic when relying on the isolation of pure cultures from contaminated diagnostic samples. Some diagnostic markers previously developed (McMillen et al., 2006; Abril et al., 2007) were also found to cross react with closely related species such as *C. hyointestinalis* (Spence et al., 2011; Sanhueza et al., 2014) and other species commonly found in the bull prepuce (Lew-Tabor et al., 2013). Until the genomes of these species were sequenced, it became evident that species sharing the same niche were potentially more likely to exchange mobile genetic elements containing virulence genes which are thus unreliable diagnostic markers (Gorkiewicz et al., 2010; van der Graaf-van Bloois et al., 2013, 2014; Lew-Tabor et al., 2013; Barrero et al., 2014). This suggested that conserved housekeeping genes could be the best diagnostic assay targets however none to date have been exploited by other groups. Other questions arose from our research including, do other species found in the bull prepuce cause reproductive diseases? or does the microbiome favour conditions for the persistence of *C. fetus venerealis* particularly in asymptomatic bulls?

This project planned to define the microbiomes of the reproductive tracts in healthy and diseased tropical cattle and investigate the host genetics associated with reproductive disease susceptibility/resistance (within the same cattle). This would lead to the development of specific diagnostic tests for BGC and determine whether the microbiome is associated with increased susceptibility to BGC. As a result of understanding genetic variations between cattle we will also pilot the development of genetic markers that can be used in breeding programs to select for BGC resistant genotypes.

Potential Outcomes Planned included:

- Novel methods for differentiating *C. fetus* subspecies *fetus* and *C. fetus* subsp. *venerealis* (the latter subspecies is the cause of BGC).
- Identification of novel genital pathogens.
- Diagnostic test for distinguishing BGC-infected from vaccinated animals.
- EBV for resistance/susceptibility to genital infections.

Objectives

By 1 Dec 2023 (originally drafted in 2017):

a) Sampled at least 1,000 cattle (heifers, cows and bulls) from healthy and diseased northern beef herds for genotyping and microbiome analysis.

One thousand and sixteen cattle were sampled for this study thus the objective was met successfully.

b) Defined the microbiomes of the reproductive tracts in healthy and diseased cattle.

The microbiome data from the field samples from Objective a) (2.1) were difficult to interpret, thus two controlled vaccine and *C. fetus venerealis* challenge studies were undertaken in heifers and bulls respectively. The microbiomes of unvaccinated cattle were successfully compared to vaccinated cattle in each trial to define the microbiomes of healthy and diseased (unvaccinated challenged) cattle.

c) Investigated the host genomics associated with reproductive disease susceptibility/resistance.

Despite access to nine herds which did not vaccinate with VibroVax®, too few *C. fetus venerealis* cattle were identified using qPCR methods (Objective d) or 2.4) to undertake host genomics associated with BGC susceptibility/resistance. Instead, we determined that the host genome potentially plays a role in influencing the reproductive microbiome associated with disease susceptibility/resistance.

d) Developed a validated specific molecular diagnostic test for BGC.

Following *C. fetus* genome analyses, the MraY gene TaqMan SNP Assay was validated as a specific molecular diagnostic test for BGC meeting this objective successfully. However, for low positive or ambiguous samples in qPCR, the application of Oxford Nanopore Technology (ONT) sequencing on clinical samples was shown to be more reliable for the detection of *C. fetus venerealis*.

e) Determined whether the genital microbiome is associated with increased susceptibility to BGC

Differences in the genital microbiome from different herds is an indicator of exposure to different environments and a reflection of the gastrointestinal microbiome in those environments. Susceptibility to BGC could only be associated with the detected presence of *C. fetus* in the genital microbiome of cattle from unvaccinated herds. Herds identified with *C. fetus* demonstrated a more diverse microbiome than those without *C. fetus* successfully meeting this objective.

f) Determined whether BGC immune biomarkers (post infection or vaccination) can be identified for assay development

The metagenome has no clear differences between vaccinated and unvaccinated bulls, however challenge with *C. fetus venerealis* leads to an increase in *Histophilus* spp. consistently. Vitamin D binding protein (DBP), haptoglobin (HP) and serum amyloid A (SAA) were identified as biomarkers for *C. fetus venerealis* post infection (DBP, HP, SAA) and BGC vaccination (DBP) successfully.

g) Piloted the development of genomic markers that can be used in breeding programs to select for resistant genotypes.

Objective c) (2.3) demonstrated that due to a lack of qPCR positive cattle, genomics studies for BGC resistance was not possible. Preliminary data also demonstrated that the host genome potentially plays a role in influencing the reproductive microbiome associated with disease susceptibility/resistance, however we were unsuccessful in securing sufficient samples from diseased herds towards genomic marker development.

Methodology

Reproductive tract samples from the northern genomics project were screened using new *C. fetus venerealis* qPCR assay and metagenomics methods. Inconclusive results led to the undertaking of two controlled trials in heifers and in bulls to compare host responses to *C. fetus venerealis* challenge in vaccinated vs unvaccinated animals. Reproductive samples were measured in qPCR, metagenomics, and long read sequencing; and serum was used for quantitative proteomics to identify BGC associated biomarkers.

Results/key findings

The reproductive microbiome is a product of the environment and future studies to identify unhealthy microbiomes can only be undertaken at the herd level.

Bacterial genomics guided the identification of gene (mraY) which was exploited to develop a *C. fetus venerealis* TaqMan SNP assay.

The mraY qPCR assay had only transient positivity in clinical samples as observed in samples from bull and heifer vaccination challenge trials.

Controlled bull and heifer VibroVax® vaccination and challenge trials demonstrated that *C. fetus venerealis* can be detected in both vaccinated and unvaccinated animals after challenge.

Long read sequencing methods are much more sensitive and specific in detecting *C. fetus venerealis* in clinical samples compared to qPCR when the bacterial load is low.

Histophilus somni bacteria co-infect with *C. fetus venerealis* as confirmed by metagenomic studies and during *C. fetus venerealis* artificial challenge – it is not known if this pathogen is contributing to infertility.

Metagenomics is a useful method that can identify sporadic or opportunistic pathogens associated with abortion e.g. *Escherichia coli* and *Histophilus somni*.

Vitamin D Binding protein (DBP), Haptoglobin (HP) and Serum Amyloid A (SAA) were identified as biomarkers for *C. fetus venerealis* infections (all 3 biomarkers) and BGC vaccination (DBP).

The project could not develop EBVs for BGC resistance in heifers as too few qPCR positive cattle (BGC sensitive) could be identified.

Benefits to industry

We were able to develop a new specific qPCR for *C. fetus venerealis* detection, however we also discovered that qPCR positivity is transient. Using novel genomic sequencing tools is more sensitive and reliable than qPCR, however, we will need to identify an external provider for this service to be made available to producers.

BGC biomarkers were identified, and ELISA tests could be made available to industry as a diagnostic laboratory test or a crush side rapid antibody.

The tools developed here will ultimately enable red meat producers to manage vibriosis/BGC.

Future research and recommendations

During this research program which commenced in 2018, the development of rapid antigen tests as a standard COVID19 test has been demonstrated. We had not planned to develop tools like this; however, the technology is now available, and our biomarkers could be delivered as crush side antibody tests for vibriosis/BGC if production of these tests is feasible. Through our thorough investigations including controlled experiments in bulls and heifers, as well as field sampling, we found that qPCR for vibriosis/BGC is far too transient and can yield false negative results. Advances in genomic sequencing are such that we could deliver a methodology which detects both *C. fetus venerealis* and *T. foetus* (and potentially *Histophilus somni*) in mucus samples as well as cattle genotyping records if required. Technology transfer discussions are currently underway with a State Government Laboratory and a cattle genotyping company.

For immediate adoption, the qPCR assay could be used however biomarker ELISA assays require further evaluations. For the long term, a service provider who can apply novel long read sequencing diagnostic data would be ideal for the industry.

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1. Background

Economic assessments of bovine genital campylobacteriosis (BGC, commonly called vibriosis) caused by *Campylobacter fetus* subsp. *venerealis* have been difficult to perform due to the lack of reliable diagnostic methods and the fastidious nature of the pathogen hindering its successful isolation from clinical samples. The accepted dogma is that BGC outbreaks are rare due to herd immunity, however this could also be because accurate diagnosis is currently not possible. Currently producers manage reproductive disease through culling empty cows, vaccination of bulls (and sometimes heifers) with Vibrovax® (Zoetis), and/or by pre-testing bulls using an IgA ELISA (Hum et al., 1991) prior to heifer mating. The IgA ELISA method does not accurately indicate current infection and is recommended for use as a herd test as false positives are known to occur in individual animals (Hum et al., 1994).

Tritrichomonas foetus and *C. fetus venerealis* are the main causes of bovine venereal diseases leading to infertility and abortion, while *C. fetus fetus* is highly related to *C. fetus venerealis* and only causes sporadic abortions. Diagnostic assays need to discriminate between these subspecies which has been problematic as currently pure cultures need to be isolated from heavily contaminated diagnostic samples. Some diagnostic markers previously developed (McMillen et al., 2006; Abril et al., 2007) were found to cross react with closely related species such as *C. hyointestinalis* (Spence et al., 2011; Sanhueza et al., 2014) and other species commonly found in the bull prepuce (Lew-Tabor et al., 2013). When the genomes of these species were sequenced, it became evident that species sharing the same niche were potentially likely to exchange mobile genetic elements containing virulence genes which are thus unreliable subspecies specific diagnostic markers (Gorkiewicz et al., 2010; van der Graaf-van Bloois et al., 2013, 2014; Lew-Tabor et al., 2013). This suggested that conserved housekeeping genes could be diagnostic assay targets however none to date have been exploited by other research groups.

Currently no predictive methods exist to determine the potential susceptibility or resistance to BGC. In industries such as dairy, research has examined the microbiome associated with mastitis, the genetics of mastitis disease resistance, and the identification of biomarkers to identify cattle at risk of developing mastitis (Cuccato et al., 2022; Rambault et al., 2023; Steinberg et al., 2022; Worku et al., 2023). Selecting for enhanced immune responsiveness to mastitis has been shown to improve responses to vaccination, increased milk production, and improved colostrum quality (Thompson-Crispi et al., 2014). Microbiome studies have also been examined in the context of host-pathogen interactions for example, comparing bovine immune cell transcriptomes in infected vs non-infected macrophages for *Mycobacterium bovis*. This led to the identification of a predictive biomarker of bovine tuberculosis vaccination efficacy in British calves (Aranday-Cortes et al., 2012; Bhuju et al., 2012). Host serum proteins such as Complement component 5 and serotransferrin have been linked to bovine tuberculosis resistant phenotypes (Mazorra-Carrillo et al., 2021). It thus is feasible to identify biomarkers for BGC resistance, immunity and or vaccination using microbiome and proteomics approaches.

For reproductive diseases and “empty cow” syndromes related to BGC and potentially other microorganisms the pathway to improved control is not clear without improved diagnosis and the confirmation of resistance and/or immunity. An effective vaccine is available for BGC/vibriosis; however, uptake is not 100% and producers usually only vaccinate bulls and not heifers. The mixture

of bacterial species found in previous studies is compounding the current recommended diagnostic methods and is also suggesting that perhaps these other pathogens are associated with reproductive disease either directly or indirectly. This has not been researched to date apart from the preliminary studies within a previous ARC Linkage project (Lew-Tabor et al., 2013). Laboratories in the northern hemisphere readily isolate *C. fetus venerealis* from clinical samples as transport to laboratories is relatively short compared to laboratory submissions from northern Australia. Combined with warm conditions, the delayed transport (48 hours +) leads to overgrowth of contaminants in transport culture media compromising the ability to isolate pure *C. fetus venerealis*.

Previous research has indicated:

- The current culture phenotyping methods for *C. fetus venerealis* are not reliable and require the isolation of a pure culture
- Using gold standard phenotyping and molecular testing, we isolated several closely related species with either *C. fetus venerealis* phenotype or molecular assay positive,
- Unlike for *T. foetus*, the direct real time PCR of preputial rinses is not indicative of the presence of *C. fetus venerealis* due to the current lack in assay specificity
- Unlike *T. foetus*, a pure colony of *C. fetus venerealis* needs to be isolated prior to molecular testing
- If a high 'cut off' (Ct <22) is used for the *C. fetus venerealis* parA (McMillen et al., 2006), real time PCR is specific for the pathogen, but at these low Ct scores sensitivity is compromised
- The cpn60 (~heat shock protein 60- a house keeping gene) gene sequence can differentiate between the *C. fetus* subspecies as well as closely related *Campylobacter* spp. (Hill et al., 2006)
- The UQ team has a collection of ~60 *Campylobacter fetus*- like cultures isolated from the bull prepuce which can be used in further assay development and evaluations

Other questions arising from our research include: do other species found in the bull prepuce cause reproductive diseases? does the microbiome favour conditions for the persistence of *C. fetus venerealis* particularly in asymptomatic bulls? Are vaccinated animals as protected from subsequent infection as previously infected animals? How do we know if an animal is immune to BGC? The ability to accurately diagnose BGC and determine the impact of the host microbiome on cattle reproduction will assist to develop better tools to manage infertility. In addition, if a genetic predisposition to reproductive diseases could be identified, this could assist to improve breeding programs for health and fertility.

This project planned to define the microbiomes of the reproductive tracts in healthy and diseased tropical cattle and investigate the host genetics associated with reproductive disease susceptibility/resistance (within the same cattle). This will lead to the development of specific diagnostic tests for BGC, determine whether the microbiome is associated with increased susceptibility to BGC. There are also no biomarkers available to determine if heifers are immune following vaccination and/or natural infection. As a result of understanding genetic variations between cattle we will also pilot the development of genetic markers that can be used in breeding programs to select for BGC resistant genotypes.

Potential Outcomes Planned included:

- Novel methods for differentiating *C. fetus* subspecies *fetus* and *C. fetus* subsp. *venerealis* (the latter is the cause of BGC).
- Identification of novel genital pathogens.
- Diagnostic test for distinguishing BGC-infected from vaccinated animals.
- EBV for resistance/susceptibility to genital infections.

2. Objectives (Original)

2.1 Sampled at least 1,000 cattle (heifers, cows and bulls) from healthy and diseased northern beef herds for genotyping and microbiome analysis.

One thousand and sixteen cattle were sampled for this study thus the objective was met successfully.

2.2 Defined the microbiomes of the reproductive tracts in healthy and diseased cattle.

The microbiome data from the field samples from Objective 2.1 were difficult to interpret, thus two controlled vaccine and *C. fetus venerealis* challenge studies were undertaken in heifers and bulls respectively. The microbiomes of unvaccinated cattle were successfully compared to vaccinated cattle in each trial to define the microbiomes of healthy and diseased (unvaccinated challenged) cattle.

2.3 Investigated the host genomics associated with reproductive disease susceptibility/resistance.

Despite access to nine herds which did not vaccinate with VibroVax®, too few *C. fetus venerealis* cattle were identified using qPCR methods (Objective 2.4) to undertake host genomics associated with BGC susceptibility/resistance. Instead, we determined that the host genome potentially plays a role in influencing the reproductive microbiome associated with disease susceptibility/resistance.

2.4 Developed a validated specific molecular diagnostic test for BGC.

Following *C. fetus* genome analyses, the MraY gene TaqMan SNP Assay was validated as a specific molecular diagnostic test for BGC meeting this objective successfully. However, for low positive or ambiguous samples in qPCR, the application of Oxford Nanopore Technology (ONT) sequencing on clinical samples was shown to be more reliable for the detection of *C. fetus venerealis*.

2.5 Determined whether the genital microbiome is associated with increased susceptibility to BGC

Differences in the genital microbiome from different herds is an indicator of exposure to different environments and a reflection of the gastrointestinal microbiome in those environments. Susceptibility to BGC could only be associated with the detected presence of *C. fetus* in the genital microbiome of cattle from unvaccinated herds. Herds identified with *C. fetus* demonstrated a more diverse microbiome than those without *C. fetus* successfully meeting this objective.

2.6 Determined whether BGC immune biomarkers (post infection or vaccination) can be identified for assay development

The metagenome has no clear differences between vaccinated and unvaccinated bulls, however challenge with *C. fetus venerealis* leads to an increase in *Histophilus* spp. consistently. Vitamin D binding protein (DBP), haptoglobin (HP) and serum amyloid A (SAA) were identified as biomarkers for *C. fetus venerealis* post infection (all 3 biomarkers) and BGC vaccination (DBP only) successfully.

2.7 Piloted the development of genomic markers that can be used in breeding programs to select for resistant genotypes.

Objective 2.3 demonstrated that due to a lack of qPCR positive cattle, genomics studies for BGC resistance was not possible. Preliminary data also demonstrated that the host genome potentially plays a role in influencing the reproductive microbiome associated with disease susceptibility/resistance, however we were unsuccessful in securing sufficient samples from diseased herds towards genomic marker development.

3. Methodology

3.1 Sampled at least 1,000 cattle (heifers, cows and bulls) from healthy and diseased northern beef herds for genotyping and microbiome analysis.

Samples collected from the reproductive tracts of cows and bulls from herds were previously recruited for a QAAFI research project led by Prof Ben Hayes: P.PSH.0833 “Accelerating genetic gain for productivity and profitability in Northern beef cattle with genomic technologies” with access in early 2018 to 49 properties across Queensland (see Appendix 8.1).

The health condition of the animals was assessed by experienced veterinarians prior to sample collection. The preputial samples from bulls were labelled as “Bull”. Using transrectal ultrasound, the stages of pregnancy and reproductive status of the heifers and cows were determined. The female vaginal samples were categorised into “Prepubertal”, “Cycling”, “Pregnant” and “Weaned”. “Prepubertal” were the animals which had not reached puberty during sampling; “Cycling” referred to animals which reached puberty but not pregnant during sampling; “Pregnant” were the animals which were bearing a calf during sampling, while “Weaned” were the animal which had delivered a calf during sampling. The details of each animal including gender, breed, weight, hip height, body condition score, reproductive status and Vibrovax® (Zoetis, Australia) vaccination history, were recorded.

The vaginal and preputial samples were collected using the Tricamper™ (DAF Queensland, Australia) sampling tool following the manufacturer’s protocol. For vaginal samples, the Tricamper™ was inserted into the vagina with the leading edge in contact with the dorsal wall of the vagina. The Tricamper™ was moved back and forth in the vagina to collect the swab. For preputial samples, the Tricamper™ was inserted into the prepuce with the end adjacent to the penis. The Tricamper™ was moved back and forth to scrape across the preputial mucosa and the surface of the penis. Upon removing the Tricamper™ from the vagina or prepuce, the other end of the Tricamper™ was blocked to prevent spillage. The vaginal or preputial sample was immediately preserved in a 10 ml tube preloaded with 5 mL phosphate buffered saline (PBS) by excising the head of the Tricamper™ device. The samples were kept on ice during delivery and were processed within 6 h upon arrival to the laboratory.

A systematic review of published protocols used for bovine microbiome studies (Ong et al., 2021) led to an evaluation of several extraction and sequencing methods to determine the best protocols (Ong et al., 2022a). The methods described here are those which produced the best data. Each sample was first vortexed for 15 s and followed by an additional 15 s of vortex after the Tricamper™ head was removed from the tube. The mucus samples were then transferred into a new sterile tube. Slow and short centrifugation (Soft-spin) was performed (1000 × g 1 min) at 4°C. Without disturbing the pellet, the supernatant was collected into a new tube for DNA extraction. DNA was extracted using two different extraction kits (Ong et al., 2022b). For the QIAGEN DNeasy Blood & Tissue kit, the sample was centrifuged (4000 x g 15 min) to obtain a cell pellet. The supernatant was discarded, and DNA was extracted from the cell pellet according to the manufacturer's instructions for Gram-positive bacteria. Briefly, the pellet was treated with enzymatic lysis buffer and proteinase K digestion prior to column precipitation. Precipitated DNA was washed and eluted in 60 µl of TE buffer provided with the kit. For the QIAGEN QIAamp DNA Microbiome Kit, the sample was first centrifuged (4000 x g 15 min) to obtain the cell pellet. Briefly, the pellet was treated with lysis buffer, benzonase enzyme, bead beating and pathogen lysis buffer before column precipitation. The precipitated DNA was eluted in 60 µl of TE buffer provided with the kit.

3.2 Defined the microbiomes of the reproductive tracts in healthy and diseased cattle

Field sampling was difficult to interpret, we thus decided to undertake two controlled vaccine-challenge trials in heifers and bulls to provide microbiome data following infection with and without prior Vibrovax® vaccination. Blood samples were also collected for serum biomarker research.

3.2.1 Heifer study

A small pilot trial was undertaken under UQ Animal Ethics Approval 2021/AE000056 – approved through the Production and Companion Animal (PCA) animal ethics committee at UQ. The vagina houses a complex microbiome which recent studies have indicated may be dependent on the normal oestrus cycle and hormonal profile of the animal, specifically progesterone and oestrogen levels. Identifying biomarkers in relation to disease therefore require an understanding of variation in response to oestrous cycle. It is also likely that changes in the microbiome and other biomarkers for *C. fetus venerealis* may occur in response to vaccination status. The overall objective of the trial was to identify potential biomarkers in vaginal and serum samples for infection and vaccination status for *C. fetus venerealis*.

The aim of the first part of the study was to identify the composition of microorganisms in the lower reproductive tract of normal cycling virgin heifers, and to identify changes in the microbiome over the oestrous cycle in heifers which were vaccinated against *C. fetus venerealis* (Vibrovax®, Zoetis vaccine) compared to non-vaccinated animals. The aim of the second part of the study was to identify these changes and thereby potential biomarkers in the vaginal mucus and serum, after an experimental challenge with a pure *C. fetus venerealis* culture, in heifers which are vaccinated against *C. fetus venerealis* (Vibrovax®, Zoetis) compared to non-vaccinated animals. Two diagrammatic plans of the trial are shown in Figure 1.

Twelve cycling Droughtmaster heifers aged approximately 18 months, selected by ultrasound scanning for presence of a *corpus luteum*, were sourced from Spyglass Beef Research Facility, Charters Towers. The heifers were trucked on 28th April 2021 to Columba Catholic College (CCC) in

Charters Towers where they were managed as a single group in a paddock of approximately one hectare and were fed with *ad libitum* Rhodes grass hay.

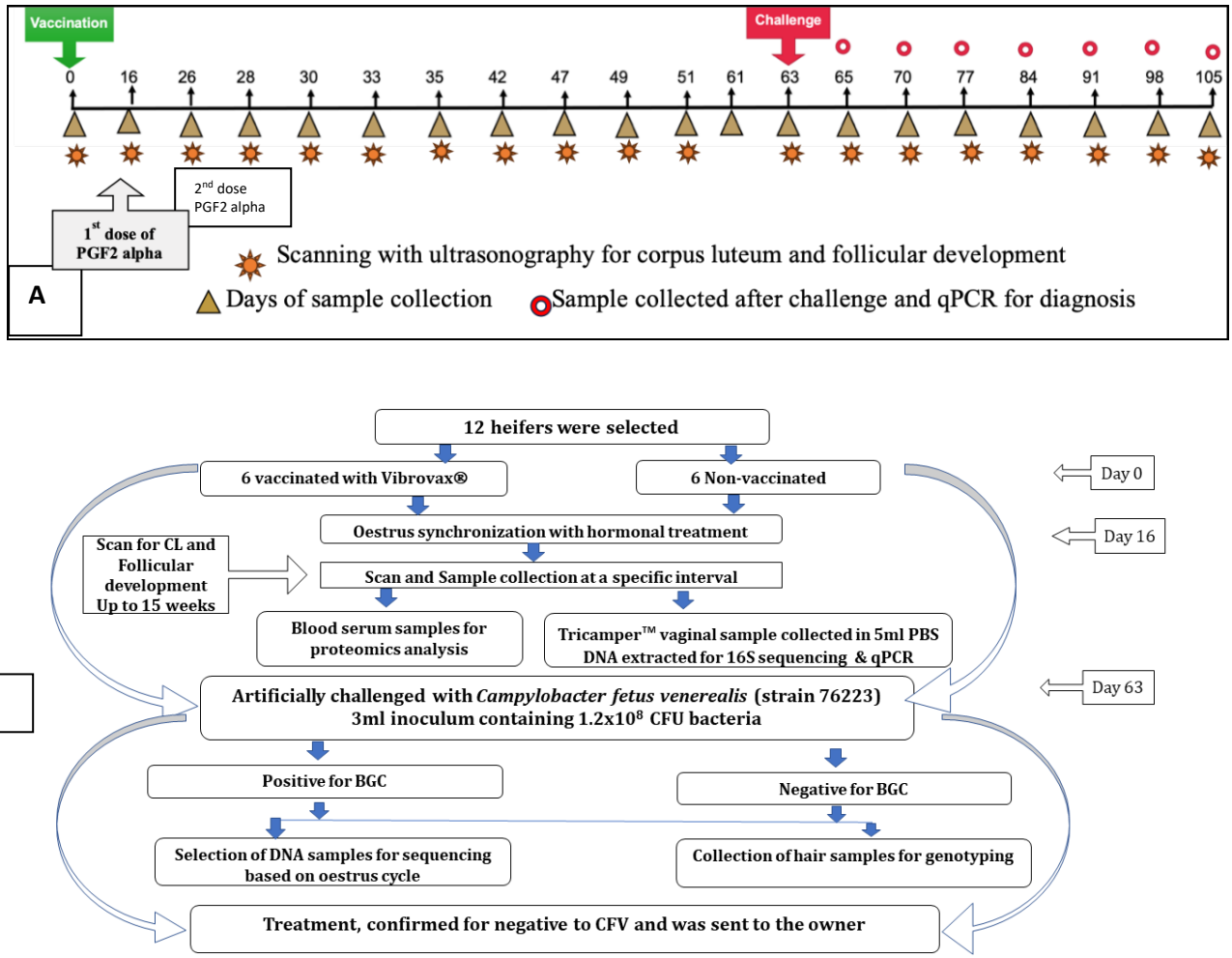


Figure 1. Diagrammatic representations of the *C. fetus venerealis* heifer vaccination challenge trial. A. Summary of sample collections. B. Details of treatments and sample collections for different experiments including serum proteomics, and qPCR.

In phase 1, six heifers were randomly selected and vaccinated (Vibrovax®, Zoetis) on arrival at CCC on the 28th April 2021. All heifers were then synchronised using two prostaglandin (PGF2α) injections (Lutalyse®; Zoetis) on 14th and 24th May 2021. The heifers were then assessed and sampled twice weekly for four weeks (Figure 1).

In phase 2, heifers were again synchronised with a single PGF2α injection on 28th June 2021. Two days later (30th June 2021) each heifer was inoculated intravaginally using an AI insemination catheter with a 3 ml inoculum containing freshly grown *C. fetus venerealis* 76223 (field isolate obtained from an aborted foetus). A 45 ml inoculum was prepared for the 12 heifers. Freshly grown bacterial colonies were collected with disposable cotton tips and vigorously mixed in 45 ml PBS. Subsequently, a 5 ml aliquot was transferred into fresh tube and the turbidity compared with the McFarland No. 3 standard. The bacterial suspension was diluted to match the McFarland standard corresponding to 9 x 10^8 colony forming units (CFU)/mL. This adjusted suspension was then used for the challenge. To confirm the CFU/mL count, 10-fold dilutions of the adjusted inoculum were prepared, and 0.1 ml of each dilution was spread onto 5% sheep blood agar plates using plastic disposable spreaders. For each dilution, two blood agar plates were used. The plates were incubated

at 37°C for 72 h in a micro-aerophilic atmosphere. After 72 h of incubation, the viable count was determined.

Post inoculation, the heifers were assessed and sampled weekly for the following six weeks (Figure 1). Assessments included real time ovarian ultrasound scanning using a 10 MHz transducer (Honda 2100V, Honda Electronics, Toyohashi City, Japan) to monitor the stage of the oestrous cycle through recording presence and size of *corpora lutea* and large follicles. Body condition was assessed (1-5 scale) at each assessment. Heifers were weighed at the start and end of each phase. At each assessment, vaginal mucus was collected into 5 mL of chilled PBS using a Tricamper™ as described by the manufacturer (QDAF: Bovine infertility – Sample collection – Culture/PCR (publications.qld.gov.au), which was then transported to the laboratory. Coccygeal vein blood samples were collected, allowed to clot, and then chilled overnight. Sera was collected following centrifugation and stored frozen for subsequent serum biomarker research (proteomics).

Immediately after the last assessment and sampling, all heifers were treated with an intra-uterine infusion of Metricure® (MSD Animal Health) and the unvaccinated heifers were vaccinated with Vibrovax® on the 11th August 2021. Heifers were confirmed clear of *C. fetus venerealis* by qPCR on 1st September 2021 from the samples collected on 18th August 2021, and returned to Spyglass.

3.2.2 Bull study

To date, inconclusive data was obtained from our field bull-sampling strategies thus we decided to undertake a bull Vibrovax® (Zoetis) vaccination and *C. fetus venerealis* challenge experiment using mature uncastrated bulls. In addition, in most cases heifers are not vaccinated (3.2.1) and primarily only bulls are vaccinated. This research was undertaken under UQ Animal Ethics 2021/AE001138 entitled 'Res-Biomarkers and the preputial microbiome for bovine genital campylobacteriosis' at QASP UQ Gatton campus. Twenty-four bulls with known backgrounds for vaccination, breed and performance were sourced from the NT Department of Industry, Tourism and Trade's experimental herd located at Douglas Daly Research Farm. However, one bull was deemed unfit for transport and thus only 23 bulls were provided. As Animal Ethics Approval required the mandatory sourcing of bulls from a single herd (to manage bull behaviour, additional study animals were not sought. The 23 bulls used in this study were a mix of Brahman and Brahman composite containing approximately 50% *Bos indicus* genetics and were 2-6 years of age.

Using commercial transport, the study bulls were relocated by road to QASP over a period of approximately one week with regular spelling. Soon after arrival and prior to the commencement of the trial, bulls were inducted using a standard prophylactic QASP protocol which included: vaccination with '7 in 1', treatment for coccidiostats (toltrazuril, Baycox®) and worms (fenbendazole, Panacur®). Faecal egg counts were undertaken through the Queensland Department of Agriculture & Fisheries/QDAF Biosecurity Sciences Laboratory. The bulls were randomised using excel [fx=RANDBETWEEN(1,6)] into groups of 4 groups of bulls with young and old bulls mixed into vaccinated and unvaccinated groups of 11 and 12, respectively. The cattle were maintained in pasture paddocks and monitored by QASP staff. Water was available ad libitum throughout the course of the trial. Table 1 summarizes the bull ages, assigned group and previous Vibrovax® vaccination status. It took some time to source a herd of bulls which met our requirements and as such eight of the bulls had Vibrovax® vaccination history as they had been previously used for mating.

Table 1. Data for mature bulls obtained for bull Vibrovax® vaccination and challenge trial.

QASP tag	*Trial Group	Breed	Weight at QASP	Year of Birth	Date of Birth	Vibrovax® History
3766	2U	Brahman	491	2019	30/10/2018	2021
3767	1U	Composite	407	2020	18/11/2019	Nil
3768	1U	Brahman	431	2020	16/11/2019	Nil
3769	2V	Brahman	682	2016	1/12/2015	2021
3770	1V	Composite	323	2020	14/12/2019	Nil
3771	2U	Brahman	642	2017	6/11/2016	2021
3772	1V	Brahman	313	2020	3/01/2020	Nil
3773	1U	Brahman	366	2020	10/01/2020	Nil
3774	2U	Brahman	307	2019	21/10/2018	Nil
3775	2V	Brahman	508	2019	19/11/2018	Nil
3776	2U	Brahman	670	2016	5/12/2015	2021
3777	1U	Composite	407	2020	20/10/2019	Nil
3778	2V	Brahman	502	2019	25/10/2018	2021
3779	2V	Brahman	446	2019	21/10/2018	2021
3780	1U	Brahman	427	2020	3/11/2019	Nil
3781	1V	Brahman	340	2020	1/12/2019	Nil
3782	2V	Brahman	498	2019	23/10/2018	2021
3783	1V	Composite	355	2020	31/10/2019	Nil
3784	2U	Composite	530	2019	20/10/2018	Nil
3785	1V	Composite	384	2020	8/11/2019	Nil
3786	2U	Composite	498	2019	24/10/2018	2021
3787	1V	Brahman	363	2020	18/10/2019	Nil
3788	1U	Brahman	390	2020	2/01/2020	Nil

*1=young <3 years; 2=old ≥3years; U=un-vaccinated; V=vaccinated

Three weeks after their induction to QASP, the bulls were vaccinated (n=11) as described by the manufacturer for Vibrovax®, Day 0. Samples were obtained from each bull at the time of vaccination including: two Tricamper™ preputial mucus samples (as described by the manufacturer, QDAF: Bovine infertility – Sample collection – Culture/PCR (publications.qld.gov.au)) and blood samples. One Tricamper™ sample was collected into PBS for microbiome processing and blood samples were collected into serum tubes for proteomics analysis. These samples were collected weekly until 2 weeks post-challenge, whereby samples continue to be sampled fortnightly until Day 105 (Week 15). Four weeks after the initial vaccination, the vaccinated bulls were boosted with Vibrovax® and three weeks later were challenged with 2.7 x 10^9 live *C. fetus venerealis* strain 76223 in a 3 mL volume PBS prepared as described for the Heifer study above. Following the final sampling the bulls were sold.

3.2.3 Microbiome methods

After vortexing and removal of the Tricamper™, PBS vaginal or preputial mucus samples were centrifuged at 1000 x g for 1 minute to pellet host material and the supernatant was centrifuged 4000 x g for 15 minutes to obtain the microbial pellet for extraction. The samples were then processed using the QIAamp DNA Microbiome kit as described by the manufacturer (QIAGEN) and submitted to the Australian Centre for Ecogenomics (ACE) for sequencing and analysis. The samples were processed by Australian Centre for Ecogenomics (ACE) Sequencing by amplifying the V5-V8

regions of the 16S rRNA genes using primers 803F (5'- TTAGAKACCCBNGTAGTC -3') and 1392wR (5'- ACGGGCGGTGWGTRC -3') (Engelbrektson et al., 2010) modified to contain Illumina specific adapter sequence (803F: 5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTAGAKACCCBNGTAGTC -3' and 1392wR: 5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGGGCGGTGWGTRC-3') in NEBNext® Ultra™ II Q5® Mastermix (New England Biolabs). The resulting amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter) and indexed with unique 8bp barcodes using the Nextera XT 384 sample Index Kit A-D (Illumina) in standard PCR conditions with the above NEB mastermix. Indexed samples were pooled and sequenced on MiSeq Sequencing System using paired end sequencing and V3 300bp chemistry at ACE according to the manufacturer's protocol (Illumina).

ACE undertook the bioinformatics analyses following the pipeline in Figure 2.

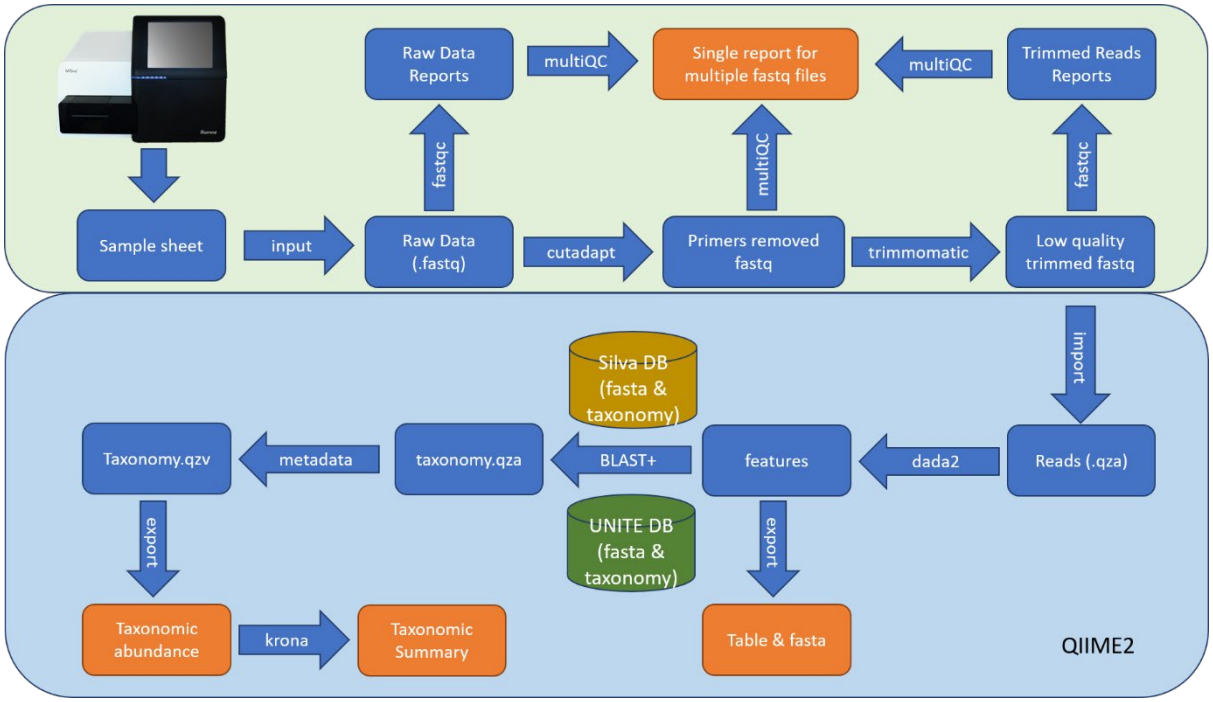


Figure 2. Bioinformatics pipeline developed by the Australian Centre for Ecogenomics (UQ).

3.3 Investigated the host genomics associated with reproductive disease susceptibility/resistance.

3.3.1 Genomic markers. Tail hairs were collected from cattle and submitted to Neogen for genotyping using the GGP_TropBeef50kx array as part of the Northern Genomics project (P.PSH0833). A comparison of un-vaccinated herds was undertaken comparing qPCR positive herds with qPCR negative herds. Targeted herds are presented in Appendix 8.2. qPCR was undertaken using assay 1 described in 3.4.2 below. Herds NG001, NG003, NG008, NG011, NG022, NG023, NG033, NG036 were un-vaccinated but tested negative for *C. fetus venerealis*, only herd NG034 (un-vaccinated) had positive cases (Appendix 8.2). A further 67 Roma (BGC outbreak) cattle were sourced for genotyping using GGP_TropBeef50x test through Neogen.

A pipeline was developed to analyse the genotypes and *C. fetus venerealis* test outcomes, for both genomic estimated breeding value (GEBV) prediction and genome wide association studies, to identify if any individual SNP markers are involved in host resistance to *C. fetus venerealis*.

3.3.2 Genomic:metagenome markers.

To address the question of whether the host genome affects the profile of the vaginal microbiome genome, we compared host genomes derived from 709,768 SNP markers from the bovine HD chip to microbiome profiles assayed with adaptive Nanopore sequencing, see 3.5 below. For 28 animals (from herds NG036 and NG037), the genomic relationship matrix was constructed among individuals following the algorithm of Yang et al (2011). The microbiome relationship matrix was constructed for the same animals using the approach of Ross et al (2013). The correlation of off-diagonal elements of the two matrices was compared with a Mantel test, and permutation testing (shuffling matrix elements across pairs of animals) was used to derive a null distribution for significance testing.

3.4 Developed a validated specific molecular diagnostic test for BGC.

3.4.1 Genome sequencing

A publication draft is under journal submission and is added to Appendix 8.3.

In total, four *C. fetus fetus* (M20-08756/1A, M20-04752/1B, BT268/06 and BT376/03), three *C. fetus venerealis* (A8, 957 and 76223) and two *C. fetus venerealis* biovar intermedius (924 and 926) strains were used in this study (Table 2). These strains were phenotyped using standard biochemical assays and their subspecies identity was confirmed by cpn60 gene sequencing (Hill et al., 2006). The type strains for *C. fetus fetus* (ATCC 27374^{T}) and *C. fetus venerealis* (ATCC 19438^{T}), which had their complete genome published on the National Center for Biotechnology Information (NCBI) database (Home - Genome - NCBI (nih.gov)), were also sequenced in this study to serve as internal controls. The bacteria were cultured on the tryptone soya agar supplemented with 5% defibrinated sheep blood (Thermo Scientific, Delaware, USA) under micro-aerophilic conditions at 37°C for 48 h. Colonies of each bacterial strains were resuspended in sterile phosphate-buffered saline to reach an optical density measured at wavelength of 600 nm (OD_{600}) to yield approximately 1 x 10^9 CFU/mL. Genomic DNA extraction of the pure bacterial culture was conducted using the Genomic-tip extraction kit as described by the manufacturer (QIAGEN, Hilden, Germany). The quantity and quality of extracted gDNA was assessed using Qubit™ 4 fluorometer (Thermo Scientific, Delaware, USA) and pulsed-field gel electrophoresis (Pippin Pulse, Sage Science, Massachusetts, USA).

Table 2. List of *Campylobacter fetus* strains sequenced and analysed in this study.

Strain	Accession	Organism	Country of origin	Year of isolation	Source
Strains analysed in this study					
M20-08756/1A	CP100646	*Campylobacter fetus* subsp. *fetus*	New Zealand	1986	Ovine (fetal stomach contents)
BT376/03	CP076387	*Campylobacter fetus* subsp. *fetus*	United Kingdom	2003	Bovine
BT268/06	CP076388	*Campylobacter fetus* subsp. *fetus*	United Kingdom	2006	Ovine
M20-04752/1B	CP100645	*Campylobacter fetus* subsp. *fetus*	Australia	2020	Ovine (fetal liver)
A8	CP075536-CP075537	*Campylobacter fetus* subsp. *venerealis*	Australia	2011	Bovine
957	CP075538-CP075539	*Campylobacter fetus* subsp. *venerealis*	Australia	2011	Bovine (bull prepuce)

Strain	Accession	Organism	Country of origin	Year of isolation	Source
76223	CP075534-CP075535	*Campylobacter fetus* subsp. *venerealis*	Australia	2012	Bovine (aborted foetus)
924	CP075542-CP075543	*Campylobacter fetus* subsp. *venerealis* bv. Intermedius	Australia	2011	Bovine (bull prepuce)
926	CP075540-CP075541	*Campylobacter fetus* subsp. *venerealis* bv. Intermedius	Australia	2011	Bovine (bull prepuce)
Strains sequenced in previous studies					
ATCC 27374^T	GCA_900475935.1	*Campylobacter fetus* subsp. *fetus*	France	1952	Ovine (fetus)
82-40	GCA_000015085.1	*Campylobacter fetus* subsp. *fetus*	USA	1982	Human
00A031	GCA_011600945.2	*Campylobacter fetus* subsp. *fetus*	Canada	2000	Bovine (bull prepuce)
02A725-35A	GCA_011600855.2	*Campylobacter fetus* subsp. *fetus*	Canada	2002	Bovine (bull prepuce)
04-554	GCA_000759485.1	*Campylobacter fetus* subsp. *fetus*	Argentina	2004	Bovine (aborted fetus)
09A980	GCA_011600995.2	*Campylobacter fetus* subsp. *fetus*	Canada	2009	Bovine (bull prepuce)
INIA/17144	GCA_007723545.1	*Campylobacter fetus* subsp. *fetus*	Uruguay	2017	Ovine (placenta)
ATCC 19438^T	GCA_008271385.1	*Campylobacter fetus* subsp. *venerealis*	United Kingdom	1962	Bovine (vaginal mucus)
84-112	GCA_000967135.1	*Campylobacter fetus* subsp. *venerealis*	USA	1984	Bovine
97-608	GCA_000759515.1	*Campylobacter fetus* subsp. *venerealis*	Argentina	1987	Bovine
08A948-2A	GCA_011601005.2	*Campylobacter fetus* subsp. *venerealis*	Canada	2008	Bovine (bull prepuce)
08A1102-42A	GCA_011600845.2	*Campylobacter fetus* subsp. *venerealis*	Canada	2008	Bovine (bull prepuce)
ADRI545	GCA_011601375.2	*Campylobacter fetus* subsp. *venerealis* bv. Intermedius	Australia	1984	Bovine (reproductive tract)
ADRI1362	GCA_011600955.2	*Campylobacter fetus* subsp. *venerealis* bv. Intermedius	Argentina	1989	Bovine (vaginal mucus)
01/165	GCA_001686885.1	*Campylobacter fetus* subsp. *venerealis* bv. Intermedius	Argentina	2001	Bovine (vaginal mucus)
03-293	GCA_000512745.2	*Campylobacter fetus* subsp. *venerealis* bv. Intermedius	Argentina	2003	Bovine (fetus lung)

The Ligation Sequencing Kit SQK-LSK-109 (Oxford Nanopore Technologies/ONT, Cambridge, UK) was used to prepare sequencing libraries from the double-stranded high molecular weight genomic DNA. The sequencing libraries were loaded onto MinION (ONT) for long-read sequencing with MinKNOW software (ONT). Approximately 1 Gbp of data were generated for each isolate and modified base-calling from raw signal data with minimum quality score filtering of 8 was performed using Guppy 5.0.7 (available to customers only at community site https://community.nanoporetech.com).

Extracted DNA was sent to the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia) for short-read sequencing at the coverage of 4 million read pairs or 1 Gbp of data. The libraries were prepared using Nextera DNA Flex library prep kit (Illumina, California, USA) and

the paired end sequencing was executed on iSeq 100 i1 sequencer with > 80% bases higher than Q30 at 2×150bp. The quality of the reads was assessed using FastQC 0.11.4 (Andrews 2010) and were trimmed with Trimmomatic 0.39.1 using the paired-end mode (Bolger et al., 2014).

Porechop 0.2.4 (Wick et al., 2017a) was utilised to first remove the adapters while NanoFilt 2.7.0 (De Coster et al., 2018) was implemented to select for reads which were greater than 8000 bp in length and greater than 10 in quality score. The quality of the Nanopore long-read sequencing data was assessed and visualised using FastQC 0.11.4 (Andrews 2010) and NanoPlot 1.3.0 (De Coster et al., 2018).

Quality long reads were assembled into contigs using Trycycler 0.5.0 (Wick et al., 2017b). The expected genome size of each bacterial strain was referred to their respective published reference genome on the NCBI database (GenBank Overview (nih.gov)). Medaka 1.4.2 and Nanopolish 0.13.2 (Loman et al., 2015) were applied to polish the complete genomes generated from Nanopore long reads, genome polishing was speed up with the help of GNU Parallel (Tange 2011). The draft assemblies were further improved by polishing with their Illumina short read data using Pilon 1.24 (Walker et al., 2014). The quality of the complete genomes was evaluated using Samtools 1.10 (Li et al., 2009) and QUAST 5.0.2 (Gurevich et al., 2013) with their respective long and short reads. The quality assessments generated for each polished assembly were combined and presented using MultiQC 1.10.1 (Ewels et al. 2016). The polished genomes were visualised and validated on Artemis (Carver et al. 2011). The identities of the complete genomes were verified by BLAST (Altschul et al., 1990). The polished assemblies were submitted to NCBI and annotated with Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016).

For a more comprehensive whole-genome comparison, other complete genomes of *C. fetus fetus* (n=7) and *C. fetus venerealis* (n=9) were downloaded from NCBI Genome database (Table 2). The whole-genome average nucleotide identity (ANI) of the 25 *C. fetus* subspecies genomes was computed using FastANI 1.33 (Jain et al., 2018). The correlation of the 25 *C. fetus* subspecies complete whole genomes based on their ANI was computed in R (R Core Team 2018) using the 'corrplot' package (Wei and Simko, 2021).

Prokka 1.14.6 (Seemann 2014), a two-step wrapper which incorporated Prodigal 2.6.3 (Hyatt et al., 2010), was utilised to annotate the complete genome sequences (n=25). *Campylobacter fetus fetus* 04/554 (GCA_000759485.1) and *C. fetus venerealis* ATCC 19438^{T} (GCA_008271385.1) were provided as reference genomes for Prokka annotations to minimise the biases in annotation files for downstream analysis. The annotated assemblies were submitted to Roary 3.13.0 (Page et al., 2015) for pan genome calculation. The gene content and differences between the two closely related subspecies were also computed with GenAPI 1.0 (Gabrielaite and Marvig, 2020). The virulence factors known to be associated with *Campylobacter* were downloaded from the Virulence Factor Database (VFDB) (Chen et al., 2005) for building a *Campylobacter*-specific VFDB database using ABRicate 1.0.1 (Seemann 2016). The virulence factor encoding regions in each of the *C. fetus* subspecies were identified using the ABRicate 1.0.1 programme.

Single nucleotide polymorphisms (SNPs) were identified using Parsnp 1.5.6 (Treangen et al., 2014) from the core genome generated by aligning the complete genomes of the 25 *C. fetus* subspecies. The putative recombination regions with high SNPs density were detected and the SNPs in these regions were masked using Gubbins 3.0.0 (Croucher et al., 2014). A phylogenetic tree based on the recombination-filtered SNPs in the core genomes of the 25 *C. fetus* strains was generated in R (R Core Team 2018) using the 'ape' package (Paradis and Schliep, 2018). The recombination-filtered SNPs were analysed and annotated with SnpEff 4.3t (Cingolani et al., 2012) to filter out SNPs which

potentially induce synonymous amino acid changes. The amino acid change was verified by examining the translated protein sequences of the SNP-encoding coding sequences (CDS). The recombination-filtered synonymous SNPs which were different between *C. fetus fetus* and *C. fetus venerealis* and which not present in putative Genomic Islands (GIs) were labelled as "candidate SNPs". Additionally, SNPs which were different between *C. fetus venerealis* and its biovar intermedius variant were labelled as "biovar SNPs". Any CDS which encoded for candidate SNPs were functionally annotated using eggNOG-mapper 2.1.6 (Cantalapiedra et al., 2021). The annotated proteins of the SNP-encoding CDS were retrieved from Prokka 1.14.6 and the interactions between the proteins were computed using STRING v11 (Szklarczyk et al., 2019) with *C. fetus venerealis* set as the organism of interest.

Whole-genome alignment of the 25 *C. fetus* complete genomes were computed and visualised using the Blast Ring Image Generator (BRIG) 0.95 (Alikhan et al., 2011), which incorporated BLAST+ 2.10.1 (Camacho et al., 2009) for genome alignment. The putative genomic islands (GIs) were predicted using IslandViewer 4 (Bertelli et al., 2017). Additionally, the genes which were used in the published *C. fetus* subspecies identification PCR assays, including sodium/hydrogen exchanger family protein (*nahE*) (Abril et al. 2007), *ISCfe1* (Abril et al., 2007), peptide transporter carbon starvation (*cstA)* (Hum et al., 1997) and *parA* (Hum et al., 1997; McMillen et al., 2006) were downloaded from the NCBI nucleotide database. The location of putative GIs, candidate SNPs and existing PCR targets for *C. fetus* subspecies identification were identified and labelled on the alignment image generated by BRIG (Alikhan et al., 2011).

3.4.2 Assay 1. The first assay *C. fetus venerealis* reported in this research project was based on a single SNP difference between *C. fetus venerealis* and *C. fetus fetus* in the conserved heat shock protein 60 or chaperonin protein 60 (*cpn*60) gene. Custom Applied Biosystems™ TaqMan® MGB (minor groove binder) probes incorporate a 5' reporter and a 3' nonfluorescent quencher (NFQ). Custom TaqMan MGB probes for SNP assays were designed using the Thermo Fisher Scientific online tool (Custom TaqMan™ SNP Genotyping Assay) targeting the *C. fetus venerealis* and *C. fetus fetus* cpn60 genes and labelled with FAM™ and VIC™ reporter dyes, respectively. The two probes and the forward and reverse primers were pre-mixed by Applied Biosystems and provided as a 20x mix for use in custom assays (Thermo Fisher Scientific, Australia). The mastermix was the AgPath-ID™ One-Step RT-PCR Reagent (Thermo Fisher Scientific) without the Reverse Transcriptase step and was provided as a 2x concentrated buffer. The 10 µl reaction consisted of 1x buffer (5 µl), 900 nM of the forward (5' CCGTATTTCATCACAAACGCTGAA 3') and reverse (5' CCGGAAGTAGATCTTTTAAATTTGTAATTTTCTTATCA 3') primers, 200 nM of the *C. fetus venerealis* FAM probe (6FAM-TGCAAGTCGAGTTAAGT-3MGBNFQ), 200nM of the *C. fetus fetus* VIC probe (VIC-TGCAAGTCGAATTAAGT-3MGBNFQ), 0.4 µl of the 25X RT-PCR Enzyme Mix (AmpliTaq Gold™ DNA Polymerase at 0.025 units per reaction). Assays were run in duplicate and the mastermix dispensed into tubes or plates. For clinical field samples, 4.1 µl total DNA was added to each reaction, with 1 µl for the positive controls (2 ng DNA and 3.1 µl nucleases free water) and nuclease-free water was added to the No Template Control (NTC, negative control) tubes or plates. The assays were run in the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System for the two fluorophores under the following conditions: activation at 95^{o}C 10 min, followed by 45 cycles of 95^{o}C 15 s, 67^{o}C 1 min; and a final extension at 67^{o}C for 7 min.

The Bio-Rad Optical data was received after the sample RUN was opened in CFX maestro Software and done log corrections. This Bio-Rad optical file displayed data in charts and spreadsheets when opened using the CFX Maestro Software. The Quantification tab showed chart data regarding the relative fluorescence (RFU) for each well at every cycle. The spreadsheet tab showed the Cq data

collected from each well after log correction. Data from a single fluorophore in a single well was represented by each trace in the graph. These numbers were used to calculate Cq values for each fluorophore in each well by the software. This was achieved by the single threshold mode, where a single threshold value was used to calculate the Cq value based on the threshold crossing point of each fluorescent trace. The threshold if fitted, subtracted the NTC and other backgrounds, giving the best fit data for each sample. The data from the spreadsheet tab was exported and tabulated in Excel. The average of the two Cq scores provided a numeral of up to 40 or negative. The cut-off for a positive sample was a Cq score of 33, with those identified at Cq 34-40 considered background due to non-specific positives. This was determined by mixing DNA from other non-specific bacterial species and bovine DNA. The non-specific bacterial species included: *Campylobacter hyointestinalis*, *Arcobacter cryaerophilus*, *Campylobacter ureolyticus* and *Campylobacter sputorum* which were isolated in a previous study screening abattoir bull penises (Lew-Tabor et al., 2013) and have been shown to cross-react in previous *C. fetus venerealis* molecular assays (McMillen et al., 2006; Spence et al., 2011).

3.4.3. Assay 2. Following preliminary genomics analyses of *C. fetus* subspecies sequences, a second assay was designed which targeted a hypothetical protein gene (RS04330) found to contain more than a single SNP to differentiate *C. fetus venerealis* and *C. fetus fetus*. Assay sequences designed using Thermo Fisher Scientific's online tool (Custom TaqMan™ SNP Genotyping Assay) were:

Cfv RS04330 FAM Assay: Forward Primer: 5'ATAAGCGATAATCGCTACCCTTATTG; Reverse Primer: 5'CGCTCAAGAAAATGGAGGTAGATCT; MGB Probe: 5' 6FAM TTGATCTAGCATCATAGGA-3'MGBNFQ

Cff RS04330 VIC Assay: Forward Primer: 5'ATAAGCGATAATCGCTACCCTTCTTT; Reverse Primer: 5'CGCTCAAGAAAATGAAGGTAGATCC; MGB Probe: 5'VIC-TTGATCTAACATCATAGGAT-3'MGBNFQ

The assays were screened using gradient PCR to determine the optimal annealing temperature. The same concentrations for primers and probes for assay 1 above were used as recommended by the manufacturer (Thermo Fisher Scientific, Australia). The concentrations for the 10 µl reactions were the same as described for assay 1. The assays were first screened using DNA from both *C. fetus fetus* and *C. fetus venerealis* controls and both sets of primers and probes to determine if the assays could be multiplexed. As there was a cross-reaction in the *C. fetus venerealis* assay, the multiplex approach was not applied, and separate assays were used for each subspecies. The assay conditions were the same as Assay 1 except 50^{o}C was determined to be the optimal annealing temperature. The same Assay 1 bacterial species and mixtures were used to determine the specificities of these assays.

3.4.4. Assay 3. Once genome sequencing comparisons were completed, a SNP in the *mraY* gene was found to consistently differentiate *C. fetus venerealis* from *C. fetus fetus* at the genomic level. Custom TaqMan MGB probes for SNP assays were designed using the Thermo Fisher Scientific online tool (Custom TaqMan™ SNP Genotyping Assay) targeting the *C. fetus venerealis* and *C. fetus fetus mraY* genes and labelled with VIC™ and FAM™ reporter dyes, respectively:

mraY Forward primer: 5' AAAATGATGATGAATTGGCGCCATT 3'; *mraY* Reverse primer: 5' TGTGATGGAAACCTTATCTGTTATATTGCA 3'; Cff *mraY* Probe: 5'FAM- CGTTTTTTGCGTATTTT-3'MGBNFQ; Cfv *mraY* Probe: 5'VIC- CCGTTTTTTGTGTATTTT 3'MGBNFQ (yellow highlight is the SNP targeted which differentiates the subspecies). Assay reagents and conditions were as described for Assay 1 except that cycling conditions differed to incorporate a 69^{o}C annealing temperature: activation at 95^{o}C 10 min, followed by 45 cycles of 95^{o}C 15 s, 69^{o}C 1 min; and a final extension at 69^{o}C for 7 min.

3.4.5. Diagnostic assay adoption by State Government Veterinary Diagnostic laboratories

Assays 1 and 2 were trialled by Biosecurity Queensland Laboratories by their diagnostic staff using RotorGeneQ (Qiagen) qPCR equipment in 2021. Since this time, relevant staff have retired and currently the Queensland laboratory are not trialling assay 3 for *C. fetus venerealis*. Assay 3 is currently under evaluation by staff at Elizabeth Macarthur Agricultural Institute from NSW Department of Primary Industries (2023) with a plan to co-publish their data with our manuscript describing this new assay.

3.4.6. Other diagnostic approaches.

3.4.6.1 Digital Droplet PCR

Assays 1 and 2 above were also tested using the fully integrated nano-plate based digital PCR system in a QIAGEN's QIAcuity instrument on trial at the University of Queensland in 2021. For the digital PCR (dPCR), the QIAcuity Probe Master mix was used was provided as a 4 x mix, thus 10 µl was used in a 40 µl reaction (QIAGEN). Each primer was used at 0.8 µM concentration and each probe at 0.4 µM concentration. The total reaction volume was 40 µl with the addition of sterile water, and 1 µl of template DNA per reaction. Standard PCR plates were used to set up the assays and the reaction was transferred to the nanoplate. The dPCR assays were performed on a 24 well nanoplate with 26,000 partitions in total. The nanoplate was sealed with QIAcuity Nanoplate Sealant and inserted into the QIAcuity device to run the dPCR program. The assay conditions used: PCR initial heat activation 95^{o}C 2 mins, followed by 40 cycles of denaturation at 95^{o}C 15 s, annealing/extension at 56^{o}C for 30 s. As this was a demonstration by QIAGEN, the company representative did the analyses and provided the results shown in this report.

The plate analysis option in the software QIAcuity Software suit shows the result in different modes. The Absolute Quantification tab option has a list tab tabulating the sample name, assay type, target, concentration of the sample as well as the number of positive and negative partitions per sample. The number of positive & negative partitions were compared and the larger the output positive partitions number, the greater the reaction's positivity (as opposed to normal Cq in normal qPCR assays described above).

3.4.5.2 Nanopore enrichment methods

We have optimised an ‘adaptive sampling’ technique where DNA samples (extracted from the reproductive tracts of heifers and bulls) are sequenced by enriching for *Campylobacter* genetic material in the sample. Two approaches were taken, the first simply enriched for all Campylobacter genomic material, whereas the second enriched specifically for the three *C. fetus venerealis* specific SNPs identified in this research programme cpn60 and mraY. Initial experiments were undertaken using DNA prepared from *C. fetus venerealis* negative clinical samples which were spiked with known concentrations of *C. fetus venerealis* DNA (4 ng to 40 fg in serial dilutions). Briefly, 100 ng of DNA from a preputial sample from a *C. fetus* negative sample (NG034-211) was mixed with *C. fetus fetus* (ATCC27374) or *C. fetus venerealis* (ATCC19438) DNA samples from 4 ng to 40 fg in serial dilutions. Sequencing libraries were constructed from each sample using the Nanopore Native Barcoding kit (SQK-NBD114.24) as described by the manufacturer (ONT, UK). Eight libraries were multiplexed on a single 10.4 Flow Cell and sequenced for 24 hours on the Nanopore GridION with enrichment adaptive sampling and high accuracy basecalling turned on. This process was replicated, but sequencing using depletion of the bovine genome (rather than enrichment for *Campylobacter*). This enabled the two techniques to be directly compared and provided field data to compare to the bull

trial described above. Samples were enriched for the *C. fetus* subspecies genomes or regions of interest in a .bed file with the following co-ordinates ATCC-19438-contig1:818000-819000, ATCC-19438-contig1:655000-656000, ATCC-19438-contig1:332000-333000. Once validated the technique was trialled on DNA from animals known to be *C. fetus venerealis* negative, positive, or suspected positive using the same workflow as above. To analyse the data, sequences were trimmed, and quality checked using the NanoPack package and all reads with a quality score below 10 were removed. Reads were mapped against the bovine genome using Minimap2 and all reads that failed to map (non-bovine) were extracted using Samtools. Non-bovine reads were classified using the software package Kraken2 and a custom database of all Campylobacter genomes characterised previously (Appendix 8.3).

3.4.6 Field assay evaluations

Field samples were collected using the Tricamper™ tool in PBS and processed as described above for DNA extraction and for qPCR assays. Field samples collected from Roma Veterinarians (n=95, see Appendix 8.2), AaCo (n=639), Jandowae (n=38) and random abattoir samples (n=9). The samples collected from AaCo were DNA preparations provided by the Qld Government Biosecurity Sciences Laboratory prepared for the *Tritrichomonas foetus* qPCR. Briefly, 1 mL of the Tricamper™ mucus samples in PBS were pelleted at 14,000 x g for 5 minutes and resuspended in 100 µL for extraction using the MagMax CORE Nucleic Acid Purification Kit (as described by the manufacturer, Thermofisher) with DNA eluted into 90 µl. Samples provided directly to our laboratory (Roma Vets, Jandowae, and abattoir samples) were processed using the DNA extraction protocol described in section 3.1 or the MagMax CORE Nucleic Acid Purification Kit according to the manufacturer (Thermofisher). Briefly the PBS vaginal or preputial mucus samples were centrifuged at 1,000 x g for 1 minute to pellet host material and the supernatant was centrifuged 4,000 x g for 15 minutes to obtain the microbial pellet for extraction. DNA was processed using either the QIAamp DNA Microbiome kit (QIAGEN) (section 3.1) or the MagMax Kit (Thermofisher) as described by the manufacturers.

For the 9 abattoir samples, blood samples were also provided for proteomics to confirm biomarkers using methods described in section 3.6.2.

Long read sequencing *C. fetus venerealis* detection was undertaken using 10 Roma samples, 4 AaCo samples and 9 abattoir samples. Sequencing was carried out using both the adaptive sampling by depletion of the bovine genome and adaptive sampling by enrichment for *Campylobacter* genomes. This enabled the two techniques to be directly compared and provided field data to compare to the metagenomics data from the bull trial described above. This dataset also allowed comparison to and validation of qPCR results.

3.5 Determined whether the genital microbiome is associated with increased susceptibility to BGC

Methods were developed using Oxford Nanopore Technologies for shotgun long read sequencing of the entire metagenome using adaptive sampling technologies to minimise bovine host sequence contamination. A preliminary study has been published (Ong et al., 2022c, Appendix 8.7) and is described here.

Long read adaptive sampling enables the removal of host material during sequencing through the recognition of bovine reads which are ejected from the pore which will preferentially process non-

bovine reads. Two herds with different reproductive rates were sequenced using this method, see Table 3.

Table 3. Annual pregnancy rates for the 2 cohorts in herd NG036 (Composite) and NG037 (Droughtmaster).

ClbCode	Year	Total	Pregnant	% Pregnant
NG036	2018	82	14	17.1
NG036	2019	175	86	49.1
NG036	2020	155	89	57.4
NG037	2018	20	4	20.0
NG037	2019	20	1	5.0

Samples were collected from two cattle properties, which were approximately 200 km apart, in Northern Queensland by an experienced veterinarian. The cattle breeds in Station A and B were Crossbred and Droughtmaster respectively and the *Bos indicus* content of the herds in these two stations were 49 and 54% respectively (Appendix Supplementary 8.8.1). In total, four hundred and fifty-two samples were collected from 2018 to 2021. For heifers and cows, the stages of pregnancy and reproductive status were determined using transrectal ultrasound as previously described (Corbet et al., 2016). Descriptive terms: 'prepubertal' were heifers which did not have a corpus luteum (CL); 'cycling' referred to animals that had a CL present, but no foetus or embryo was detected; 'pregnant' were the animals in which a foetus or embryo was detected; 'postpartum' were cows which were known to have delivered a calf in the past 12 months. Samples were collected into PBS using the Tricamper™ tool as described above and transported chilled to the laboratory. DNA was extracted using the DNeasy Blood & Tissue kit as described by the manufacturer (QIAGEN). The quantity of extracted DNA was measured using QubitTM 4 fluorometer (Invitrogen™) with the Qubit™ dsDNA Broad Range assay kit (Invitrogen™). The extracted genomic DNA were examined using the Pippin Pulse (Sage Science) pulsed-field electrophoresis gel to determine the size and integrity.

Libraries for Oxford Nanopore Technologies (ONT) adaptive sampling were prepared using the ONT SQK-LSK109 kit (ONT, Cambridge, UK) according to the manufacturer's instructions Briefly, 48 uL of the extracted DNA was added to the end-repair reaction mix. Then, adapters were ligated to the end-repaired DNA. The ligated DNA library was loaded to be sequenced on an individual MinION flowcells FLO-MIN106D (ONT, Cambridge, UK). Each time, three to five libraries were running concurrently on the ONT GridION Mk1 sequencer with software MinKNOW version 21.05.8 (ONT, Cambridge, UK). The adaptive sampling mode was applied to deplete genomes of both ARS-UCD1.2 *Bos taurus* genome (GCA_002263795.2) and Brahman genome (Ross et al., 2022). Each sample was sequenced for 24 h and the raw data was transferred to Linux system for base calling using Guppy version 5.0.11 (ONT, Cambridge, UK). The quality of the long reads generated using ONT were evaluated using NanoPlot 1.3.0 (De Coster et al., 2018). Adapters on the long reads were removed using Porechop 0.2.4 (Wick et al. 2017a) while NanoFilt 2.7.0 (De Coster et al., 2018) was applied to filter out reads which were less than 5 in quality score. The long reads were mapped against the ARS-UCD1.2 *Bos taurus* genome (GCA_002263795.2) and Brahman genome (Ross et al., 2022) using Minimap2 2.17 (r941) (Li 2018) to examine the percentage of cattle DNA in the sample sequenced with different technologies.

Read-based taxonomic classification of the reads was performed using Kraken v2.1.2 (Wood et al., 2019). To ensure a more targeted and efficient search for the metagenome samples in this study, instead of the standard database suggested by Kraken2, a customised database was constructed with the build script provided by Kraken2. The customised database used in this study was built with

the complete genomes of archaea, bacteria and fungi, which were downloaded from NCBI RefSeq (Pruitt et al., 2005) with their low complexity sequences masked. The abundances of the organisms in the metagenomic samples sequenced with different methods were estimated using the Bayesian Re-estimation of Abundance with KrakEN (Bracken) v2.6.2 (Lu et al., 2017). The downstream bioinformatic analyses and visualisation of the results were conducted on R studio (R Studio 2020) with R packages including vegan 2.5.7 (vegan 2020), phyloseq 1.34.0 (McMurdie and Holmes, 2013), DEseq2 1.30.1(Love et al., 2014), dplyr 1.0.7 (dplyr 2020) and ggplot2 3.3.5 (ggplot2 2016). The pipeline MetaErg 1.2.0 (Dong and Strous, 2019) was performed to functionally annotate the assembled contigs of each sample. Briefly, the predicted ORFs were subjected to HMMs profile similarity searches or DIAMOND (double index alignment of next-generation sequencing data) searches against several databases, including Pfam-A (Finn et al., 2016), TIGRFAM (Haft et al., 2013), FOAM (Prestat et al., 2014), metabolic-hmms (Anantharaman et al. 2016), *cas*genes.hmm (Burstein et al., 2017) and SwissProt (Bairoch and Apweiler, 2000). Mapping files generated from searches against SwissProt, FOAM and TIGRFAMs databases were incorporated in MinPath (Ye and Doak 2009) to infer to KEGG (Kanehisa and Goto, 2000) and MetaCyc metabolic pathways (Karp et al., 2002). Bioinformatic analyses and visualisation of the outputs were performed on R studio (RStudio 2020) with R packages including edgeR 3.32.1 (Robinson et al. 2010) and clusterProfiler 3.18.1 (Yu et al., 2012).

Ribosomal DNA amplicon sequencing. For these analyses, samples were sent to an external provider (the Australian Genome Research Facility/AGRF) who sequenced the V1-V3 variable region of the 16S ribosomal DNA using Illumina (short read) technologies. This assists to estimate the relative bacterial populations of a given sample. Herd NG034 was selected for amplicon analysis due to its bovine genital campylobacteriosis cases and 'non-Vibrovax®' vaccination history.

3.6 Determined whether BGC immune biomarkers (post infection or vaccination) can be identified for assay development

3.6.1 Microbiome biomarkers.

One hundred and twenty samples from the bull trial (3.2.2) were screened using ONT adaptive metagenomics. DNA concentrations for each sample were measured using the Qubit™ fluorometer (Invitrogen™) with Qubit™ dsDNA Broad Range assay kit (Invitrogen™). DNA samples were pooled into groups (5 individuals/group) according to vaccination status, age (old = ≥3 years; young = <3 years) and timepoint of sample collection (Table 4. Figure 3). DNA from 5 individuals (120 ng each) was combined to achieve 600ng of DNA per group. The pooled DNA was barcoded (24 barcodes) and library preparation was carried out using the Native Barcoding Kit 24 V14 (SQK-NBD114-24) according to the manufacturers protocol.

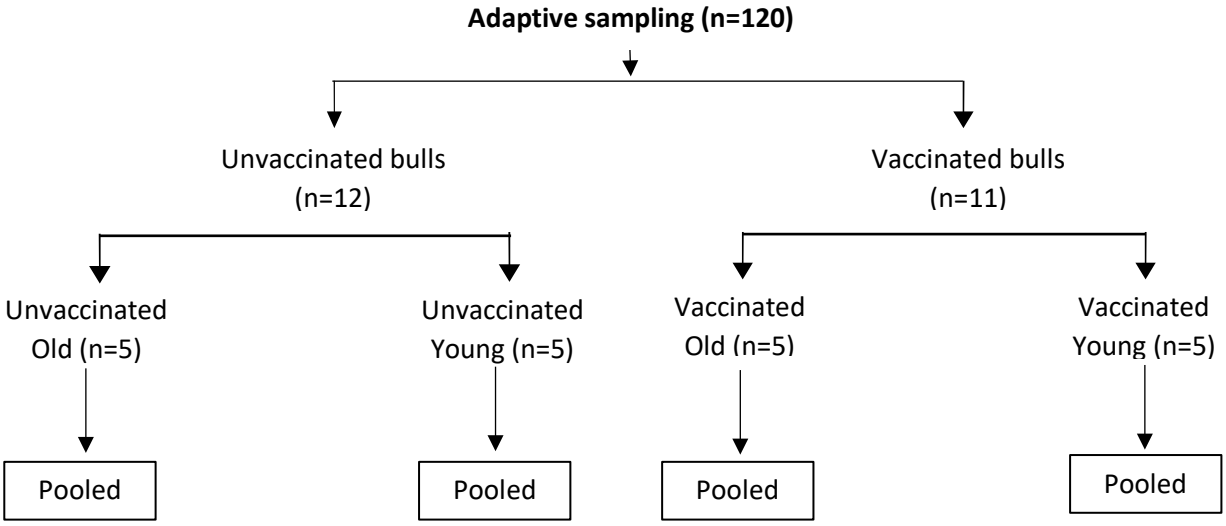


Figure 3. The figure showing the grouping of animal for adaptive metagenome sequencing. A total number of 120 samples were sequenced from four groups (unvaccinated_old, unvaccinated_young, vaccinated_old, vaccinated_young) using 6 time points of trial. Samples from 5 animals were pooled in each group (5 animals x 4 groups x 6 timepoints=120).

Table 4. Description of the timepoints and barcodes used for bull metagenome study.

Barcodes	**Status**	**Time points**	**Number of animals samples pooled**
Barcode 01	Unvaccinated old	Day 0_naive	5
Barcode 02	Unvaccinated young	Day 0_naive	5
Barcode 03	Vaccinated old	Day 0_naive	5
Barcode 04	Vaccinated young	Day 0_naive	5
Barcode 05	Unvaccinated old	Day-49_after 2nd vaccination	5
Barcode 06	Unvaccinated young	Day-49_after 2nd vaccination	5
Barcode 07	Vaccinated old	Day-49_after 2nd vaccination	5
Barcode 08	Vaccinated young	Day-49_after 2nd vaccination	5
Barcode-09	Unvaccinated old	1st week _after challenge	5
Barcode 10	Unvaccinated young	1st week _after challenge	5
Barcode 11	Vaccinated old	1st week _after challenge	5
Barcode 12	Vaccinated young	1st week _after challenge	5
Barcode 13	Unvaccinated old	7th week _after challenge	5
Barcode 14	Unvaccinated young	7th week _after challenge	5
Barcode 15	Vaccinated old	7th week _after challenge	5
Barcode 16	Vaccinated young	7th week _after challenge	5
Barcode 17	Unvaccinated old	14th week _after challenge	5
Barcode 18	Unvaccinated young	14th week _after challenge	5
Barcode 19	Vaccinated old	14th week _after challenge	5
Barcode 20	Vaccinated young	14th week _after challenge	5
Barcode 21	Unvaccinated old	2nd day_after re-challenge	5
Barcode 22	Unvaccinated young	2nd day _after re-challenge	5
Barcode 23	Vaccinated old	2nd day_after re-challenge	5
Barcode 24	Vaccinated young	2nd day_after re-challenge	5

The 24 barcoded libraries were sequenced across 4 PromethION flow cells (PromethION R10.4.1) on the PromethION 24 (A100) sequencer from Oxford Nanopore Technologies. Flow cells were primed and loaded using the Native Barcoding Kit 24 V14 (SQK-NBD114-24) according to the manufacturer's instructions. Table 5 shows the amount of DNA loaded on to the flow cell for each barcoded library.

Table 5. Amount of DNA loaded on to a flow cell for each barcode.

Libraries	Concentration of DNA	Amount of DNA loaded (ng)	Barcodes	Concentration of DNA/barcodes
Library 1	28.00 ng/µL	67.2 µL	Barcode-01	29.00
			Barcode-03	34.6
			Barcode-04	29.4
			Barcode-05	23.6
			Barcode-07	25.8
Library 2	15.50 ng/µL	91.6 µL	Barcode-02	12.5
			Barcode-06	11.3
			Barcode-08	10.1
			Barcode-13	10.4
			Barcode-16	11.0
			Barcode-17	15.4
Library 3	4.48 ng/µL	145.4 µL	Barcode-14	6.82
			Barcode-18	4.92
			Barcode-19	5.66
			Barcode-20	4.62
			Barcode-21	4.66
			Barcode-22	2.58
Library 4	1.47 ng/µL	47.0 µL	Barcode-09	0.96
			Barcode-10	0.80
			Barcode-11	1.00
			Barcode-12	1.02
			Barcode-23	2.38
			Barcode-24	2.82

The samples were sequenced using the adaptive sampling mode for 24 h with a minimum quality of 9 and a minimum length of 150 bp. Sequences were depleted against the bovine genome ARS-UCD1.2 (GCA_002263795.2) in fasta format. High accuracy basecalling was set with MinKNOW software and output files were saved as .fast5 and .fastq.

All bioinformatic analysis was carried out on the Bunya high performance computer provided by University of Queensland. Data was checked using NanoComp v.1.21.0 (De Coster & Rademakers, 2023) and reads with quality scores below 10 or lengths below 200 bp were removed using NanoFilt v2.8.0 (De Coster et al., 2018). Reads were mapped to the bovine reference genome (genome ARS-UCD1.2 – GCA 002263795.2) using minimap2 v.2.25 with alignment mode for Oxford Nanopore data (Li 2021). All reads that could not be mapped to the bovine genome were then extracted using samtools v.1.6 (Danecek et al., 2021). Species classification was performed on extracted fasta reads using Kraken2 V2.0.7 (Wood et al., 2019). A customized Kraken2 database was built containing the complete genomes of archaea, bacteria, viral, fungi and protozoa from NCBI RefSeq (Pruitt et al., 2005), with the scripts provided by Kraken2. Data was visualised using R Studio (2022.07.2) and the dplyr (Wickham et al., 2023) and ggplot2 (Wickham 2016) packages.

3.6.2 Protein biomarkers

Protein biomarkers for BGC resistance were identified from the proteomics data which were validated following analysis from the heifer and bull trials. Field samples were submitted from abattoir collections for biomarker validation.

Proteomics analysis was undertaken at UQ's School of Chemistry and Molecular Bioscience's Mass spectrometry: proteomics facility. Samples were separated using reversed-phase chromatography on a Waters M-Class UPLC system. Samples were loaded onto a Waters NanoEase HSS T3 column (100 A, 1.8 µm, 300 µm x 150 mm). Chromatography was performed at 5 µL/min and column set at 40 deg C, with LC conditions as follows: 0 - 1min = 3% B, 1- 31 min = 3-30%, 32-45 min = 30 – 97%, held at 97% for 4 min followed by equilibration for 4 min. Pump A = 0.1% FA in water, and pump B = 0.1% FA in ACN. Eluted peptides were directly analysed on a ZenoTof 7600 instrument (ABSciex) using an OptiFlow Micro/MicroCal source. Curtain gas = 35 psi, CAD gas = 7, Gas 1 = 20 psi, Gas 2 = 15 psi, source temp = 150 deg C, spray voltage = 5000 V, DP = 80, CE = 10. For data dependent acquisition analysis, MS TOF scan across 400-1500 m/z was performed for 0.2 sec followed by data dependent acquisition of up to 20 peptides with intensity greater than 150 counts, across 100-2000 m/z (0.035 sec per spectra) using dynamic collision energy. Zeno pulsing was on, with a threshold set to 100000 cps. For SWATH acquisitions, MS TOF scan across 400-1500 m/z was performed (0.1 sec). For MS2, variable windows spanning 399.5 m/z – 750.5 m/z were chosen for fragmentation (0.013 sec) with fragment data acquired across 140 – 1750 m/z with Zeno pulsing on. Dynamic collision energy was used.

The raw MS data files were converted to DIA-NN format using the DIA-NN software (Demichev et al., 2020) followed by the generation of ion library of proteins identified with DIA-NN (DIA-NN 1.8) using a representative sample from each condition and time point. The output library was reformatted using a Python script for further analysis with PeakView 2.1 (SCIEX®). The settings for PeakView were as follows: with settings: shared peptides = allowed; peptide confidence threshold = 99%; false discovery rate = 1%; peptides per protein = 6; transitions per peptide = 6; XIC extraction widow = 6 min; XIC width = 75 ppm. A python script (https://github.com/bschulzlab/reformatMS) was used to reformat the PeakView output, with a 1% peptide FDR cut-off to remove the ion measurements for low quality peptides from each sample, and reformatting appropriate for use with MSstats (Kerr et al., 2019). Differences in protein abundance were determined with a linear mixed model using MSstats (2.4) in R (Choi et al., 2014), with Benjamini and Hochberg corrections adjusting for multiple comparisons and a significance threshold of $P < 10^{-5}$.

3.7 Piloted the development of genomic markers that can be used in breeding programs to select for resistant genotypes.

This milestone intended to expand on the metagenome:genome relationship demonstrated in Figure 10 (Section 4.3) to further test that the host genome influences the microbial genome.

Available Roma herd cohorts had tail hairs collected (See Appendix 8.2, n=26) and submitted for genotyping through Neogen using GGP_TropBeef50x test.

4. Results

4.1 Sampled at least 1,000 cattle (heifers, cows and bulls) from healthy and diseased northern beef herds for genotyping and microbiome analysis.

A total of 1012 reproductive tract samples have been collected for this research (see summary Table 6, see detailed spreadsheet, Appendix 8.1). Thirty herds have been sampled with 11 also including bull tract samples. Of the 30 herds, 11 do not routinely vaccinate with Vibrovax® to protect against bovine genital campylobacteriosis (BGC) and 19 herds routinely vaccinate their bulls (Table 6).

Table 6. Summary of reproductive tract samples collected from 30 northern Australian herds.
*^Legend: X=Cross; B=Brahman; '-' represents 'none identified'

Property code	Breed^	No. bulls sampled	No. cows sampled	Vibrovax®	Reproductive Status			
					Pre-puberty	Cycling	Empty	Pregnant
NG001	Brahman Cross	10	20	Nil	5	4	2	9
NG003	Brahman	0	20	Nil	-	-	10	10
NG004	Cross	0	25	Nil	-	-	6	19
NG005	Brahman	0	16	Bulls vaccinated	-	-	7	9
NG006	Brahman	0	27	Nil	-	-	14	13
NG007	Brahman	7	26	Bulls vaccinated	-	-	5	21
NG008	Brahman	0	30	Nil			12	18
NG011	Brahman	0	18	Nil	-	-	6	12
NG021	Brahman/ Angus/Santa Gertrudis X	0	25	Bulls vaccinated	-	3	22	-
	Santa Gertrudis	10	0	Vaccinated	NA	NA	NA	NA
	Angus	7	0	Vaccinated	NA	NA	NA	NA
NG022	Brahman	4	71	Nil	5	20	43	3
NG023	Shorthorn	0	21	Nil	-	17	4	-
NG024	Droughtmaster	0	20	Bulls vaccinated	-	-	1	19
NG025	Droughtmaster	0	53	Bulls vaccinated	23	30	-	-
NG027	Cross	0	20	Bulls vaccinated	-	-	9	11
NG028	Droughtmaster	0	20	Bulls vaccinated	2	18	-	-
	Composite		30		-	-	1	29
NG031	Brahman Cross	12	25	Bulls vaccinated	5	7	-	13
NG032	Brahman Cross	0	40	Nil	-	-	40	-
NG033	Cross	0	20	Nil	11	9	-	-
NG034	Brahman and Brahman Cross	3xB 3xX	20 (B)	Nil	5	14	-	1
NG036	Brahman Cross	46	60	Bulls vaccinated	19	1	26	14

Property code	Breed^	No. bulls sampled	No. cows sampled	Vibrovax®	Reproductive Status			
					Pre-pub-erty	Cycl-ing	Empty	Preg-nant
NG037	Droughtmaster	4	65	Bulls vaccinated	17	15	27	6
NG038	Brahman	0	30	Nil	9	2	11	8
NG039	Cross and Brahman	6 Brahman	21X	Bulls vaccinated	10	2	-	9
NG040	Brahman	0	18	Bulls vaccinated	6	3	-	9
NG041	Cross	0	26	Bulls vaccinated	12	10	-	4
NG042	Brahman	0	23	Bulls vaccinated	15	6	-	2
NG043	Brahman	0	10	Bulls vaccinated	-	-	10	-
NG044	Cross	0	20	Bulls vaccinated	1	5	-	14
NG048	Cross	20	20	Bulls vaccinated	-	15	5	-
NG100	18 Droughtmaster 22 Brahman	0	40	Bulls vaccinated	-	-	40	-
TOTAL	**1012**	**132**	**880**		**145**	**181**	**301**	**253**

4.2 Defined the microbiomes of the reproductive tracts in healthy and diseased cattle

4.2.1 Heifer study

The trial was completed successfully with samples collected for analysis. qPCR results are presented in Figure 3 with details in Appendix 8.9. Prior to challenge, the heifers were *C. fetus venerealis* qPCR negative. Post challenge, 5 of the 6 unvaccinated heifers were positive by qPCR with animal number 578 subsequently positive a week later (See Figure 4 and Appendix 8.9). A few of the vaccinated cattle were mildly positive post challenge and a week later which is not surprising as the challenge bacteria were still present. At 2 weeks post challenge, there was a mix of negative, low positive and positives for all cattle and by 3 weeks post challenge all cattle had cleared the infection. Following treatment, cattle were confirmed qPCR negative. Figure 3 shows a summary of the qPCR results. Since both vaccinated and unvaccinated cattle were positive to the qPCR in the weeks immediately post challenge, this confirms the need for a BGC immunity assay as planned in this research program. It is also evident that within 3 weeks of challenge, or one oestrous cycle, the unvaccinated group cleared the *C. fetus venerealis* bacteria from the vagina. This would explain why in our previous field studies using qPCR (Appendix 8.2) we rarely found positive cows in unvaccinated herds where bulls presented as qPCR positive.

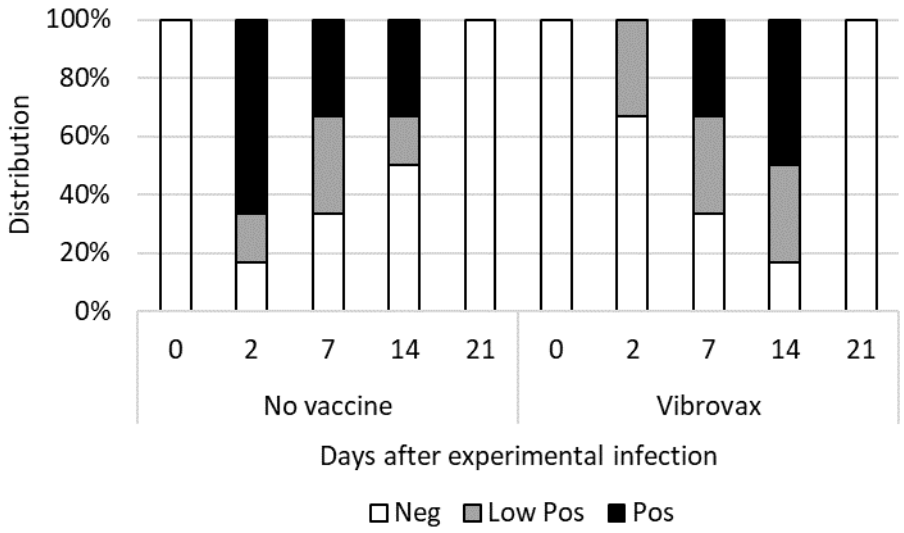


Figure 4. Distribution of infection status by qPCR of vaginal samples, in vaccinated and unvaccinated heifers following infusion of a field isolate of *C. fetus venerealis*. Details in Appendix 8.9.

4.2.2 Heifer microbiome analysis

The mean abundance of microbiome populations was determined from 16S rDNA analyses from the trial. Oestrous and dioestrous stages were examined to determine if there was a cycle effect on the microbiome, see Figure 5. The mean abundance of microbiome populations was determined from 16S analyses associated with vaccination status and challenge as demonstrated in Figure 5. The relative abundances of the microbiome were analysed to demonstrate the population changes during the trial.

Swartz et al (2014) reported that in dairy cows the vaginal microbial population is dominated by *Bacteroidetes*, followed by *Fusobacteria* and *Proteobacteria*. Other more recent studies in dairy heifers found that *Tenericutes*, *Firmicutes,* and *Bacteroidetes* represents more than 96% of the bacterial composition in the heifer vagina (Quereda et al., 2020). The relative abundance of *Bacteroidetes*, *Fusobacterium* and *Proteobacteria* have been shown to vary depending on the reproductive health status in dairy heifers. More work is required in beef heifers generally and specifically breeds of *Bos indicus* and tropical compost heifers. We need an understanding of how the microbiota behaves in healthy *Bos indicus* cross heifers to identify vaginal dysbiosis related to disease.

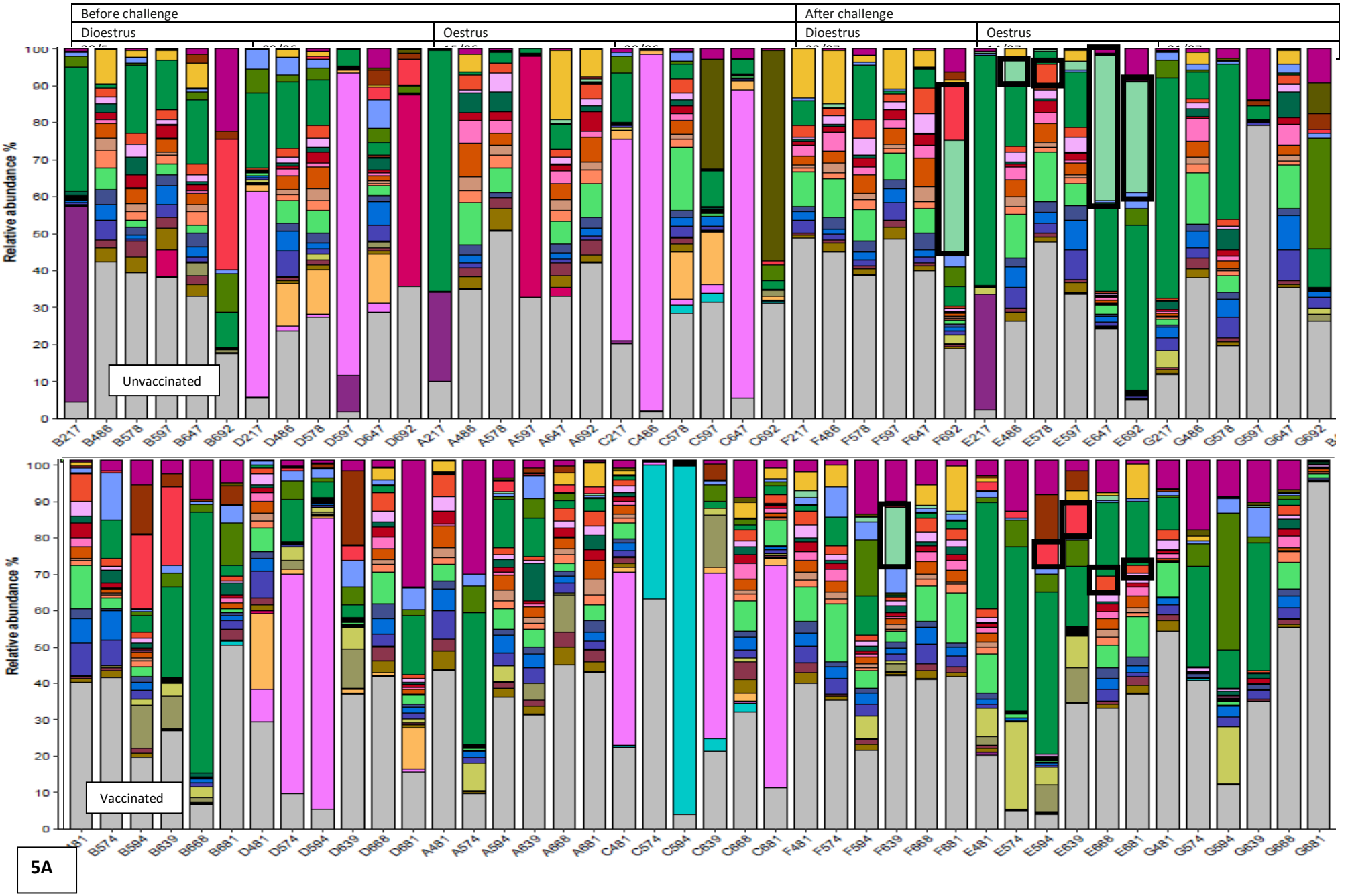
Clemmons et al (2017) reported three dominant phyla in the vaginal microbiota of Angus breed cows in Europe; *Firmicutes* (65.9%), *Bacteroidetes* (16.8%), and *Proteobacteria* (7.4%) with only 2.8% of *Tenericutes*. Vaginal microbial communities of Nellore cattle were *Firmicutes* (40–50%), *Bacteroidetes* (15–25%) and *Proteobacteria* (5–25%), while *Tenericutes* were not detected (Laguardia-Nascimento et al., 2015).

Most of the reports indicate that the bovine vaginal microbiota are dominated by similar groups of bacteria (*Firmicutes*, *Bacteroidetes* and *Proteobacteria*) but variations exist, and these are associated with the oestrous cycle. In previous studies it has been shown that microbial phyla such as *Firmicutes*, *Actinobacteri*a, and *Bacteriodetes* were in high abundance during oestrous in some cattle/buffalo, with a great reduction in the *Proteobacteria*, with are in high abundances during dioestrus.

From the current study, the most abundant phyla were the *Firmicutes* particularly *Streptococcus* spp. but also Aerococcaceae, Peptostreptococcaceae families. This genus is at a much higher abundance in oestrus than in dioestrus after the challenge in both vaccinated and unvaccinated animals (Figure 5)

and this could have been influenced by the hormonal changes in the vaginal epithelium. It has been shown that the microbiome changes in the uterus as well as the vagina in response to hormonal changes (Poole et al., 2023). In addition, *Arthrobacter* sp. changed during the oestrous cycle, consistently higher abundances in oestrus compared to dioestrus, yet was also higher in abundance in one of the vaccinated heifers. *Campylobacter* reads are highlighted in Figure 5 showing higher abundance after challenge in the unvaccinated heifers and not influenced by cycle change. Alternately *Proteobacteria* seem to increase generally after challenge, particularly *Acinetobacter* (Unassigned). The level of *Proteobacteria* seems somewhat dependent on the cycle stage with higher abundances during oestrus which differs to the published literature cited above. There is also some evidence that changes in the ruminal microbiome also affects the content of the vaginal microbiome (Ong et al., 2022c, Section 4.5.1). This could explain the abundances of different phyla across different studies of the vaginal microbiome.

In terms of *Campylobacter* spp. microbiome reads, there were more present following live challenge in the unvaccinated cattle compared with vaccinated as evidenced in Figure 5B.


Before challenge
After challenge
Dioestrus
Oestrus
Dioestrus
Oestrus
Relative abundance %
Unvaccinated
Vaccinated
B217 B486 B578 B597 B647 B692 D217 D486 D578 D597 D647 D692 A217 A486 A578 A597 A647 A692 C217 C486 C578 C597 C647 C692 F217 F486 F578 F597 F647 F692 E217 E486 E578 E597 E647 E692 G217 G486 G578 G597 G647 G692
B574 B594 B639 B668 B681 D481 D574 D594 D639 D668 D681 A481 A574 A594 A639 A668 A681 C481 C574 C594 C639 C668 C681 F481 F574 F594 F639 F668 F681 E481 E574 E594 E639 E668 E681 G481 G574 G594 G639 G668 G681

5A

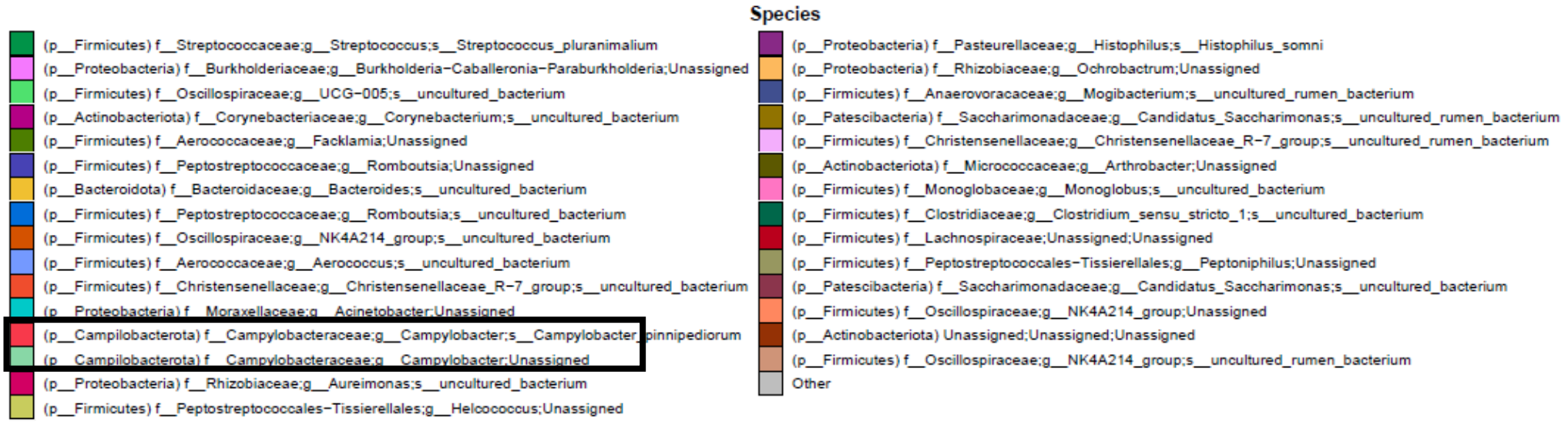


Figure 5A. Top 30 species from 16S microbiome analyses associated with heifer vaccination vs unvaccinated, *C. fetus* subsp. *venerealis* challenge, and oestrous vs diestrous cycle stage changes during the trial. Campylobacter spp. reads are highlighted in black boxes and have been represented diagrammatically in Figure 5B.

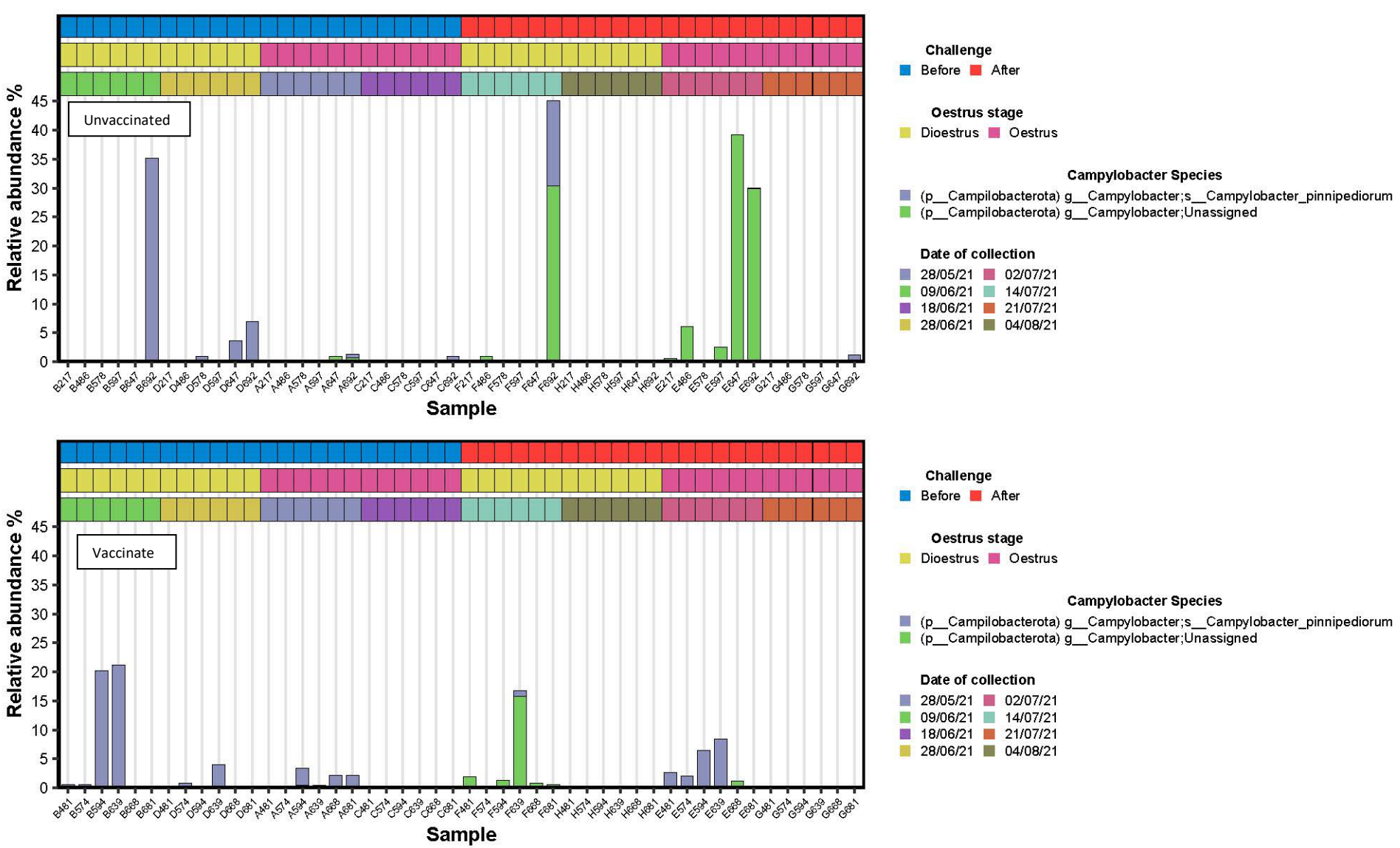


Figure 5B. *Campylobacter* spp. 16S reads only comparing vaccinated and unvaccinated heifers, *C. fetus* subsp. *venerealis* challenge, and oestrous vs diestrous cycle stage changes during the trial.

4.2.3 Bull study

To confirm our microbiome methods for BGC resistance/susceptibility, we decided to undertake a controlled bull vaccination challenge trial (2022) to compare with the data from the controlled heifer study (2021). The dates studied were pre-vaccination, after the first and second vaccinations, and post challenge. Within vaccinated and unvaccinated groups, young (<3 years) vs old (≥3 years) bulls were also compared. The results show that the diversity of the microbiome of unvaccinated bulls ('non-vaccinated' in Figures below) is generally higher than vaccinated. Figure 6 demonstrates the alpha diversity (Shannon index as a box and whisker plot) as a measure of the microbiome diversity in each of the groups of interest.

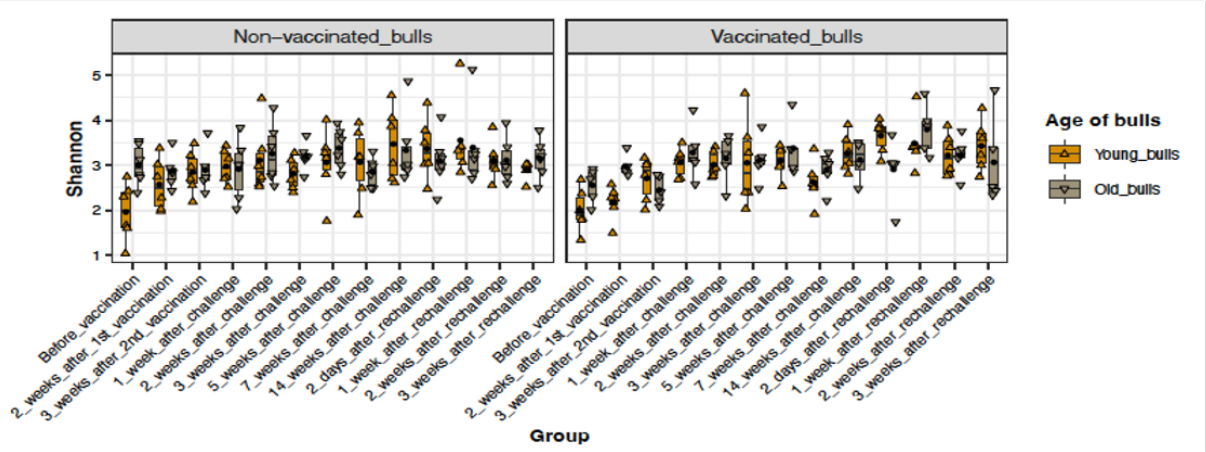
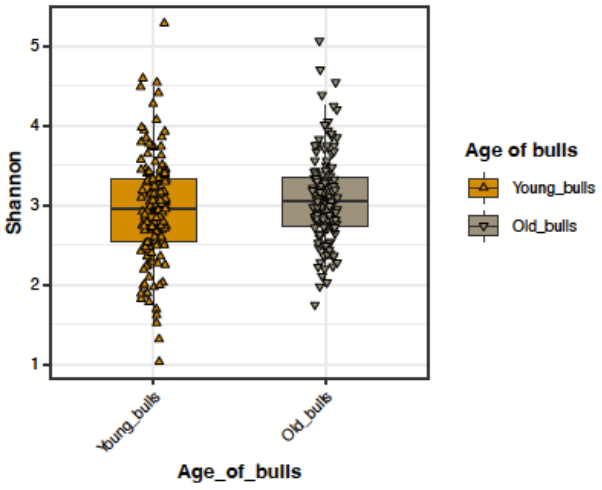


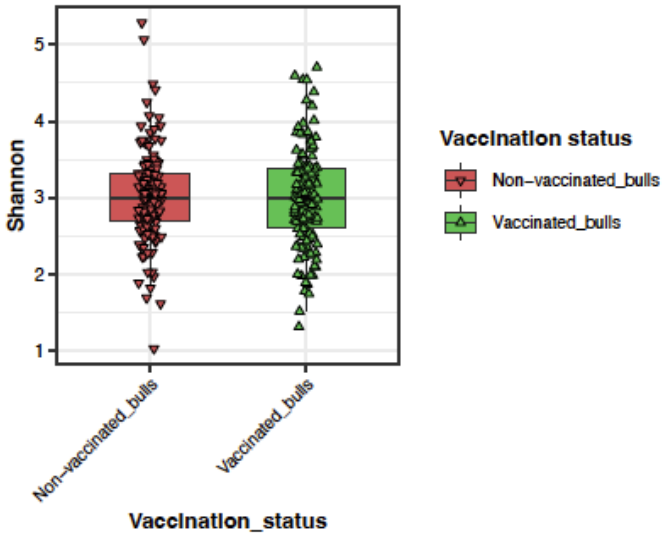
Figure 6. Alpha diversity (Shannon indices) of each group and timepoint of the reproductive tract microbiomes from the bull vaccine and challenge trial.

When different parameters were directly compared, younger bulls appear to have a broader range of alpha diversity measured as Shannon indices, compared with old bulls (Figure 7A). The alpha Shannon diversity of vaccinated bulls appeared to be broader than unvaccinated bulls (Figure 7B), yet pre-trial vaccination history showed similar diversity and slightly higher levels for previously vaccinated bulls (Figure 7C).

A



B



C

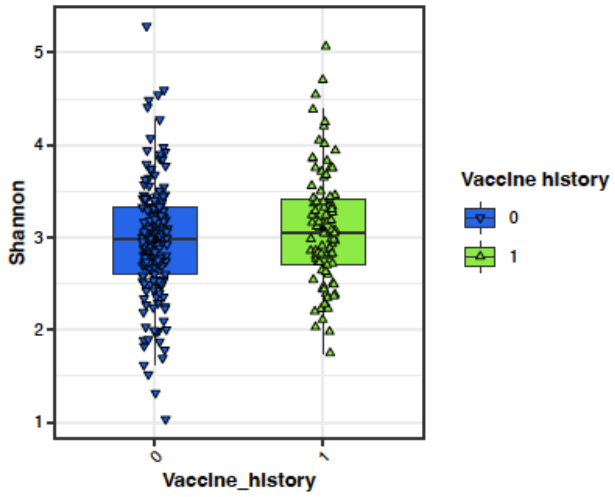


Figure 7. Shannon alpha diversity of bull microbiomes comparing A. young and old bulls, B. post Vibrovax® vaccination, and C. pre-trial vaccination status.

The microbial profiles from the bull trial are summarised in Figure 8A. These results show the increased presence of Campylobacter species in unvaccinated bulls post challenge with *C. fetus venerealis* with slightly stronger presence noted in older vs younger bulls in both groups. There was a shift in prevalent species present in the bull prepuce before (high *Pseudomonas* content) and during vaccination to populations present post challenge in either group (vaccinated or non-vaccinated), however this may have been due to re-location of the bulls to QASP, increased stress level and change of diet. One notable species is *Histophilus* spp. which can be pathogenic. The presence of the organism *H. somni* in the genital tract of both male and female cattle between 6 months and 2 years of age has been associated with increased risk of abortion in the female cattle (reviewed by Parkinson 2019). *Histophilus somni* has been reported to cause reproductive disorders, endometritis, vulvovaginitis, cervicitis, infertility associated with embryonic death and abortion in cows, and testicular degeneration, orchitis and epididymitis in bulls (reviewed by Parkinson 2019; Headley et al., 2015). *Histophilus somni* is a common inhabitant of the genital tracts of healthy male and female cattle (Eaglesome and Garcia, 1992). It is not known if the increase in abundance of *Histophilus* spp. is associated with the shift in environment for the bulls (NT to Gatton) or if it is a host response associated with BGC vaccination and *C. fetus venerealis* challenge. *Streptococcus spp.* was not noted to be present in the top 10 species in the bulls, this species was the most highly abundant in the heifer study. In comparison to the heifer study, the diversity of phyla present was very high which is also previously highlighted in the metagenomic studies (Section 4.5.1).

Although we did not study semen samples in this study, there is published evidence that shows that differences in the bacterial microbiota of healthy bulls does occur and this might be associated with the fertility potential of the bull (Cojkic et al., 2021).

For ease of observations around Campylobacter, these were stacked separately see Figure 8B. *Campylobacter fetus* was not speciated but the presence of *C. pinnipediorum* was found to be higher in the unvaccinated bulls. However, *C. pinnediorum* is unlikely as this species is found in pinnipeds (walruses, seals) and not cattle thus *C. pinnediorum*, unassigned and uncultured Campylobacter are all collectively considered to be 'unassigned Campylobacter' using the microbiome data. This correlates with the heifer data and thus we must assume that this represents *C. fetus* in our context. Note that microbiome is only guaranteed to the genus level, species level is possible only if 6/10 Blast hits are for the same species.

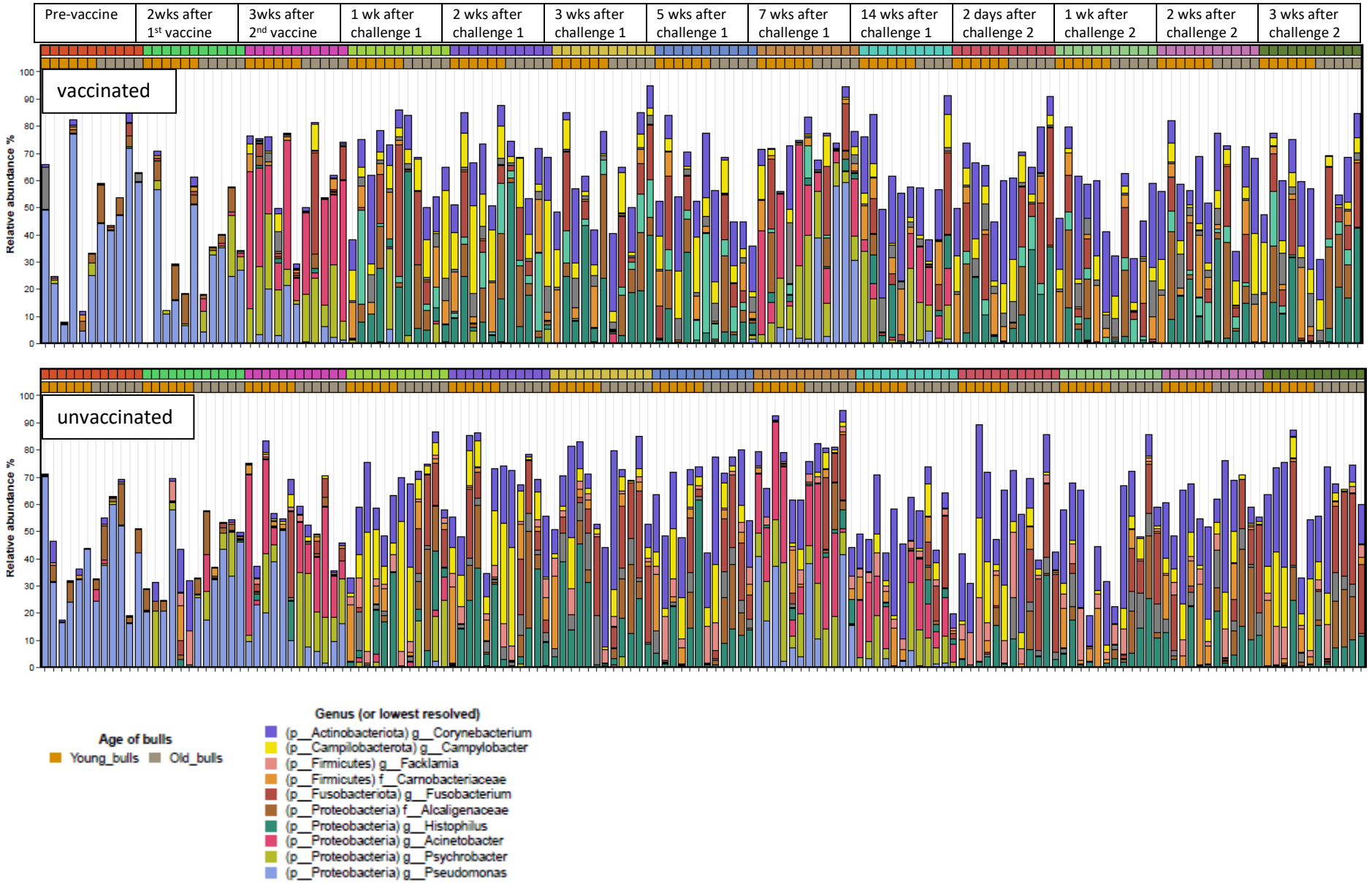


Figure 8A Stacked top 10 16S microbiome populations from the bull vaccine and challenge trial. *Campylobacter* reads are shown in yellow bars.

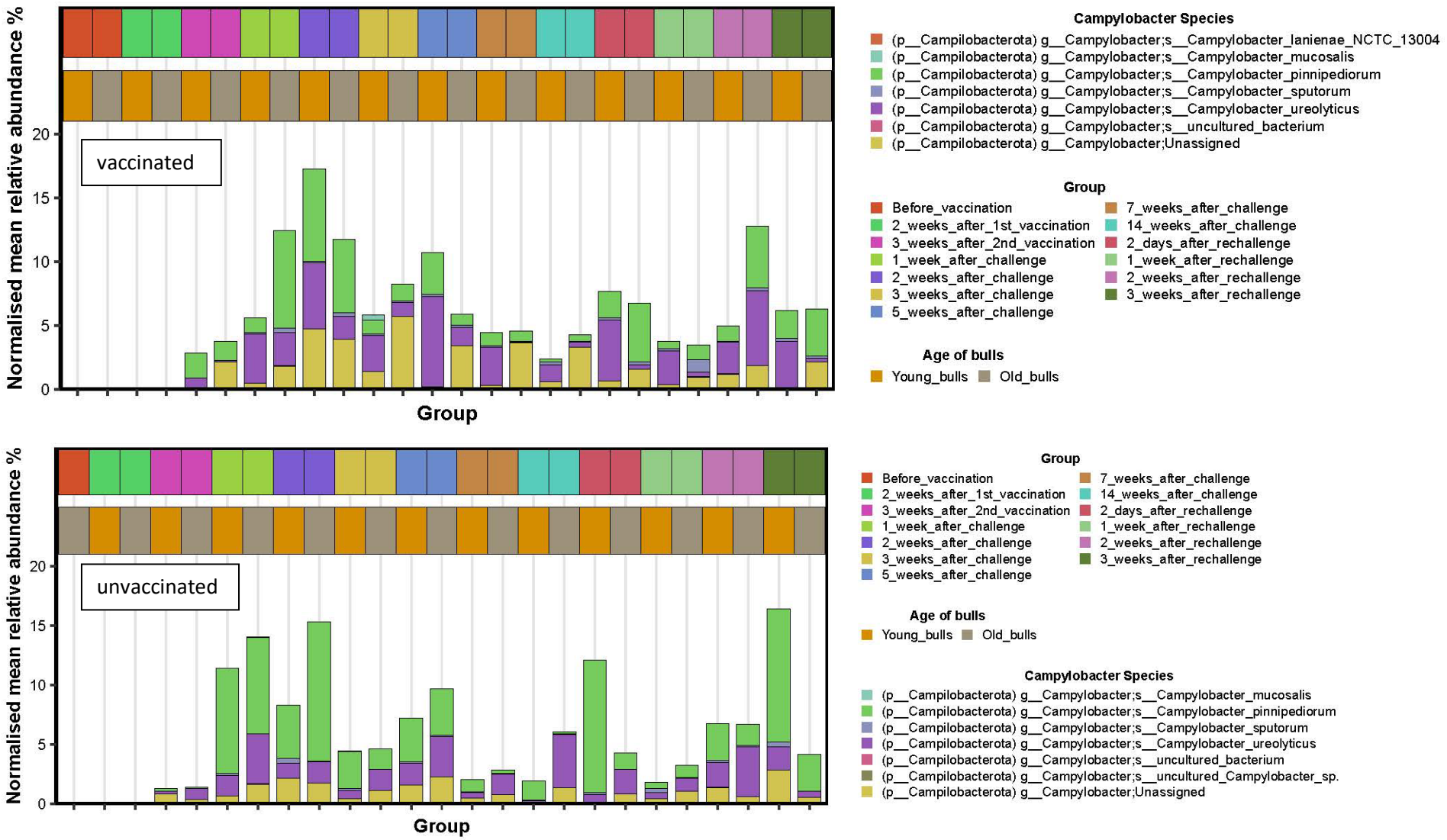


Figure 8B. Stacked Campylobacter species. The taxonomy is assigned based on consensus among the top 10 ASVs from BLAST analysis—in the cases where there is a species defined, at least 6/10 top-matching ASVs had the same species assignment.

4.3 Investigated the host genomics associated with reproductive disease susceptibility/resistance.

Despite extensive testing, we were only able to source samples from 11 cattle from unvaccinated herds that tested positive for *C. fetus venerealis* using qPCR. Having an GEBV accuracy of 0.4 would be very useful for identifying and culling animals likely to contract *C. fetus venerealis*. Sample size calculations suggested that 3,510 samples would be required to be collected from unvaccinated herds and 1,755 animals with and without BGC (50% cases and 50% controls genotyped), see Figure 8. It is important to note that the project has faced substantial challenges in accessing bulls during the screening process. This is because our timing has coincided with the typical mating period used by managers. To overcome this obstacle and increase our access to bulls, we have tried various strategies such as advertising on the FutureBeef website and collecting samples from the abattoirs known to process bulls sourced from a large geographical region.

However, despite these efforts, we were not able to access some of the herds that had tested positive for *C. fetus venerealis* through qPCR, which has limited our sample size. The current database has an insufficient number of positive outcomes to support the development of GEBVs. Our heifer and bull studies also confirmed that *C. fetus venerealis* is cleared from reproductive tracts thus it is possible we had animals that were positive, but we missed the qPCR positivity window to identify positive cattle.

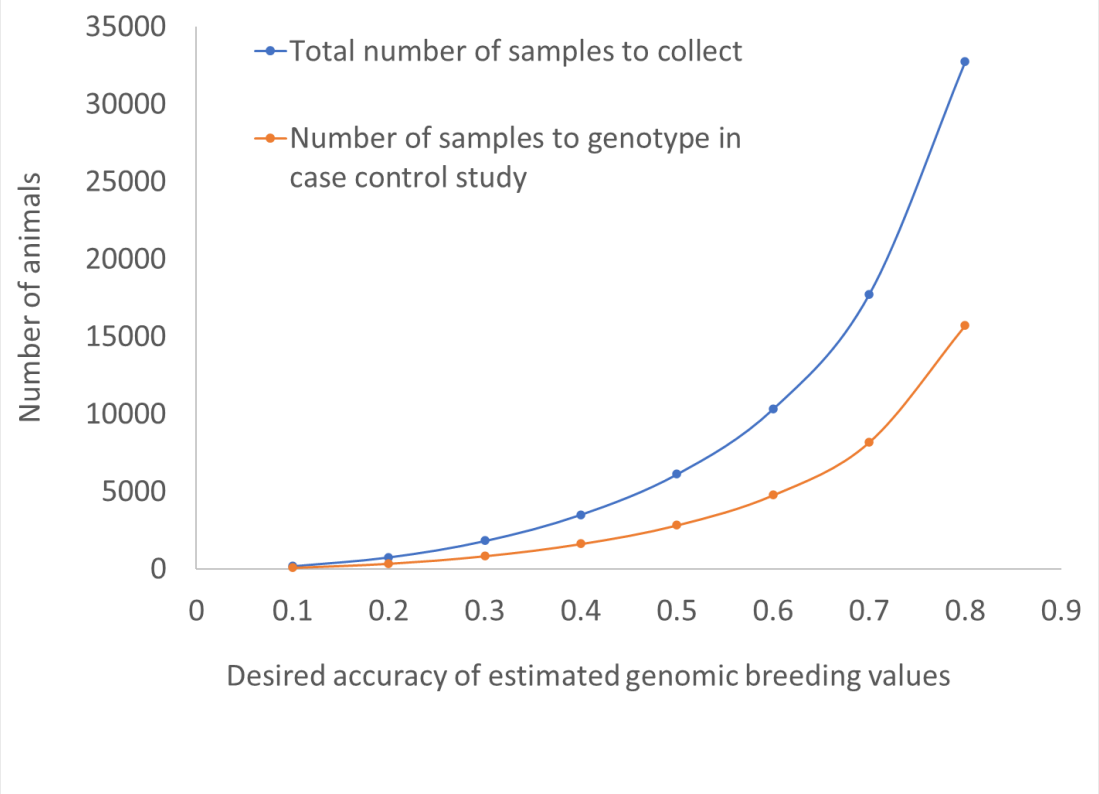
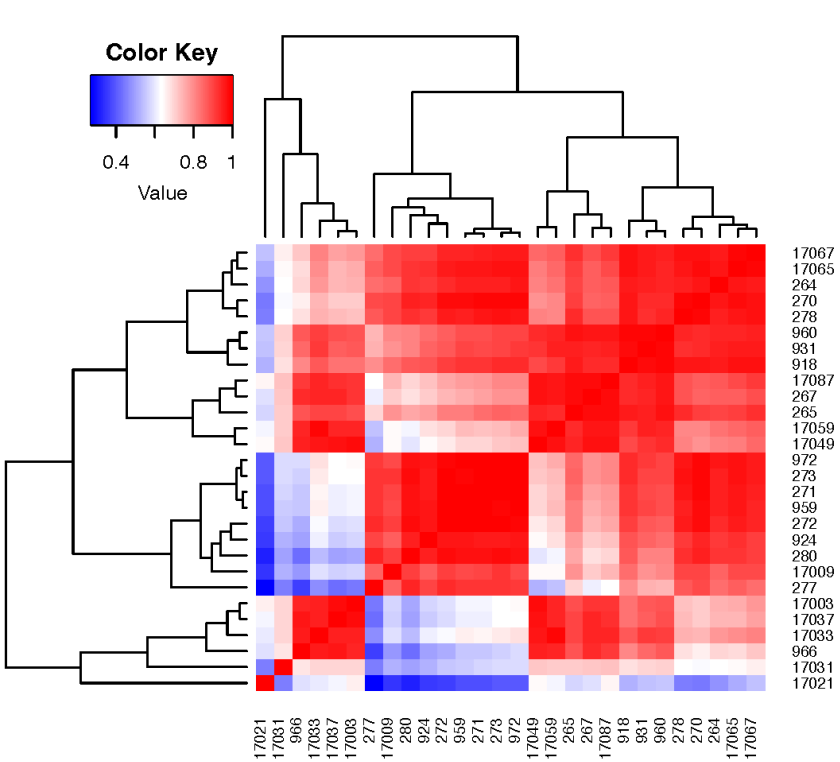
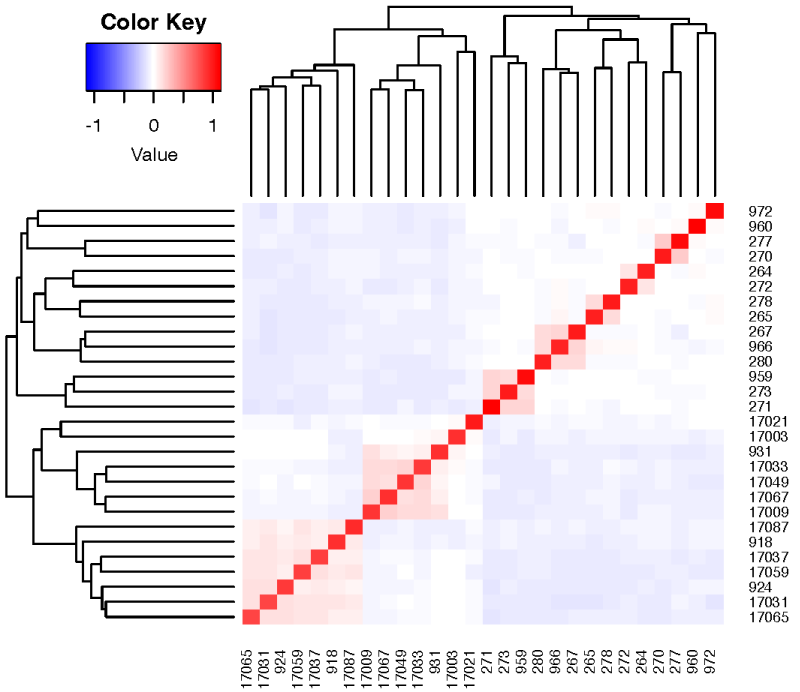


Figure 9. Number of samples to be collected and genotyped to achieve desired level of accuracy of genomic estimated breeding value for *Campylobacter fetus* subsp. *venerealis* resistance.

We examined genomic and microbiome relationship matrices to see if there were any correlation, Figure 9. This correlation was small but significant (Mantel test $P<0.05$), suggesting the host genome may play some role in influencing the microbial genome.





264	265	267	270	271	272	273	277	278	280	918	924	931	959	960	966	972	17003	17009	17021	17031	17033	17037	17049	17059	17065	17067	17087
BX preg	BX prep	BX prep	BX prep	BX Preg	BX Preg	BX Prep	BX Preg	BX Prep	BX Preg	D Postp	D Postp	D Postp	BX Postp	BX Postp	BX Postp	BX Postp	D Prep	D Cycl	D Preg	D preg	D prep	D prep	D preg	D prep	D cycl	D prep	D cycl

Figure 10. Metagenome (A) and genomic (B) relationship matrices. Animal numbers shown on both axes showing the relationship between female cattle on properties NG036 (BX; n=17) and NG037 (D; n=11). Legend: BX= Brahman cross; D=Droughtmaster; preg=pregnant; prep=prepubertal; postp=postpartum; cycl=cycling.

4.4 Developed a validated specific molecular diagnostic test for BGC.

4.4.1. Genome sequencing, see Appendix 8.3

To accurately identify differences between the *C. fetus* subspecies for diagnostic assay development, we undertook long read sequencing to obtain single complete genomes for five *C. fetus venerealis* (A8, 924, 926, 957 and 76223) and four *C. fetus fetus* ((M20-08756/1A, M20-04752/1B, BT268/06 and BT376/03) isolates. All data has been deposited at the NCBI (National Center for Biotechnology Information (nih.gov)) under BioProject PRJNA675960.

The average read length, average read quality and average N50 of the nine strains sequenced with ONT were 33.52, 12.61 and 38.43 kb respectively (Table S1, Appendix 8.3). Illumina short-read sequencing yielded a total mean of 416,870 paired-end raw reads for each sample which gave a mean coverage of 64X for each bacterial isolate (Table S2, Appendix 8.3). All *C. fetus* strains were assembled into complete circular genomes. The average assembly size of *C. fetus fetus* and *C. fetus venerealis* were 1,818,690 bp and 2,136,077 bp respectively (Table 7). The QUAST quality assessment of each assembly showed that the long reads and short reads provided an average 745.73X and 64.56X respectively to the assemblies (Table S3 and S4, Appendix 8.3). The average percentage of mapped long reads and mapped short reads were 98.56% and 97.50% respectively. The complete genomes generated in this study and published genomes of *C. fetus fetus* and *C. fetus venerealis* were annotated with an average of 1,901 and 2,177 genes respectively (Table S5, Appendix 8.3).

Table 7. Details of *Campylobacter fetus* complete genomes assembled in this study.

Sample ID	Species	Assembly size (bp)	GC content (%)
76223	*Campylobacter fetus* subsp. *venerealis*	2,105,546	33.47
924	*Campylobacter fetus* subsp. *venerealis* bv. intermedius	2,250,778	33.48
926	*Campylobacter fetus* subsp. *venerealis* bv. intermedius	2,123,600	33.48
957	*Campylobacter fetus* subsp. *venerealis*	2,088,026	33.41
A8	*Campylobacter fetus* subsp. *venerealis*	2,112,436	33.42
BT268/06	*Campylobacter fetus* subsp. *fetus*	1,909,714	33.26
BT376/03	*Campylobacter fetus* subsp. *fetus*	1,800,589	33.22
M20-08756/1A	*Campylobacter fetus* subsp. *fetus*	1,782,221	33.10
M20-04752/1B	*Campylobacter fetus* subsp. *fetus*	1,782,237	33.10

Pangenome analysis revealed that there were 1,561 core genes and 1,064 accessory genes shared among the 25 *C. fetus* strains. The *C. fetus fetus* and *C. fetus venerealis* strains were separated in the hierarchical tree generated based on the presence and absence of gene orthologs (Figure 11). The pangenome analysis demonstrated gene ortholog group_129 was exclusively encoded in all the *C. fetus venerealis* genomes but not in any of the *C. fetus fetus* genomes. Gene ortholog group_129 encoded for a peptidase S24 LexA-like protein. There were three paralogs, group_17, group_650 and group_680, which also encoded for peptidase S24 LexA-like proteins and were inconsistently expressed in some of the *C. fetus fetus* and *C. fetus venerealis* strains (Table 8).

Figure 11. Expression of the pangenome, identified using Roary analysis, among the 25 *Campylobacter fetus* strains. Dark purple indicates the presence of the gene ortholog while light purple represents absence of the gene ortholog. International Organization for Standardization (ISO) country code: Canada (CA), United States (US), France (FR), Uruguay (UY), Argentina (AR), United Kingdom (UK), Australia (AU) and New Zealand (NZ).

Table 8. Presence or absence of gene families encoding for the peptidase S24 LexA-like protein among the 25 *C. fetus* strains. International Organization for Standardization (ISO) country code: Canada (CA), United States (US), France (FR), Uruguay (UY), Argentina (AR), United Kingdom (UK), Australia (AU) and New Zealand (NZ)

Gene family	**group_129**	**group_17**	**group_680**	**group_650**
Annotation	**peptidase S24 LexA-like protein**	**peptidase S24 LexA-like protein**	**peptidase S24 LexA-like protein**	**peptidase S24 LexA-like protein**
CFF-BT268/06 (UK)	No	Yes	No	No
CFF-BT376/03 (UK)	No	Yes	No	No
CFF-GCA_000015085 (US)	No	No	No	No
CFF-GCA_000759485 (AR)	No	Yes	No	No
CFF-GCA_007723545 (UY)	No	Yes	No	No
CFF-GCA_011600855 (CA)	No	Yes	No	No
CFF-GCA_011600945 (CA)	No	No	No	No
CFF-GCA_011600995 (CA)	No	No	No	No
CFF-GCA_900475935^{T} (FR)	No	No	No	No
CFF-M20-04752-1B (AU)	No	Yes	No	No
CFF-M20-08756-1A (NZ)	No	Yes	No	No

Gene family	group_129	group_17	group_680	group_650
Annotation	**peptidase S24 LexA-like protein**	**peptidase S24 LexA-like protein**	**peptidase S24 LexA-like protein**	**peptidase S24 LexA-like protein**
CFV-76223 (AU)	Yes	Yes	Yes	Yes
CFV-957 (AU)	Yes	Yes	Yes	Yes
CFV-A8 (AU)	Yes	Yes	Yes	Yes
CFV-GCA_000759515 (AR)	Yes	Yes	No	Yes
CFV-GCA_000967135 (US)	Yes	Yes	No	Yes
CFV-GCA_008271385^{T} (UK)	Yes	Yes	No	Yes
CFV-GCA_011600845 (CA)	Yes	Yes	No	Yes
CFV-GCA_011601005 (CA)	Yes	Yes	No	Yes
CFVi-924 (AU)	Yes	Yes	Yes	No
CFVi-926 (AU)	Yes	Yes	Yes	Yes
CFVi-GCA_000512745 (AR)	Yes	No	No	No
CFVi-GCA_001686885 (AR)	Yes	No	No	No
CFVi-GCA_011600955 (AR)	Yes	Yes	No	Yes
CFVi-GCA_011601375 (AU)	Yes	Yes	Yes	Yes

On the contrary, *C. fetus venerealis* and the bv. intermedius variant did not fall into distinct branches because none of the gene orthologs was able to differentiate *C. fetus venerealis* and *C. fetus venerealis* bv. intermedius. Interestingly, the six Australian *C. fetus venerealis* strains (GCA_011601375, A8, 924, 926, 957 and 76223) formed a separated clade from the other non-Australian *C. fetus venerealis*. There were 14 gene orthologs which were identified in the Australian *C. fetus venerealis* strains but not in the non-Australian strains, of which one were present in all *C. fetus fetus* and 9 were absent in all the *C. fetus fetus* strains. On the other hand, there were 37 gene orthologs which were present in the non-Australian *C. fetus venerealis* and not the Australian strains, of which none were identified in all *C. fetus fetus* and 34 were absent in all the *C. fetus fetus* strains.

A closer look at the genomic regions encoding the *Campylobacter*-specific virulence factors demonstrated that the 25 *C. fetus* subspecies commonly expressed genomic regions encoding 88 virulence factors (Figure 12). Nine virulence factors were not expressed in some of the *C. fetus* subspecies, but not explicitly in either *C. fetus fetus* or *C. fetus venerealis*. The highest variation was observed within the S-layer proteins, which is categorised under the class of "Colonization and immune evasion" in VFDB. Nonetheless, the variations among the gene expression were not consistent within either of the subspecies or the *C. fetus venerealis* biovars.

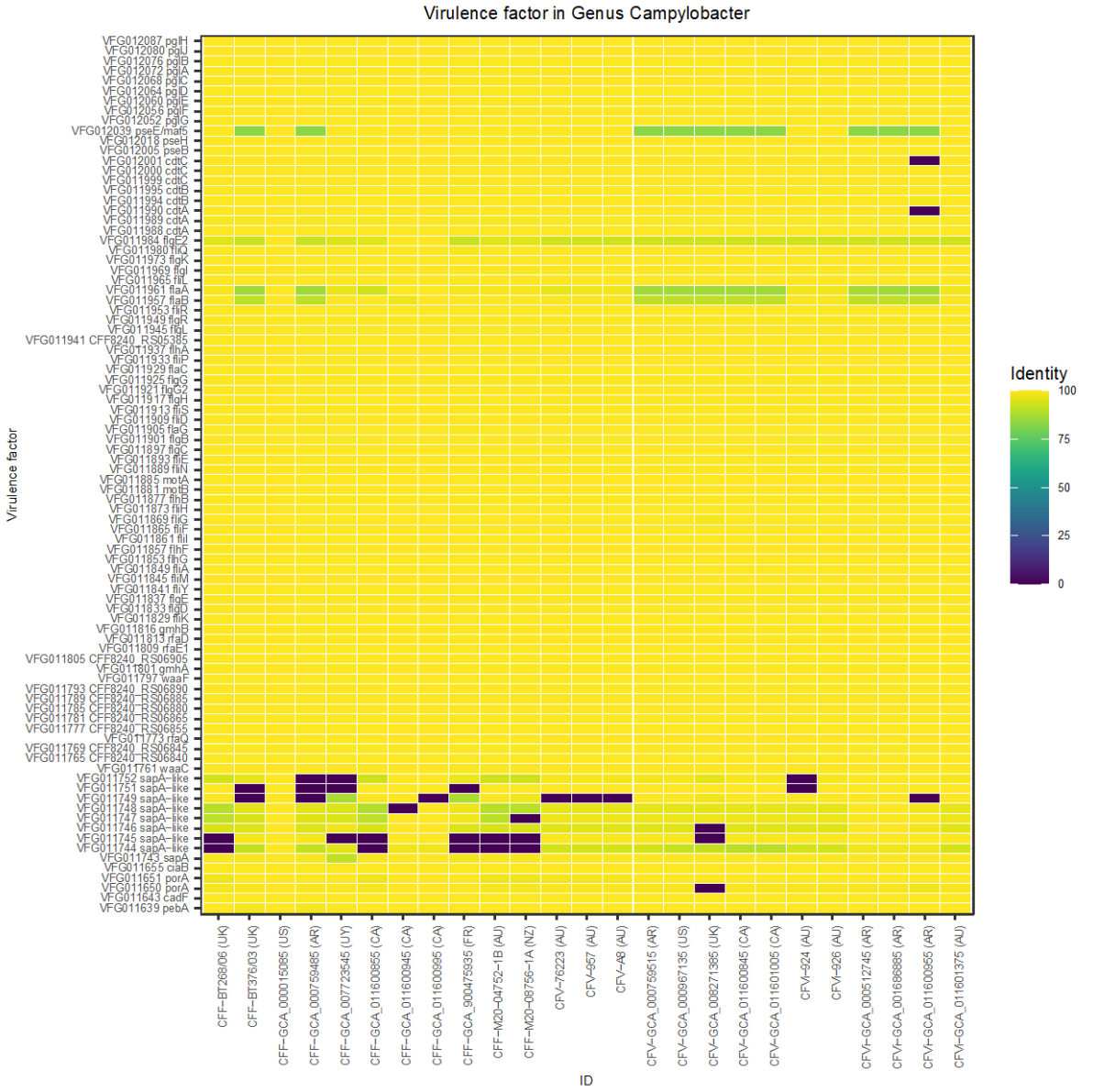


Figure 12. Expression of *Campylobacter*-specific virulence factors among the 25 *Campylobacter fetus* strains. The colour key indicates the percentage of identity between the genomic regions identify in each isolate and the *Campylobacter*-specific virulence factors, in which yellow represents 100% identity and purple represents 0% identity. International Organization for Standardization (ISO) country code: Canada (CA), United States (US), France (FR), Uruguay (UY), Argentina (AR), United Kingdom (UK), Australia (AU) and New Zealand (NZ).

The average nucleotide identity between 25 *C. fetus* strains was more than 95%. The correlation tree based on the ANI showed that eight of the *C. fetus fetus* strains, including GCA_011600855, BT268/06, M20-04752/1B, M20-08756/1A, GCA_900475935^{T}, GCA_007723545, BT376/03 and GCA_00759485 formed a separate branch from the *C. fetus venerealis* strains (Figure 13). The other three *C. fetus fetus* strains (GCA_000015085, GCA_011600995, and GCA_011600945), shared the same ancestor with three *C. fetus venerealis* bv. intermedius strains (GCA_011600955, GCA_001686885 and GCA_000512745) from Argentina. The remaining *C. fetus venerealis* strains (n=11) formed a separate clade, in which the *C. fetus venerealis* including both biovars from Australia

(GCA_011601375, A8, 924, 926, 957 and 76223) clustered separately to all other *C. fetus venerealis* from around the world.

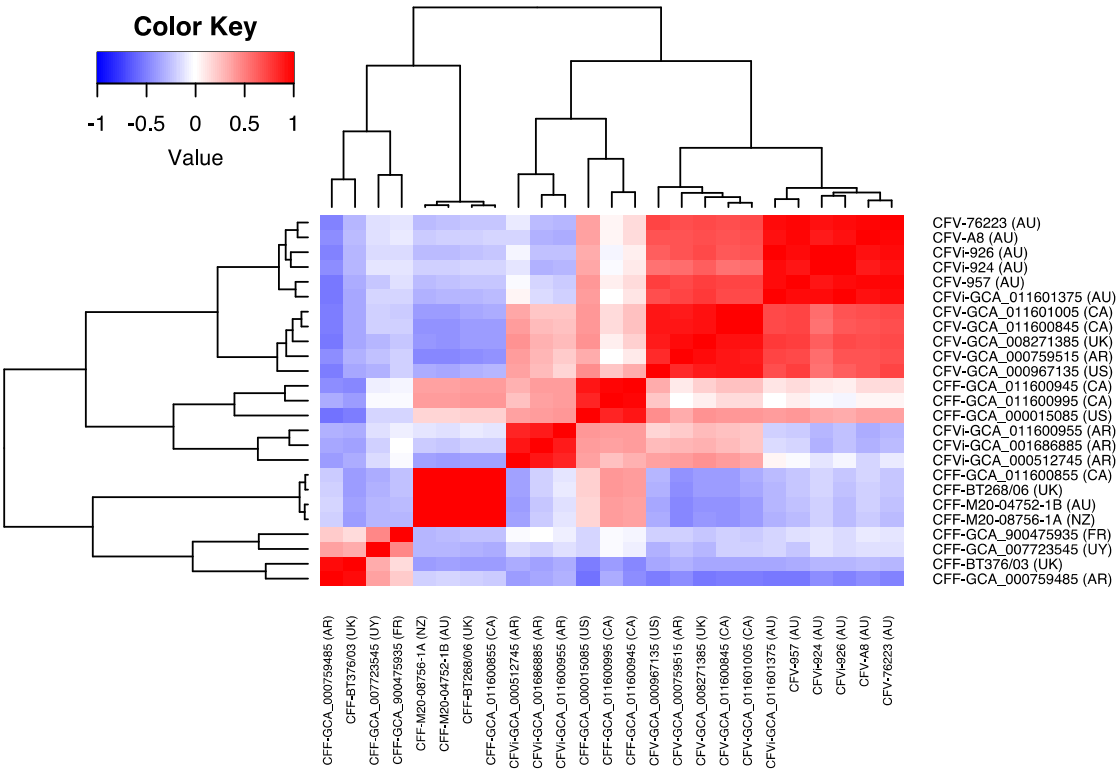


Figure 13. Correlation between the 25 *Campylobacter fetus* strains based on their average nucleotide identity. The colour key indicates the degree of correlation between the strains, in which red represents the highest correlation while the blue represents least correlation. The dendrogram demonstrates the hierarchical relationship between the 25 *Campylobacter fetus* strains based on their average nucleotide identity. International Organization for Standardization (ISO) country code: Canada (CA), United States (US), France (FR), Uruguay (UY), Argentina (AR), United Kingdom (UK), Australia (AU) and New Zealand (NZ).

Whole-genome alignment of the 25 *C. fetus* strains generated a core genome with 1,594,459 bases. The phylogenetic tree that resulted from the whole-genome alignment with recombination regions removed successfully clustered *C. fetus venerealis* into a separate branch from the *C. fetus fetus*, indicating that there were SNPs which were distinctive between *C. fetus fetus* and *C. fetus venerealis*. Nine thousand and forty-four SNPs were identified from the core genome, of which only 269 SNPs were different between all *C. fetus fetus* and all *C. fetus venerealis* strains. The SNPs which contributed to putative synonymous amino acid change, located on putative GIs and involved in recombination events were filtered out from downstream analysis. The remaining 184 SNPs were labelled as 'candidate SNPs', of which 17 were located on non-coding region and 167 were located on CDS (Table S6, Appendix 8.3). Sixteen of the CDS encoded for two candidate SNPs, one CDS encoded for three candidate SNPs and one CDS encoded for 5 candidate SNPs. In total, the 167 candidate SNPs were located on 145 CDS, of which 15 of the CDS were encoding for hypothetical proteins. The majority of the SNPs-encoding CDS were responsible for "Cellular processes and signalling" (n=45), followed by "Metabolism" (n=44) and "Information storage and processing" (n=21) (Figure 14 and Table S7, Appendix 8.3).

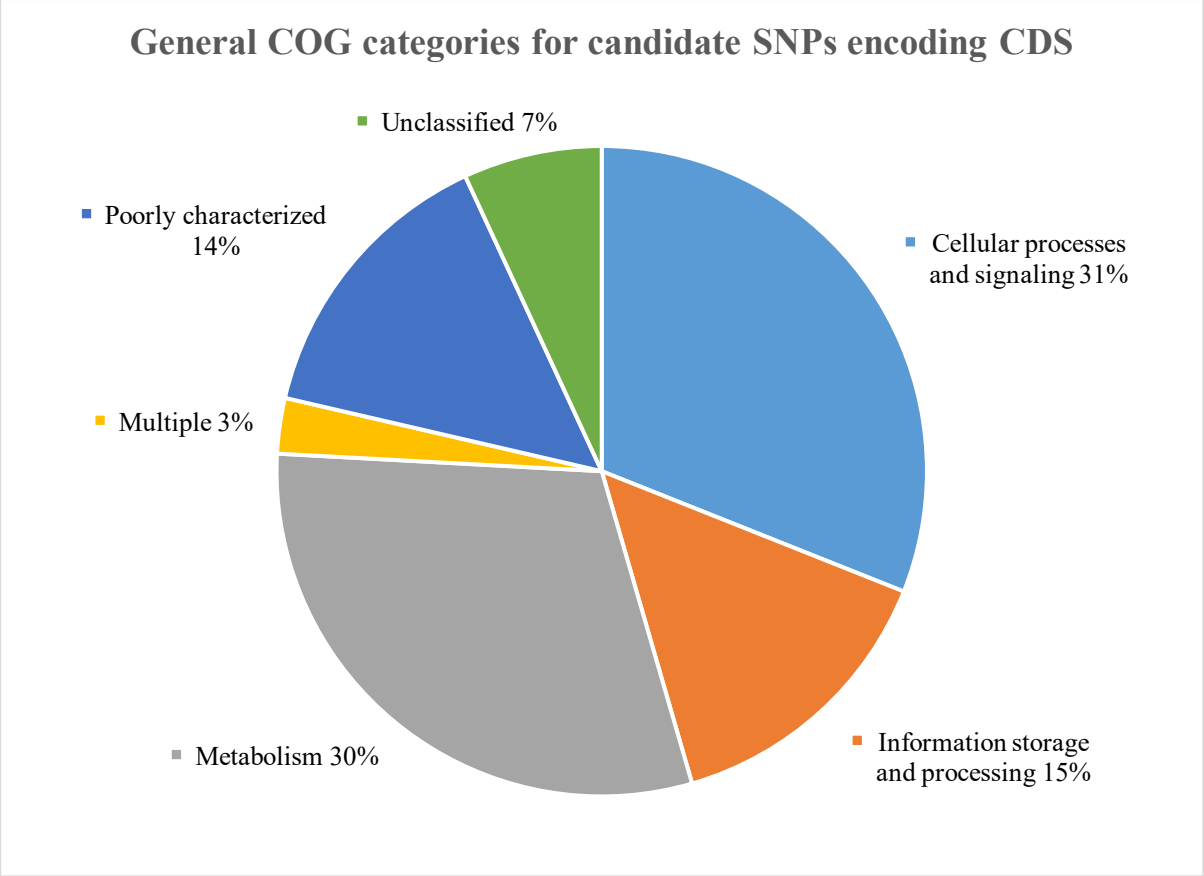


Figure 14. Candidate single nucleotide polymorphisms (SNPs) categorised into Clusters of Orthologous Groups (COGs). The blue section represents coding sequences (CDS) responsible for "Cellular processes and signalling" (n=45). The orange section represents CDS responsible for "Information storage and processing" (n=21). The grey section represents CDS responsible for "Metabolism" (n=44). The yellow section represents CDS belonging to multiple groups of COGs (n=4). Light blue section represents CDS which were poorly categorised (n=21). Green section represents CDS with no match return from querying the COG database (n=10).

The potential functional association between the SNP-encoding CDS (n=41) which had more than eight degrees of association was determined. Those CDS which were annotated with KEGG pathway "Peptidoglycan biosynthesis" (Figure S1, Appendix 8.3), including *murC*, *ftsI*, *uppP* and *mraY*, and their first neighbours, were selected for further analysis (Figure 15). Amino acid changes were on *rpoC, cysS*, *rpoB*, *flgG*, *mfd*, *mraY* and *mutS2* were verified (Table 9).

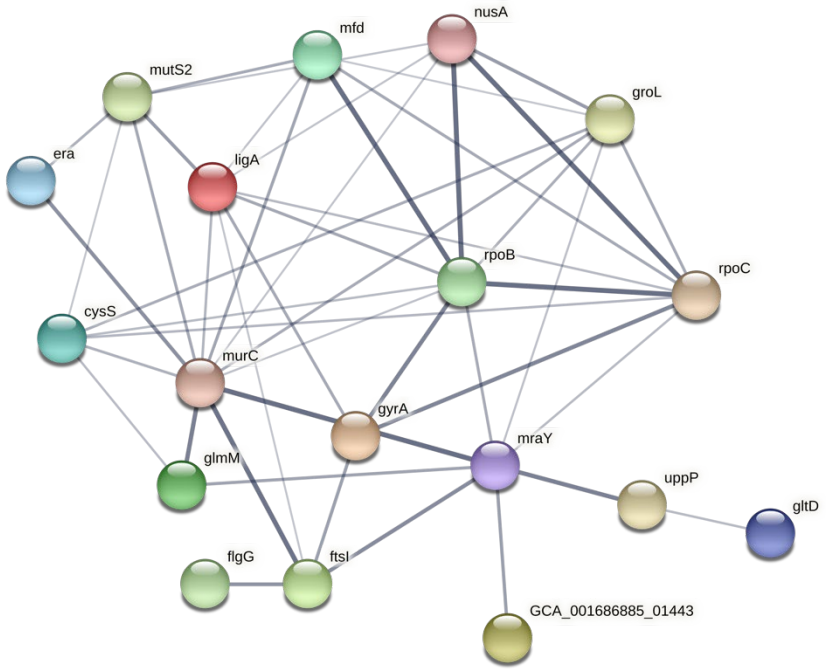


Figure 15. STRING network of SNP-encoding CDS which had more than eight degrees of association. The thickness of the network edges indicates the confidence level of the functional interactions.

Table 9. Amino acid and DNA base changes on the candidate SNP-encoding CDS encoded on *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*.

			Campylobacter fetus* subsp. *fetus		***Campylobacter fetus* subsp. *venerealis***	
Gene	**Product**	**Type**	**Amino acid**	**DNA base**	**Amino acid**	**DNA base**
cysS	cysteinyl-tRNA	Missense	Cys	C	Tyr	T
flgG	flagellar	Missense	Asn	A	Ser	G
mfd	transcription-repair	Missense	Thr	C	Ile	T
mraY	phospho-N-acetylmuramoyl-pentapeptidetransferase	Upstream variant	Arg	C	His	T
mutS2	Endonuclease	Missense	Ala	C	Val	T
rpoB	DNA-dependent RNA polymerase	Missense	Asp	C	Asn	T
rpoC	DNA-dependent RNA polymerase	Missense	Leu	G	Phe	A

Interestingly, there were no common SNPs which could absolutely separate all the *C. fetus venerealis* and its biovar intermedius variant regardless of the country of origin. The phylogenetic tree generated from whole-genome alignment also clustered the Australian *C. fetus venerealis* in a separate branch from the *C. fetus venerealis* identified in United Kingdom, United States, Canada and Argentina (Figure 16). Both within the Australian and non-Australian *C. fetus venerealis* clades, the *C. fetus venerealis* bv. intermedius were clustered separately from the normal *venerealis* variant. There were one and 7 SNPs, labelled as "biovar SNPs", which were different between *C. fetus*

venerealis and the biovar intermedius variant within the Australian and non-Australian *C. fetus venerealis* clade respectively (Table S8, Appendix 8.3).

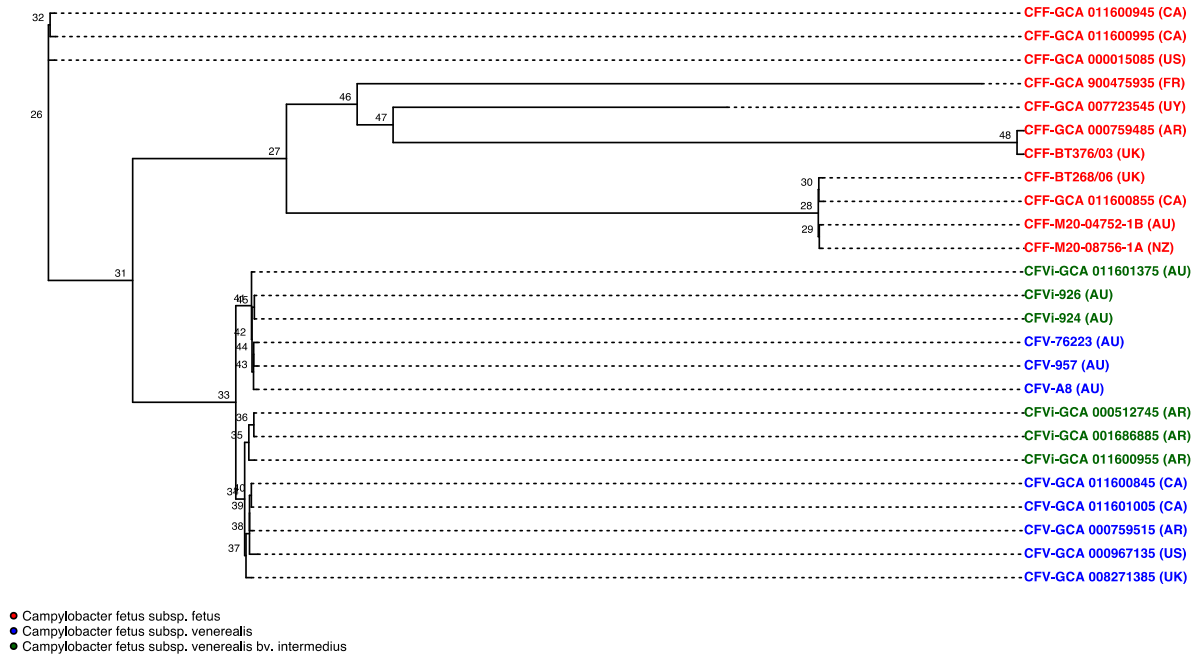


Figure 16. Phylogenetic tree based on single nucleotide polymorphisms (SNPs) between the 25 *Campylobacter fetus* strains. Strains labelled in red are *C. fetus* subsp. *fetus*, strains labelled in blue are *C. fetus* subsp. *venerealis* and strains labelled in green are *C. fetus* subsp. *venerealis* bv. intermedius. All *C. fetus* subsp. *fetus* isolates clustered into a separate branch from all *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *venerealis* bv. intermedius isolates, indicating that there were distinctive SNPs. *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *venerealis* bv. intermedius isolates from Australia clustered separately from non-Australian isolates. International Organization for Standardization (ISO) country code: Canada (CA), United States (US), France (FR), Uruguay (UY), Argentina (AR), United Kingdom (UK), Australia (AU) and New Zealand (NZ).

Comparative genome alignment of the *C. fetus* strains (n=25) illustrated the high level of genome synteny between the *C. fetus* subspecies (Figure 17). Missing genomic regions were inconsistently observed among the 25 *C. fetus* strains where putative Genomic Islands (mobile elements) were predicted. Previously published PCR target genes, including *sapB2* and *par*A, were located on putative Genomic Islands.

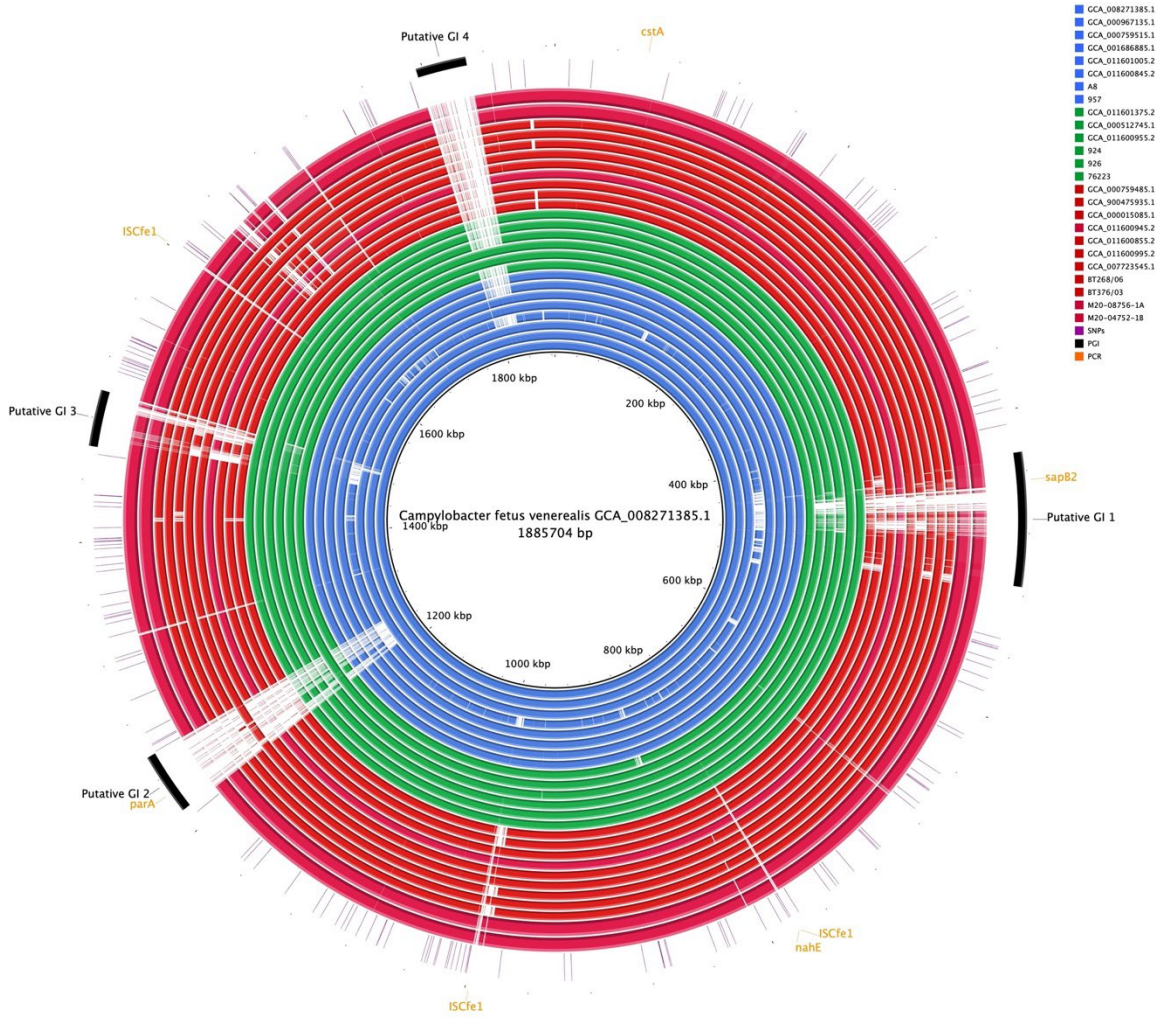


Figure 17. Whole-genome alignment of 25 *Campylobacter fetus* subspecies. Reference: published *C. fetus* subsp. *venerealis* ATCC 19438^T [GCA_008271385.1]. Query were complete genome sequences of *C. fetus* subsp. *fetus* (n=11) and *C. fetus* subsp. *venerealis* (n=14) subspecies. Strains labelled in red are *C. fetus* subsp. *fetus*, isolated labelled in blue are *C. fetus* subsp. *venerealis* and strains labelled in green are *C. fetus* subsp. *venerealis* bv. intermedius. Black arcs represent the putative genomic islands. Orange arcs represent the genes which were used in PCR *C. fetus* subspecies identification. Purple lines represent candidate SNPs identified in the 25 *C. fetus* subspecies.

4.4.2 Assay 1 cpn60

Assay 1 is based on a SNP difference within the cpn60 gene of *C. fetus venerealis* and *C. fetus fetus*. As the assay has some cross-reactions with DNA from other species of bacteria present in the bull prepuce (*A. cryaerophilus*, *C. sputorum*, *C. ureolyticus*, *C. hyointestinalis*) the cut-off for positive Cq results was determined at Cq 34. Thus results in the Cq range 34-40 are considered 'suspect' for BGC diagnostics as this could be possible due to both low *C. fetus venerealis* concentrations and/or false positive cross-reactions and thus we list them as Negative (Table 10).

Table 10. Assay Specificity Assay 1. Results of TaqMan SNP qPCR with a mixture of *A. cryaerophilus*, *C. sputorum*, *C. ureolyticus*, *C. hyointestinalis* (MIX) at annealing temperature 67°C using 2 ng DNA template. FAM assay *C. fetus venerealis* (*Cfv*) probe, VIC assay *C. fetus fetus* (*Cff*) probe; NTC- No Template Control

Sample	***Cfv* FAM probe**		***Cff* VIC probe**	
	Cq Mean	**Result**	**Cq Mean**	**Result**
Cff	0.00	Negative	20.80	Positive
Cff+Bovine DNA	0.00	Negative	19.66	Positive
Cfv	19.67	Positive	0.00	Negative
Cfv+Bovine DNA	19.25	Positive	0.00	Negative
MIX	34.00	Negative	37.93	Negative
MIX+ Bovine DNA	34.23	Negative	37.66	Negative
Cfv+*Cff*+MIX	19.39	Positive	19.94	Positive
Cfv+*Cff*+MIX + Bovine DNA	19.10	Positive	19.67	Positive
Bovine DNA	0.00	Negative	0.00	Negative
NTC	0.00	Negative	0.00	Negative

In many TaqMan assays, Cq scores at Cq ≥35 are often considered non-specific thus the cut-off used in our assay Cq ≥34 is close to the normal cut-off for this type of assay. Positive Cq results were obtained up to 20 femtograms (fg) of pure DNA from both *C. fetus fetus* (ATCC19438) and *C. fetus venerealis* (ATCC27374). From sample concentrations from 2 ng to 2 pg, the Cq value was less than the considered cut-off Cq value and thus the limit of detection of this assay is 2 pg, see Figure 18.

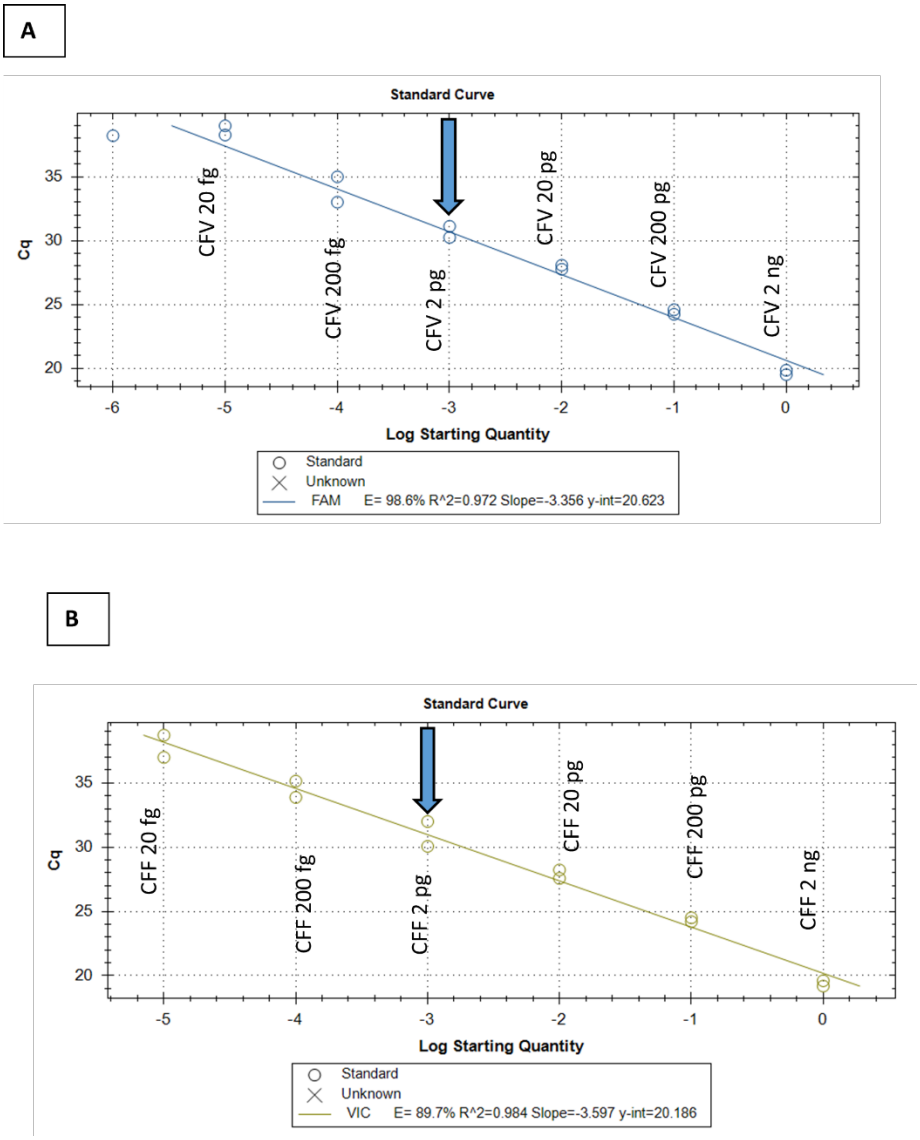


Figure 18. Assay Sensitivity Assay 1. A. TaqMan SNP standard curve of serially diluted DNA from *Campylobacter fetus venerealis* (CFV) detected on the FAM channel. B. TaqMan SNP standard curve of serially diluted DNA from *Campylobacter fetus fetus* (CFF) detected on the VIC channel. The blue arrows show the limit of detection for both assays.

4.4.3 Assay 2 RS04330

Assay 2 was developed as an alternative to assay 1 using a more heterogeneous sequence – Hypothetical protein RS04330. The assays for each subspecies could not be multiplexed (data not shown) and thus the assays were optimised separately. The assays were performed in the presence of other bacterial DNA prepuce isolates (*A. cryaerophilus*, *C. sputorum*, *C. ureolyticus*, *C. hyointestinalis*) and bovine DNA contaminants (Table 9). The contaminant bacterial species were not amplified making the specificity better than Assay 1. Since there was no cross-reaction or false positives detected for Assay 2, the usual assay cut-off was set at Cq 35. Thus, any Cq results >35 were considered as negative for Assay 2, see Table 11. The Cq scores for the *C. fetus venerealis* assay was higher than for *C. fetus fetus* indicating it is a less sensitive assay. This was confirmed when using standard curves against serially diluted DNA demonstrating that 2 ng is assay limit of detection for *C. fetus venerealis* (Figure 19). The assay for *C. fetus fetus* detected 2 pg which is the same detection limit as Assay 1. This result suggests that either this gene target is not suitable for *C. fetus venerealis*

assay development or that the assay for *C. fetus veneralis* needs to be re-designed. Subsequent finalisation of genomic data (SNP analysis) indicated that the *C. fetus veneralis* SNPs which formed the basis of this assay were not specific and thus this assay was no longer applied.

Table 11. Assay Specificity Assay 2. Results of TaqMan SNP qPCR using the FAM assay for *C. fetus venerealis* and VIC assay for *C. fetus fetus* with the MIX (*A. cryaerophilus*, *C. sputorum*, *C. ureolyticus*, *C. hyointestinalis*) of bacteria and bovine DNA at an annealing temperature of 50°C. *C. fetus venerealis* (*Cfv*) *C. fetus fetus* (*Cff*); NTC- No Template Control

Sample 2ng	Assay Type	Target	Cq Mean	Result
MIX	FAM Assay	*Cfv*	0.00	Negative
Cfv +*Cff*			29.21	Positive
MIX+*Cfv* +*Cff*			28.01	Positive
Bovine DNA			0.00	Negative
MIX+ Bovine DNA+*Cfv* +*Cff*			29.84	Positive
Positive Control- *Cfv*			30.31	Positive
Negative Control- *Cff*			0.00	Negative
NTC			0.00	Negative
MIX	VIC Assay	*Cff*	0.00	Negative
Cfv +*Cff*			21.46	Positive
MIX+*Cfv* +*Cff*			22.49	Positive
Bovine DNA			0.00	Negative
MIX+ Bovine DNA+*Cfv* +*Cff*			20.29	Positive
Positive Control			22.83	Positive
Negative Control			0.00	Negative
NTC			0.00	Negative

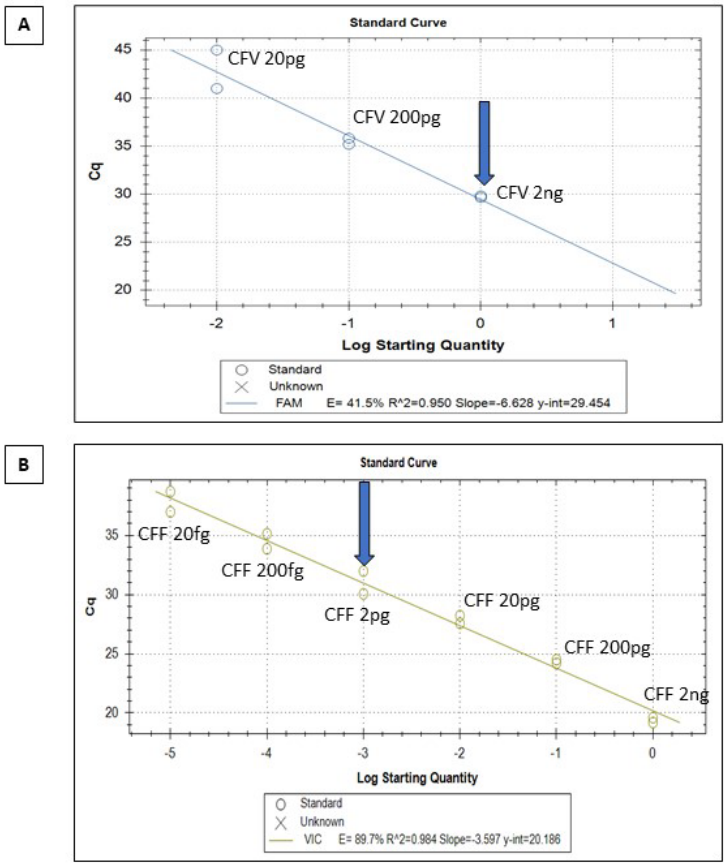


Figure 19. Assay Sensitivity Assay 2. A. TaqMan standard curve of serially diluted DNA from *Campylobacter fetus venerealis* (CFV) detected on the FAM channel. B. TaqMan standard curve of serially diluted DNA from *Campylobacter fetus fetus* (CFF) detected on the VIC channel. The blue arrows show the limit of detection for both the assays.

4.4.4 Assay 3 mraY

The mraY SNP assay has a similar sensitivity for *C. fetus venerealis* detection to the previously described cpn60 SNP assay (Figure 20). Both assays can detect *C. fetus venerealis* genetic material to 1 pg in a pure bacterial DNA sample and by testing the mraY SNP assay on mixed samples known to be positive for *C. fetus venerealis* we find that a Cq cut-off of 33 cycles for a sample to be considered positive is appropriate.

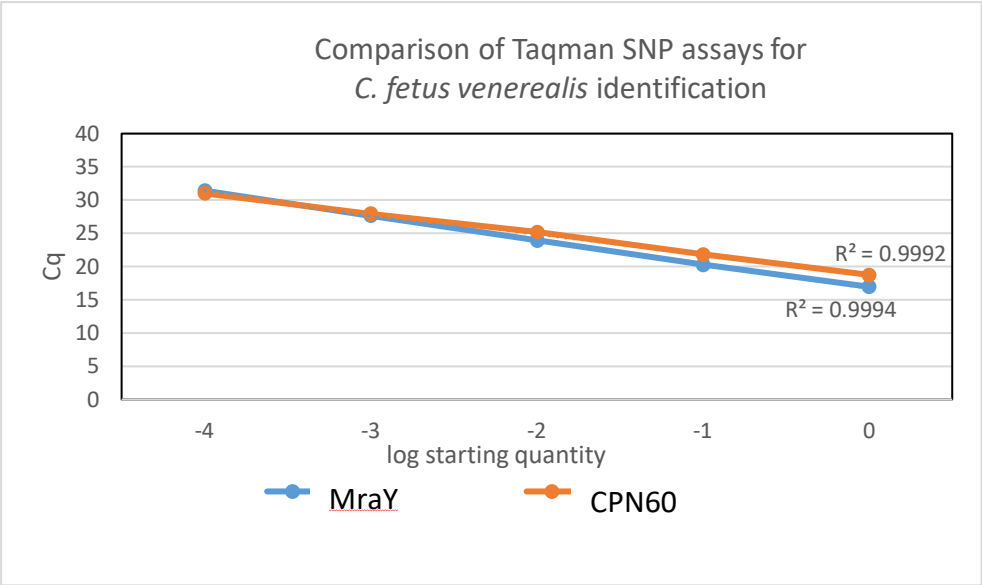


Figure 20. A standard curve for the mraY SNP assay (blue) and the CPN60 SNP assay (orange), plotting Cq value against starting quantity of CFV DNA. Data points are from 1 ng to 0.1 pg, with 1 pg indicated by an arrow. The efficiency of the qPCR is given by the R^2.

Where the *mra*Y assay is superior to *cpn*60 is in distinguishing between *C. fetus venerealis* and other closely related bacteria (*C. sputorum, C. ureolyticus, C. hyointestinalis and A. cryoaerophilus).* Figure 21 shows the discrimination between these samples using a dotplot based on the RFU in the VIC and FAM channel for both assays. The cpn60 assay incorrectly identified the *A. cryoaerophilus* sample and provides less 'distance' in RFU between *C. fetus fetus* and *C. fetus venerealis* control samples. Thus, the specificity of the mraY SNP assay will lead to less false positives.

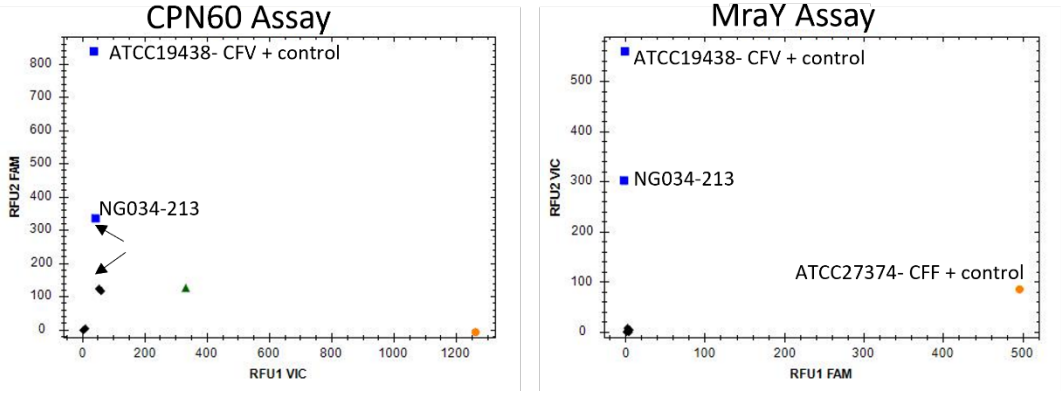


Figure 21. Allelic discrimination plots for cpn60 (left) and mraY (right) Taqman SNP assays using Relative Fluorescent Units for VIC and FAM. Blue boxes – *C. fetus venerealis* positive samples, Orange circles – *C. fetus fetus* positive samples, Black diamonds – other *Campylobacter* species (*C. sputorum, C. ureolyticus* and *C. hyointestinalis),* green triangle – *A. cryoaerophilus*. The mraY PCR shows superior separation between sample groups. Arrows indicate where the cpn60 assay fails to discriminate well between *C. fetus venerealis* and closely related species. *A. cryoaerophilus* was mistakenly classified as heterozygote.

4.4.5 Other methods

4.4.5.1 ddPCR

Assay 1 was also run using Digital PCR (dPCR) at 56°C (4°C less than the recommended temperature for dPCR, 60°C) using 4 pg DNA (note limit of qPCR above is 2pg). Both the FAM and VIC probe assays tested negative for the mix of non-*C. fetus* bacterial isolates and the negative control but produced very low positives with our positive controls (data not shown). This could be due to the use of the lower than recommended annealing temperature (56°C instead of 60°C). During this demonstration, an opportunity to repeat this assay at a higher annealing temperature was not available.

The two assays within Assay 2 (*C. fetus venerealis* FAM assay and *C. fetus fetus* VIC assay) were also tested in the dPCR machine (UQ demonstration by QIAGEN in September 2021) at an annealing temperature of 50°C and 56°C (lower than the recommended temperature 60°C). For the FAM assay, 2 ng and 200 pg of *C. fetus venerealis* DNA were strongly positive and 2 ng of *C. fetus fetus* DNA produced low positive values which could have been due to the low annealing temperature (Table 12). The VIC assay for *C. fetus fetus* DNA was specific under these conditions (Table 12) but it was decided to re-run the assays using 4 pg DNA at an increased annealing temperature of 56°C.

Table 12. Assay 2 Specificity and Sensitivity using dPCR. Results of FAM *C. fetus venerealis* TaqMan dPCR using DNA titrations and the VIC *C. fetus fetus* TaqMan dPCR at 2ng DNA at an annealing temperature of 50°C. MIX (*A. cryaerophilus*, *C. sputorum*, *C. ureolyticus*, *C. hyointestinalis*) of bacteria, *C. fetus venerealis* (*Cfv*) *C. fetus fetus* (*Cff*); NTC- No Template Control

Sample	Assay probe	Target	Concentration (copies/µL)	Positives	Negatives	Result
Cfv 2ng	FAM	*Cfv*	12106.1	25462	1	Positive
Cfv 200pg			2002.7	20217	5244	Positive
Cfv 20pg			254.4	4594	20857	Negative
Cfv 2pg			22.7	435	24420	Negative
Cff 2ng			45.8	893	24463	Negative
MIX 2ng			3	57	24689	Negative
Cfv +*Cff*+MIX 2ng			5895.1	25196	270	Positive
NTC			0	0	25467	Negative
Cff 2ng	VIC	*Cff*	4715.7	22590	672	Positive
MIX 2ng			0	0	25468	Negative
Cfv +*Cff*+MIX 2ng			7881.4	25398	70	Positive
Cfv 2ng			0	0	25450	Negative
NTC			0	0	25475	Negative

The assays were repeated at an annealing temperature 56°C using 4pg of each DNA sample including the bacterial contaminants MIX (*A. cryaerophilus*, *C. sputorum, C. ureolyticus, C. hyointestinalis*), see Table 13. The annealing temperature was not increased to 60°C as our qPCR work suggested that this temperature compromised the sensitivity of the *C. fetus venerealis* assay (Table 11, Figure 19). Under these conditions, low positivity was detected for both *C. fetus* subspecies with demonstrated specificity using 4 pg DNA. Further opportunities to optimise this assay on the dPCR equipment were not available.

Table 13. Results of FAM *C. fetus venerealis* and VIC *C. fetus fetus* TaqMan dPCR Assay 2 using 4pg/ul sample at an annealing temperature of 56°C. MIX (*A. cryaerophilus, C. sputorum, C. ureolyticus, C. hyointestinalis*) of bacteria, *C. fetus venerealis* (*Cfv*) *C. fetus fetus* (*Cff*); NTC- No Template Control

Sample 4pg	Assay Type	Target	Concentration (copies/µL)	Positives	Negatives	Result
Cfv	FAM Assay	*Cfv*	24.8	243	11829	Negative
Cff			0	0	13652	Negative
Cfv +Cff			16.2	335	25040	Negative
MIX			0.049	1	24740	Negative
Cfv +Cff+MIX			10.1	207	24932	Negative
NTC			0.049	1	24711	Negative
Cfv	VIC Assay	*Cff*	0.096	2	25456	Negative
Cff			38.8	798	24671	Negative
Cfv +Cff			17.8	369	25062	Negative
MIX			0	0	25464	Negative
Cfv +Cff+MIX			8.9	185	25264	Negative
NTC			0	0	25452	Negative

An attempt to multiplex Assay 2 using both sets of primers and probes for *C. fetus venerealis* and *C. fetus fetus* was also unsuccessful using the dPCR technology (data not shown). The MraY assay was not tested using dPCR and it is possible it would have performed better due to its improved specificity over the earlier 2 assays.

4.4.5.2 Long read sequencing

We have confirmed that the sequencing enrichment method is successful in identifying *C. fetus venerealis* in samples down to 4 pg of DNA, which makes it comparable to qPCR in terms of sensitivity (Figure 22). Enrichment for the full Campylobacter genome rather than regions containing SNPs only was a more successful strategy. Figure 22 shows the abundance of *C. fetus venerealis* and *C. fetus fetus* genetic material in the serially diluted samples. While there is some cross identification by the Kraken2 software (*C. fetus venerealis* assigned as *C. fetus fetus* and vice versa) overall the technique successfully identified *C. fetus venerealis* DNA in mixed bovine samples with high sensitivity. In addition, our results show that enriching for specific SNPs within regions of interest during sequencing produces more *C. fetus venerealis* reads and reduces the abundance of unclassified reads (reads which are not assigned to *C. fetus venerealis* or any other bacteria species) in spiked samples compared to enrichment with whole Campylobacter genomes (data not shown).

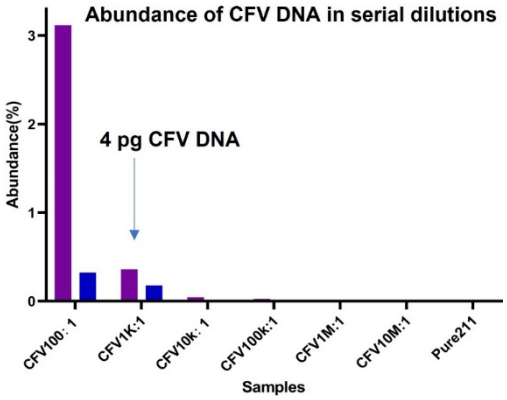


Figure 22. Abundance of *C. fetus venerealis* in spiked samples with serial dilutions from 4 ng to 40 fg with a negative mixed sample (pure211). Samples were multiplexed on a single flow cell for sequencing and enriched for Campylobacter genetic material.

4.4.6 Field evaluations of assays

4.4.6.1 AaCo samples qPCR.

DNA from AaCo herds were extracted by BSL staff at QDAF and then couriered to our laboratories at UQ St Lucia to undertake *C. fetus* qPCRs. AaCo samples (n=430) were initially screened using the cpn60 qPCR (assay 1) and then (n=170) using the mraY qPCR (assay 3). Using the cpn60 assay we identified approximately 5 positive (≤32) and 36 'suspect' positives (Cq means 33-36). When using the mraY assay none of the AaCo samples were positive.

DNA obtained from QDAF BSL had lower concentrations (mean 64.2 ng/µl) compared to our DNA extractions (122.6 ng/µl), data not shown. The DNA from BSL also had a higher content of host DNA diluting the microbial content of their extracts. We include a host cell centrifugation step to try to remove some of the non-microbial content of the sample before total DNA extraction from mucus samples. This could explain why the sensitivity of our assay was insufficient to detect *C. fetus* qPCR positive samples in many of the BSL prepared DNA extracts.

Table 14. qPCR result summary of AaCo herd samples

Sample herd IDs	cpn60			mraY qPCR		
	Pos/no. of samples	Positive	Suspect positive	Pos/no. of samples	Positive	Suspect positive
03920	25/110	4	21	*0/25	0	0
03782	5/60	0	5	-	-	-
03952	1/35	0	1	-	-	-
04177	8/39	0	8	-	-	-
04269	1/26	0	1	-	-	-
060722	1/199	1	0	-	-	-
01965	-	-	-	0/49	0	0
02334	-	-	-	0/25	0	0
02702	-	-	-	0/30	0	0
02826	-	-	-	0/26	0	0
03131				0/40	0	0

Legend: Positive=Cq average ≤32; Suspect positive=Cq average 33-<36; -=not done;
*the 25 cpn60 positive samples from Herd03920 were re-tested in the mraY qPCR assay

4.4.6.2 Roma samples qPCR

The Roma samples are summarised in Appendix 8.2 showing that for 95 samples screened using the cpn60 qPCR: 2 were positive, 8 were suspect and 85 were negative with 9 of these with a history of abortion losses.

4.4.6.3 Jindowae samples qPCR

Two samples from the Jindowae herd (n=38) were positive (Cq 29 and 27) in the mraY qPCR.

4.4.6.4 Abattoir samples qPCR

Nine abattoir samples were collected for qPCR and for serum proteomics to evaluate for the presence of biomarkers. One of the abattoir samples was qPCR positive in the mraY assay at Cq average of 25.62, the same sample was negative in the cpn60 qPCR.

4.4.6.5 Serum proteomics – Abattoir and Jindowae samples

Corresponding sera from one qPCR positive from the abattoir and the two qPCR positive from our heifer trial were subjected to serum proteomics. These were compared to three qPCR negative cattle as negative controls. Raw proteomics data is presented in Appendix 8.10. The results confirmed higher abundances of Vitamin D binding protein and Haptoglobin in the sera of the qPCR positive bulls. Three qPCR negative bulls did not have these proteins indicating that the correlation of these biomarkers to the presence of *C. fetus venerealis*. There are commercial ELISA kits for bovine Vitamin D binding protein (Millenium Science) and Haptoglobin detection (Abcam) which could be exploited to screen sera as a biomarker assay for BGC.

4.4.6.6 Comparison of qPCR with ONT methods (genomic and metagenomic analyses)

Metagenomics analysis enables the filtering of data to examine particular species. Figure 23 demonstrates the metagenomic detection of *C. fetus venerealis* in animal 2013# of herd NG034 which returned a 'suspect' qPCR result (Appendix 8.2). The diversity and abundance of Campylobacter species is higher in animal #2013 than in the individuals negative for *C. fetus*

Figure 23. A comparison of the number of Campylobacter reads found in four individuals from herd NG034. Animal #2013 returned a 'suspect' PCR result by PCR but confirmed positive by metagenomic sequencing. *C. fetus venerealis* reads are indicated by a red arrow.

We expanded this metagenomic analysis to examine diagnostic samples submitted within this project (summarised in Table 15). Figure 24 shows the genera present in the reproductive tracts of animals which were positive or negative for *C. fetus venerealis* using qPCR and enrichment sequencing. Interestingly, bacteria of the genus *Histophilus* are present in all *C. fetus venerealis*

positive animals (except for ROMA3) which supports the data from our study on the bull metagenome (Section 4.2.3).

Roma samples 62-70 did not contain detectable *C. fetus venerealis* through either qPCR or enrichment sequencing however the metagenome has a higher content of *Escherichia* compared to the other samples in Figure 24. Roma 62-70 cows all had history of abortion (Appendix 8.2) and although not commonly reported, *E. coli* can cause abortion in cattle (Kirkbride 1993) but is considered a 'sporadic or opportunistic' as abortive pathogen (Henker et al., 2022).

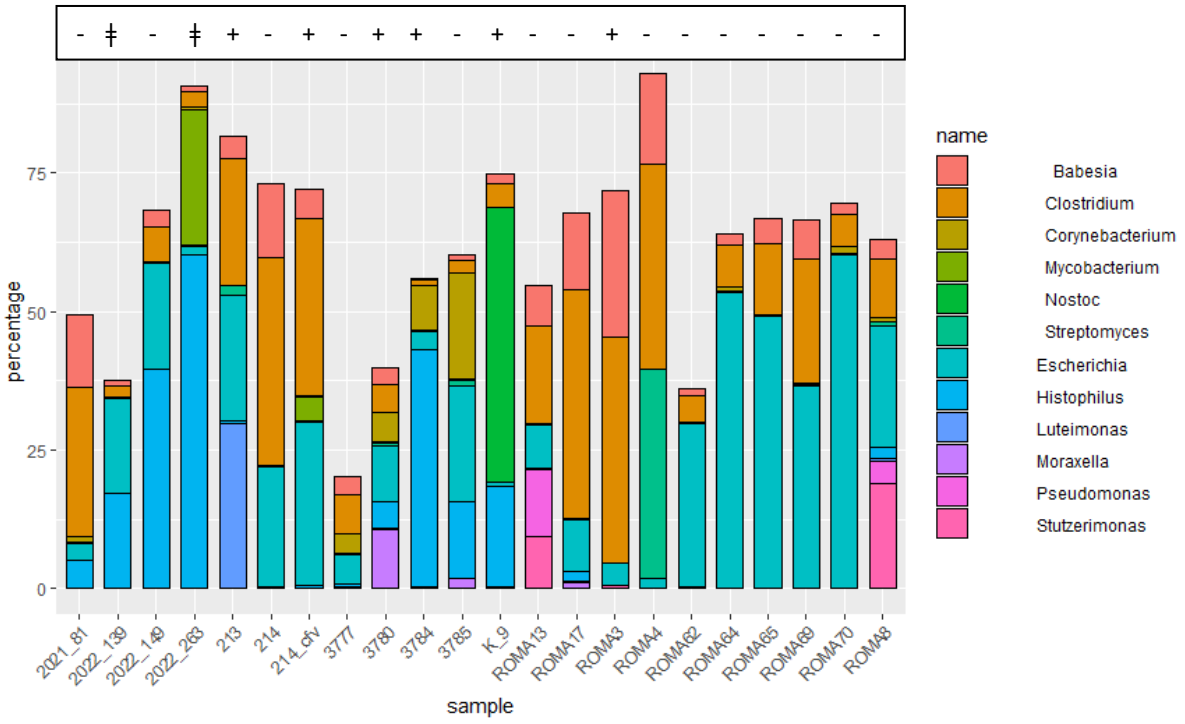


Figure 24. Metagenomics analysis of diagnostic samples described in Table 15. Legend: ‡ indicates that the sample is positive in long read enrichment sequencing and not qPCR; + indicates that the sample is positive in qPCR and enrichment sequencing; - indicates that the sample negative by qPCR and enrichment.

ONT sequencing using enrichment for the *Campylobacter* genome demonstrated a higher sensitivity when using field samples compared to qPCR (Table 15). Note that two very low positives with only one *C. fetus venerealis* read through enrichment sequencing were summarised as 'inconclusive' in Table 15 were entered as 'negative' in Figure 24. This analysis reveals that while the qPCR assays are not identifying false positives in the samples (i.e. all samples identified as *C. fetus venerealis* positive by qPCR have *C. fetus* reads in the metagenome), these assays can give false negatives. Thus, enrichment sequencing offers several advantages. First, 'suspect' samples can be classified with confidence due to sequence identity, this is of particular use where herds have reproductive issues, but the qPCR results are not definitive. Second, the portable nature of the sequencing means there is potential to undertake diagnosis yard-side in the future. The disadvantage of the method remains cost, but with new methods of multiplexing this will likely be reduced in the future.

Table 15. Comparison of qPCR methods with long read genomic sequencing data.

Sample	mraY (CFV)	CPN60 (CFV)	Sequencing result	Number of CFV reads	Total reads	Notes
ATCC-19438	*Positive*	*Positive*	Not run (NR)	NR	NR	*C. fetus venerealis* control
ATCC-27374	Negative	Negative	Not run (NR)	NR	NR	*C. fetus fetus* control
NG034-210	Negative	Negative	Negative	0	Check	
NG034-211	Negative	Negative	Negative	0	Check	
NG034-213	*Positive*	*Positive*	*Positive (low)*	2	1.19M	
NG034-214	Negative	Negative	Negative	0	0.49M	
214 spiked	*Positive*	*Positive*	*Positive*	9	0.65M	
KM.9	*Positive*	*Positive*	*Positive*	385	4.7M	
3777-06/09/22	Not done	Negative	Inconclusive	1	0.66M	Young unvaccinated challenged 30/08/22
3780-01/09/22	Not done	*Positive*	*Positive*	3	0.21M	Young unvaccinated challenged 30/08/22
3784-01/09/22	Not done	Negative	*Positive*	50	0.44M	Old unvaccinated challenged 30/08/22
3785-01/09/22	Not done	Negative	Negative	0	0.06M	Young vaccinated challenged 30/08/22
2021_81	Positive	Not done	*Positive*	4	5.26M	Reproductive issues
2022_263	Negative	Not done	*Positive*	9	0.69M	Reproductive issues
2022_139	Negative	Not done	*Positive*	43	1.15M	Reproductive issues
2022_149	Negative	Not done	Negative	0	2.44M	Reproductive issues
ROMA3	Not done	*Positive*	*Positive*	83	1.32M	
ROMA4	Not done	Negative	Negative	0	1.46M	
ROMA8	Not done	Negative	Negative	0	1.49M	
ROMA13	Not done	Negative	Negative	0	2.05M	
ROMA17	Not done	Negative	Negative	0	0.78M	
ROMA62	Not done	Negative	Inconclusive	1	1.2M	Abortion
ROMA64	Not done	Negative	Negative	0	0.63M	Abortion
ROMA65	Not done	Negative	Negative	0	2.32M	Abortion
ROMA69	Not done	Negative	Negative	0	5.05M	Abortion
ROMA70	Not done	Negative	Negative	0	0.78M	Abortion

4.5 Determined whether the genital microbiome is associated with increased susceptibility to BGC

4.5.1 Metagenome analysis of herds NG036 and NG037

In total, ONT adaptive sampling yielded an average of 12.67 Gb and 6,299,065 Kbp of raw data per sample. After host contamination removal and quality filtering, there was an average of 417, 843 Kbp of data, in each of the bovine metagenome samples. This data has now been published (Ong et al., 2022c, Appendix 8.7). NG036 consisted of Brahman crosses while NG037 were Droughtmaster cattle (Appendix 8.1).

While alpha diversity is a measure of microbiome diversity applicable to a single sample, beta diversity is a measure of similarity or dissimilarity of two communities. As for alpha diversity, many indices exist each reflecting different aspects of community heterogeneity. The alpha diversity, represented by Shannon index, indicated that the bovine reproductive metagenomes from different representative groups in NG036 had similar alpha diversities, regardless of gender and reproductive stages (Anova, P=0.129) (Figure 25). On the other hand, the bovine reproductive metagenomes posed significantly different (Anova, P=2.0×10^{-3}) diversity in herd NG037. Particularly, the reproductive metagenome of postpartum animals had significantly lower diversity than prepubertal, cycling and pregnant animals (T-test, P= 4.43×10^{-4}, 1.64×10^{-3} and 1.0×10^{-2} respectively). In general, the bovine reproductive metagenomes in herd NG037 were significantly more diverse (T-test, P=7.12×10^{-4}) than those in herd NG036.

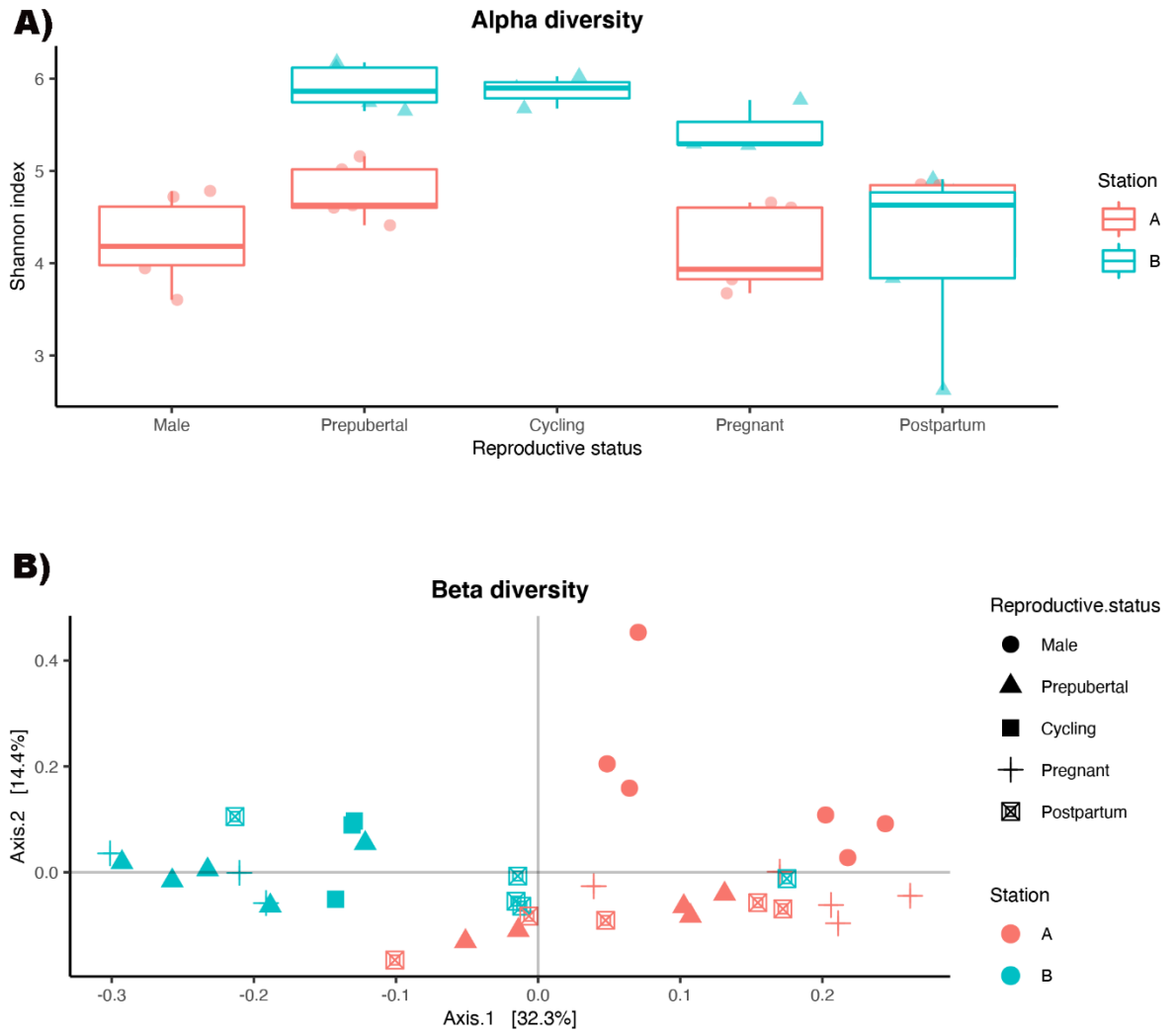


Figure 25. (A) Alpha diversity and (B) beta diversity of the reproductive tract metagenomes collected from NG036 (Station A=red) and NG037 (Station B=blue). The shapes represent animals of different genders or reproductive status: circle represents male, triangle represents prepubertal animals, square represents cycling animals, cross represents pregnant animals and square cross represents postpartum animals.

The beta diversity represented by principal coordinate analysis ordination (PCoA) of the Bray-Curtis dissimilarity matrix demonstrated that there were significant dissimilarities (PERMANOVA, $P=9\times10^{-4}$) between the bovine reproductive tract metagenomes collected from NG036 and NG037 (Figure 25). Within NG036, the dissimilarity between bovine male and female reproductive tract metagenomes was also significant (PERMANOVA, $P=2\times10^{-3}$). Nonetheless, in either of the stations, the bovine female vaginal metagenomes were not significantly different between different reproductive stages (Permutation test, $P > 0.05$).

Figure 26 depicts the abundant phyla, including those with more than 5% abundances in the bovine reproductive tract metagenome. *Ascomycota*, *Firmicutes* and *Proteobacteria* each constituted more than 20% of the bovine reproductive tract metagenome, regardless of gender or reproductive stage. *Bacteroidetes* was only abundant in the bovine vaginal metagenomes collected from NG037 while phyla *Fusobacteria* was only abundant in the postpartum bovine vaginal metagenome also collected from NG037. *Actinobacteria* and *Tenericutes* were identified in all samples, however their

abundances were not consistently higher than 5% at different stations, gender and reproductive stages.

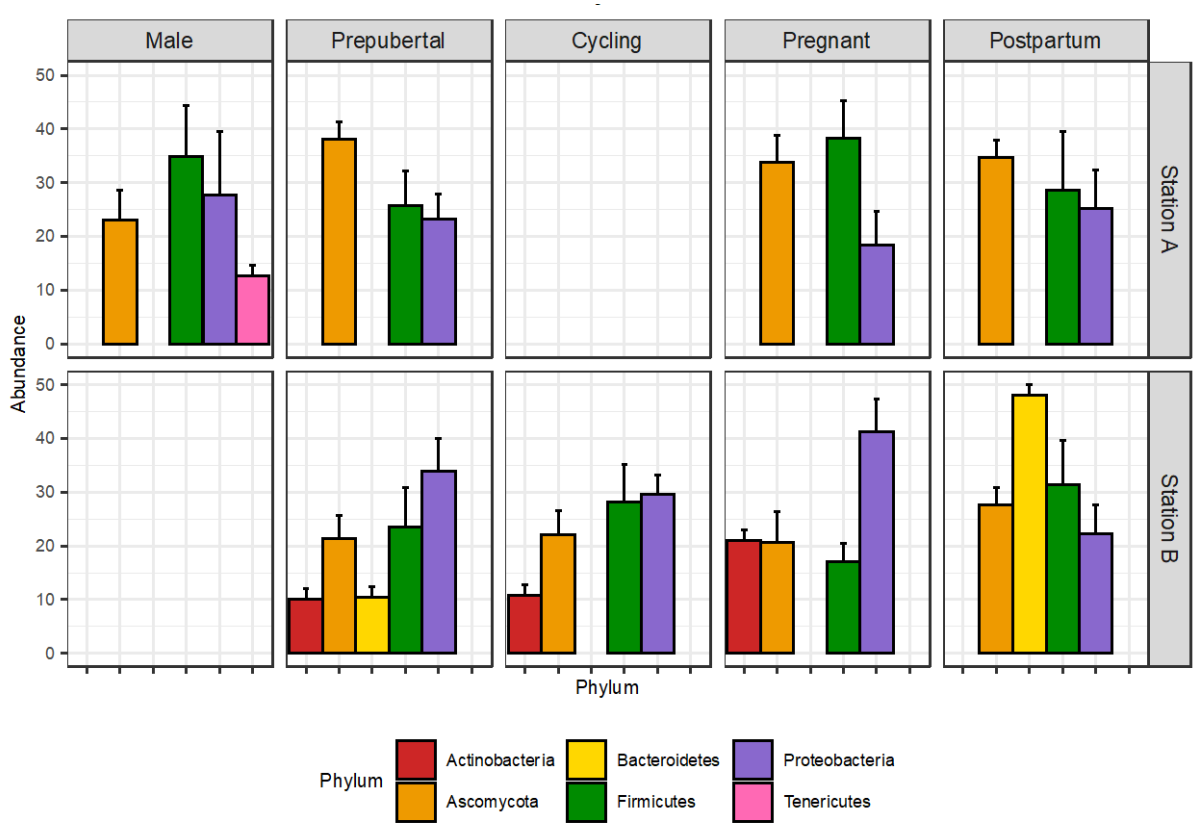


Figure 26. Phyla with more than 10% of abundance in the bovine reproductive metagenomes collected from NG036 (Station A) and NG037 (Station B). Empty panels indicate no animal was sampled in that category and empty columns indicate phyla less than 10%.

At the species level, all the top abundant species were bacteria (Figure 27, see also Table 16). In NG036, *Clostridium botulinum* (ubiquitous in the environment) was commonly abundant regardless of gender and reproductive status. *Escherichia coli* (natural colonizer of urogenital mucosa, can cause abortion) was only commonly abundant in the female reproductive metagenomes, while *Histophilus somni* (regarded as a secondary respiratory tract pathogen but a potential cause of abortion) and *Mycoplasmopsis californica* (can cause mastitis) were the abundant species in the preputial samples only. *Staphylococcus agnetis* (can cause mastitis) was abundant in pregnant animals but not in prepubertal and postpartum animals. Similarly, *E. coli* was commonly abundant in all female reproductive metagenomes in herd NG037. Except for pregnant animals, *C. botulinum* was the common abundant species in the vagina metagenomes collected from NG037. The pregnant animals in NG037 had more than 10% of *Acinetobacter ursingii* (opportunistic pathogen) and *Microbacterium sp.* CBA3102 (fermentative species). *Bacteroides fragilis* (gastrointestinal microbiota) was the most abundant species in the postpartum vaginal metagenomes collected from NG037.

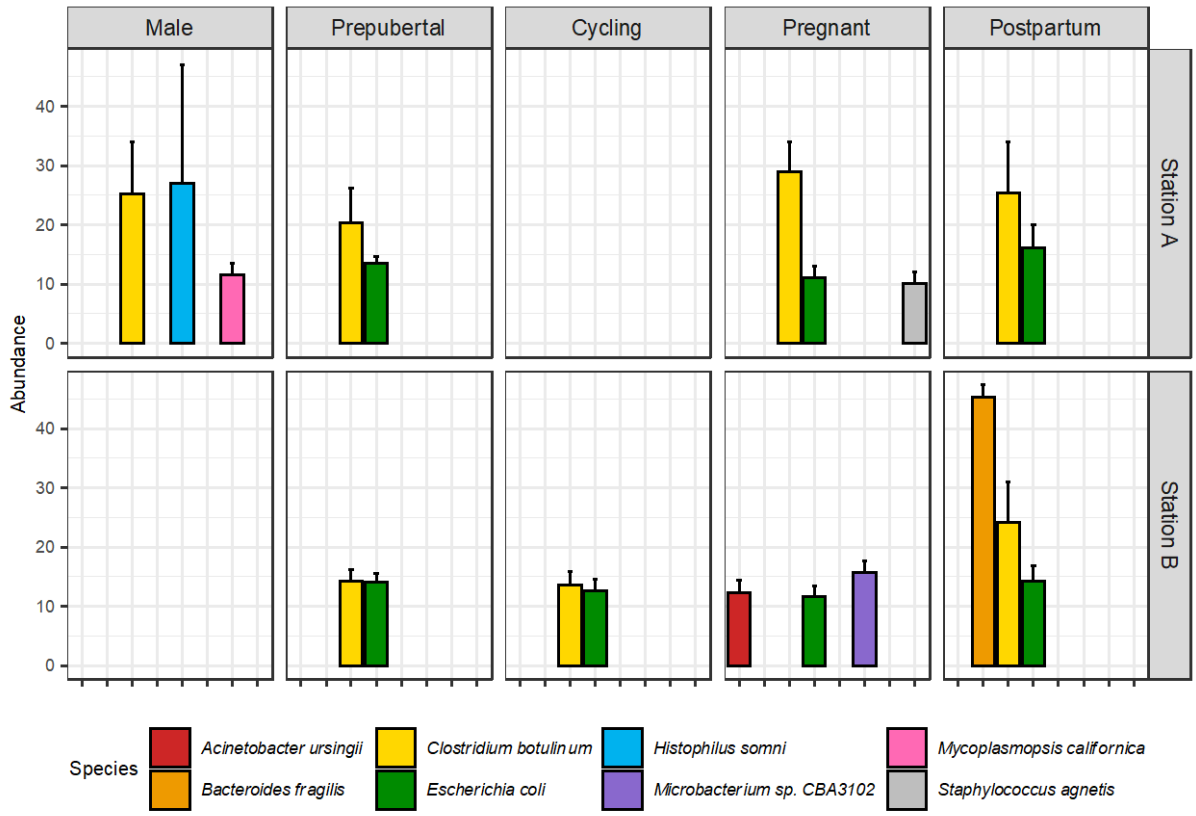


Figure 27. Species with more than 10% of abundance in the bovine reproductive metagenomes collected from NG036 (Station A) and NG037 (Station B). Empty panels indicate no animal was sampled in that category and empty columns indicate species less than 10%.

We compared and identified the species which had significantly different abundances between NG036 and NG037 (Figure 28A) and between the two genders in NG036 (Figure 28B). The species which were more significantly abundant in NG036 were *C. botulinum* (Wald test, P=2.57×10^{-3}), *Pycularia pennisetigena* (Wald test, P=2.48×10^{-2}) (plant fungal pathogen) and *Talaromyces rugulosus* (Wald test, P=4.83×10^{-2}) (mycoparasitic fungus), while the species which was more significantly abundant in NG037 was *Microbacterium sp.* CBA3102 (Wald test, P=8×10^{-3}). Within NG036, metagenome samples collected from bull prepuce had a significantly higher abundance of *Histophilus somni* (Wald test, P=4.21×10^{-7}) and *Aerococcus urinaehominis* (Wald test, P=8.36×10^{-3}) (novel bacterial species isolated from urine of mammals). The female vaginal metagenomes had significantly higher abundances of *Escherichia coli* (Wald test, P=1.47×10^{-2}) than the bull preputial samples. Results are summarised in Table 16.

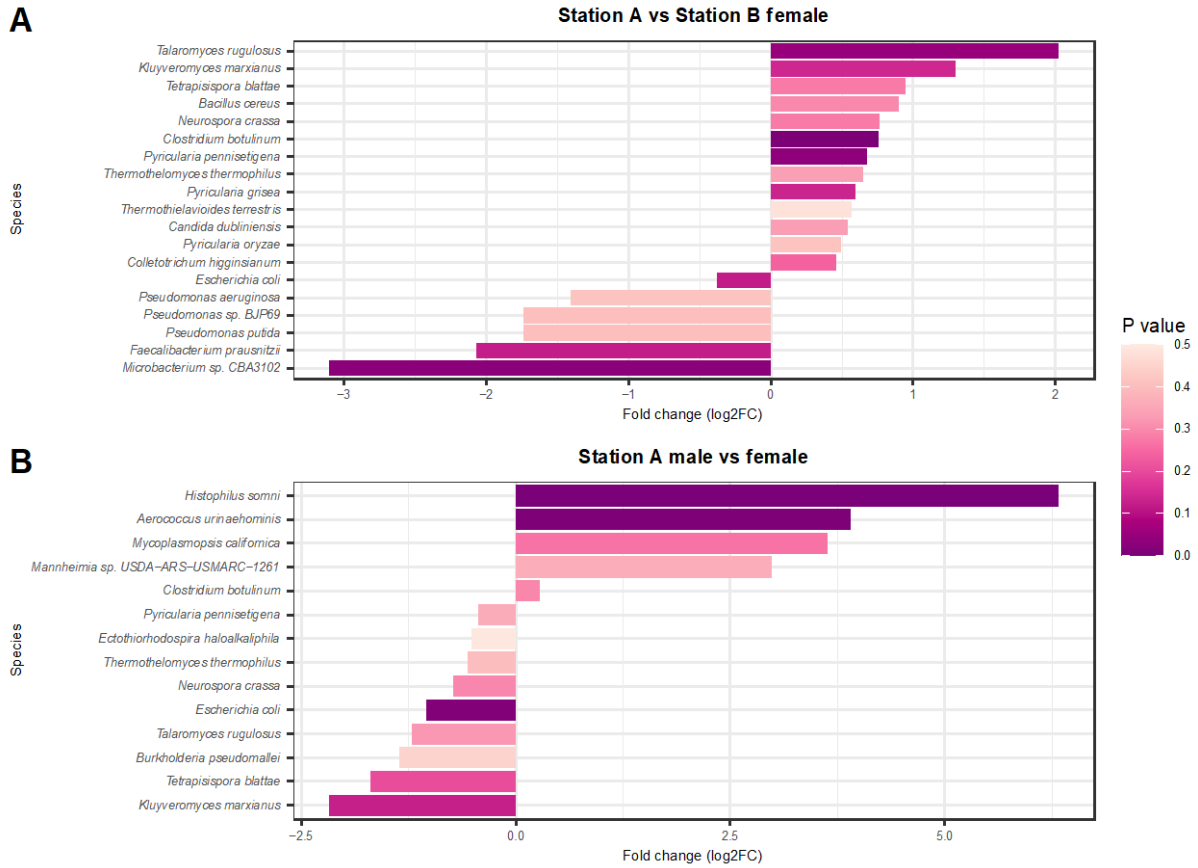


Figure 28. Microbes log_2 fold change comparing (A) Station A (NG036) against Station B (NG037) and (B) male against female in Station A (NG036).

In the multidimensional scaling (MDS) analysis, which depicted the dissimilarity based on the leading log_2 fold change of the functional annotations, it was observed that most of the bovine vaginal metagenomes collected from NG037 were clustered together. Similarly, the MDS analysis demonstrated that there were higher dissimilarities between bovine reproductive tract metagenomes collected from different genders.

Table 16 summarises the most abundant bacterial species identified in this analysis. Specific mining of the metagenomic data was undertaken to identify other species which were abundant in the rDNA amplicon study (see Section 4.5.2). Herds NG036 and NG037 had low abundance of *Ureaplasma diversum* ranging from 0.01-1.4 abundance while it was relatively more abundant in herd NG034 (Table 16). Also, comparatively, *Mycoplasma bovigenitalium* was not detected in herds NG036 or NG037, however several other Mycoplasma species were lowly abundant (<2.5) including *M. mycoides*, *M. hyopneumoniae*, *M. capricolum*, *M. conjunctivae*, *M. dispar*, *M. iowae*, *M. canadense* (high in NG034, Table 17).

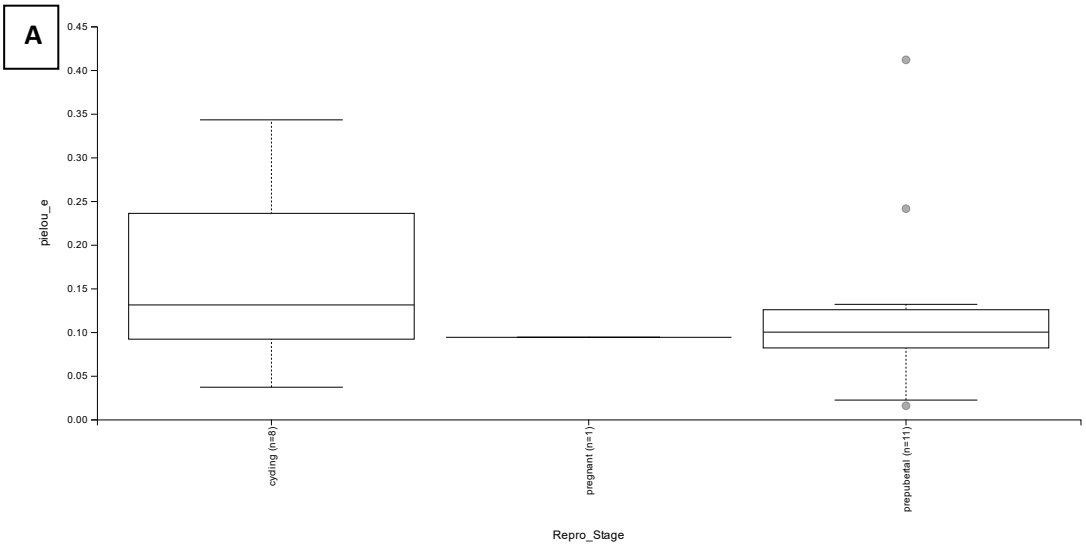
The main observations indicate that the dominant female microbiome content is related to their nutritional environment and do not seem correlate with the metagenomes of the male reproductive tracts. It is known that nutrition has the highest impact on health of cows in northern Australian properties and diet or the environment seems also be reflected by their metagenome content.

Table 16. The most abundant bacterial species from metagenomics analyses of cattle from herds NG036 and NG037 at a 10% abundance cut off.

Species	Source	Significance	Reference
Escherichia coli	Female cattle NG036 & NG037	Gut/faecal/urogenital mucosa; can cause abortion	Torres Luque et al., 2017
Mycoplasma californicum	Bull prepuce, NG036 only	Mastitis	Mackie et al., 1986
Histophilus somni	Bull prepuce, NG036 only	Abortion, usually BRD	O'Toole and Sondgeroth, 2015
Staphylococcus agnetis	Pregnant females NG036 & NG037	Mastitis	Taponen et al., 2012
Clostridium botulinum	Non-pregnant females NG036 & NG037	Environment/gastrointestinal; not previously identified in bovine reproductive tracts	Kruger et al., 2014
Acinetobacter ursingii	Pregnant females, NG037 only	Opportunistic human pathogen, not previously identified in cattle	Chiu et al., 2015
Microbacterium sp. CBA3102	Pregnant females, NG037 only	Gastrointestinal/fermentative	
Bacteroides fragilis	Postpartum females, NG037 only	Gastrointestinal/rare cause of abortion in cattle	Kraipowich et al., 2000

4.5.2 Microbiome analysis of herd NG034

A herd with known cases of BGC was analysed through an external provider to attempt to see if this service could be used for on-going microbiome analyses. No bull samples were available from NG034 at the time of sample collection and only one female sampled was pregnant. *Campylobacter fetus venerealis* had previously been detected using qPCR from two cows in this herd. Higher alpha diversity was observed in cycling cows compared to prepubertal (Figure 29A) which was also found in herd NG036 above using metagenomics (Figure 25A). Similarly for beta diversity for NG036 and NG037 above (Figure 25B), there was no significant differences between prepubertal or cycling stages in herd NG034 (Figure 29B).



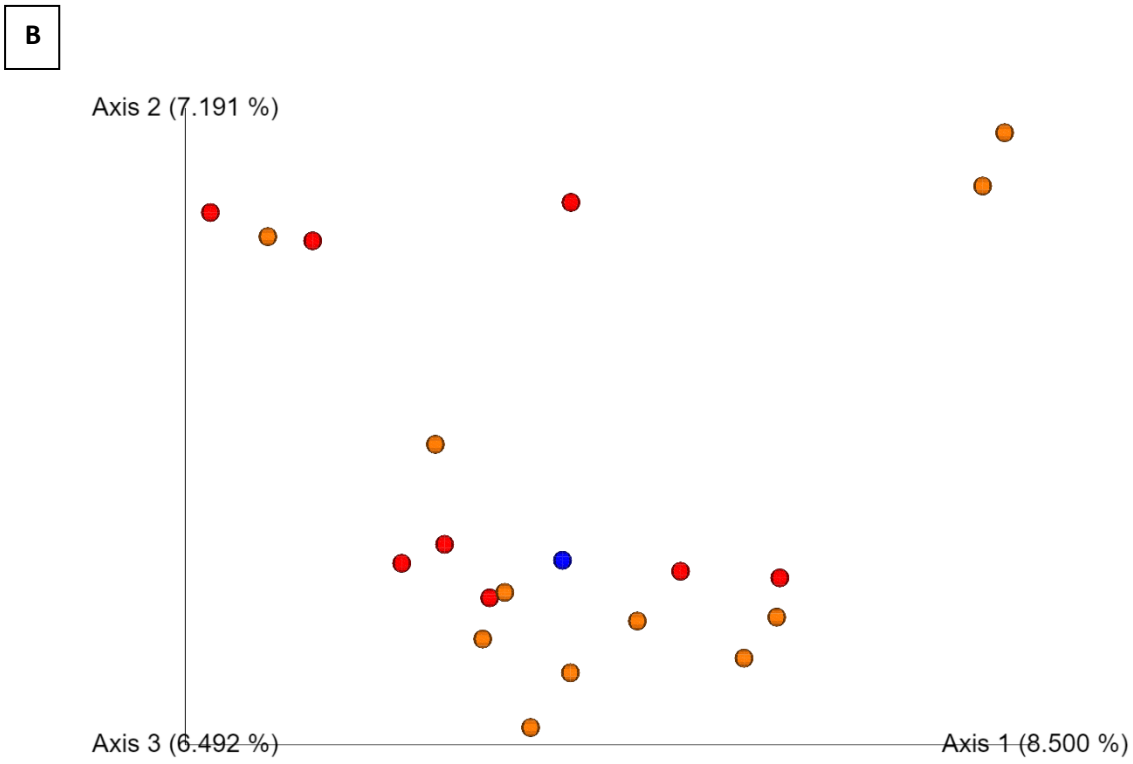


Figure 29. A) Alpha diversity and, B) Beta diversity (red=cycling, blue=pregnant, orange=prepubertal) of female reproductive tract microbiomes collected from herd NG034.

Figure 30 depicts the most abundant phyla with an abundance of *Proteobacteria* in all reproductive stages demonstrated over *Firmicutes* and *Tenericutes* which were also relatively abundant in the metagenomics analyses above. Note in the adaptive metagenomics above a cut off of 10% was used, however using amplicon microbiome analyses here the cut off is 2%.

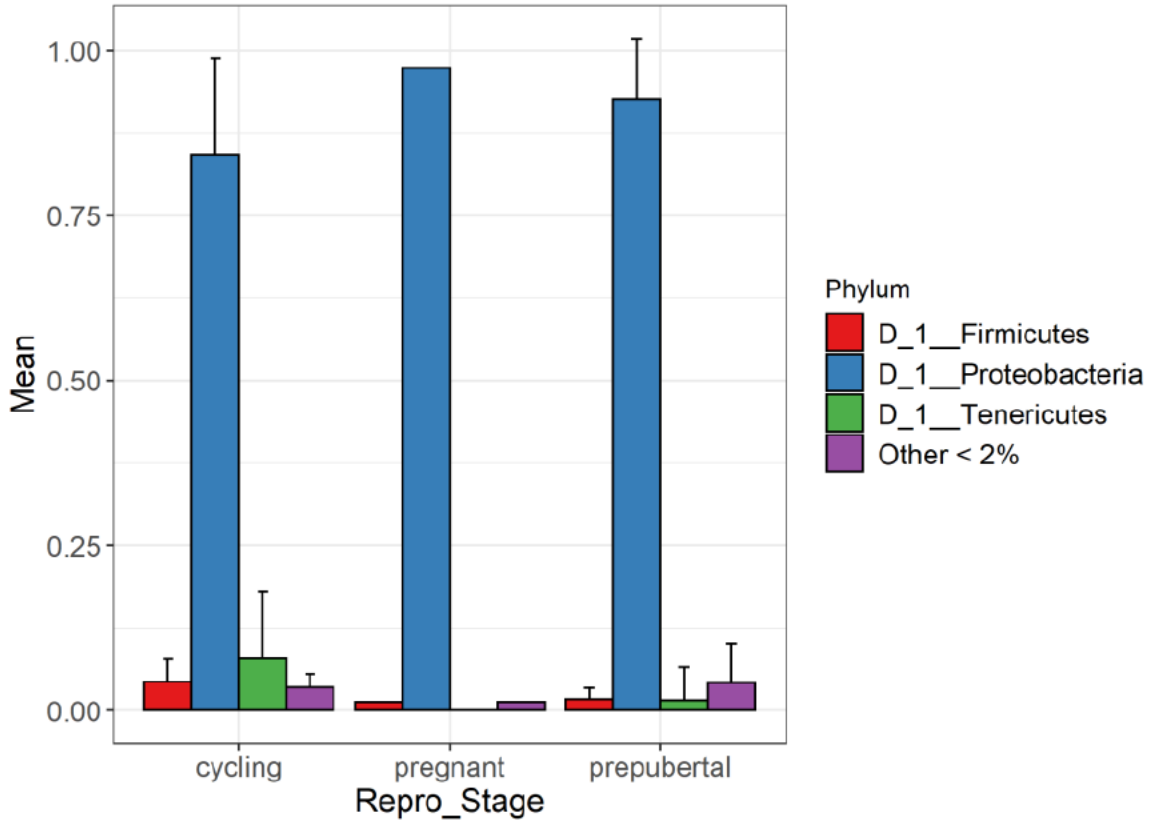


Figure 30. Phyla abundance for herd NG034 female cattle at a threshold of >2%.

Figure 31 demonstrates the most abundant bacterial species in herd NG034. An unknown *Ochrobactrum* spp. and unknown *Histophilus* spp. contributed to the majority of all sequenced reads in each sample. To identify higher diversity in the microbiome data as within the metagenomics data, lower abundances were explored to examine the bacterial species present, see Figure 32. *Ochrobactrum* and *Histophilus* species were the most abundant including several uncultured or unclassified species. Table 17 lists the most abundant species after removing uncultured and unclassified species. With such low profiles compared to the metagenomic analyses in 4.5.1, it is difficult to compare this data. *Histophilus* species could be similarly aligned with *Histophilus somni* (abundant in metagenomics) and possibly *Escherchia coli* (see Table 16 above) as 'Escherichia-Shigella' was identified below (Figure 32). Due to the differences in this type of analysis the depth is poorer. Two potential pathogens were identified which have been reported to cause granular vulvovaginitis and abortion in cattle, *Mycoplasma bovigenitalium* and *Ureaplasma diversum* (Macedo et al., 2018), Table 17. Comparatively, the metagenomic analysis of herds NG036 and NG037 demonstrated low abundance of *Ureaplasma diversum* and no *Mycoplasma bovigenitalium* however a large array of *Mycoplasma* spp. were also lowly abundant (not shown). The impact of the presence of potential pathogens at low to high levels in metagenomes and/or microbiome data is currently unknown. The high abundance of *Ochrobactrum* in NG034 (Figure 32) may be environmental as this species is commonly found in a wide variety of environments including water, soil, plants and animals (reviewed by Ryan and Pembroke, 2020). *Ochrobactrum* spp. are considered to be of low virulence and capable of causing infections in immunocompetent human hosts (Ryan and Pembroke, 2020) with no reports associated with bovine reproductive health reported.

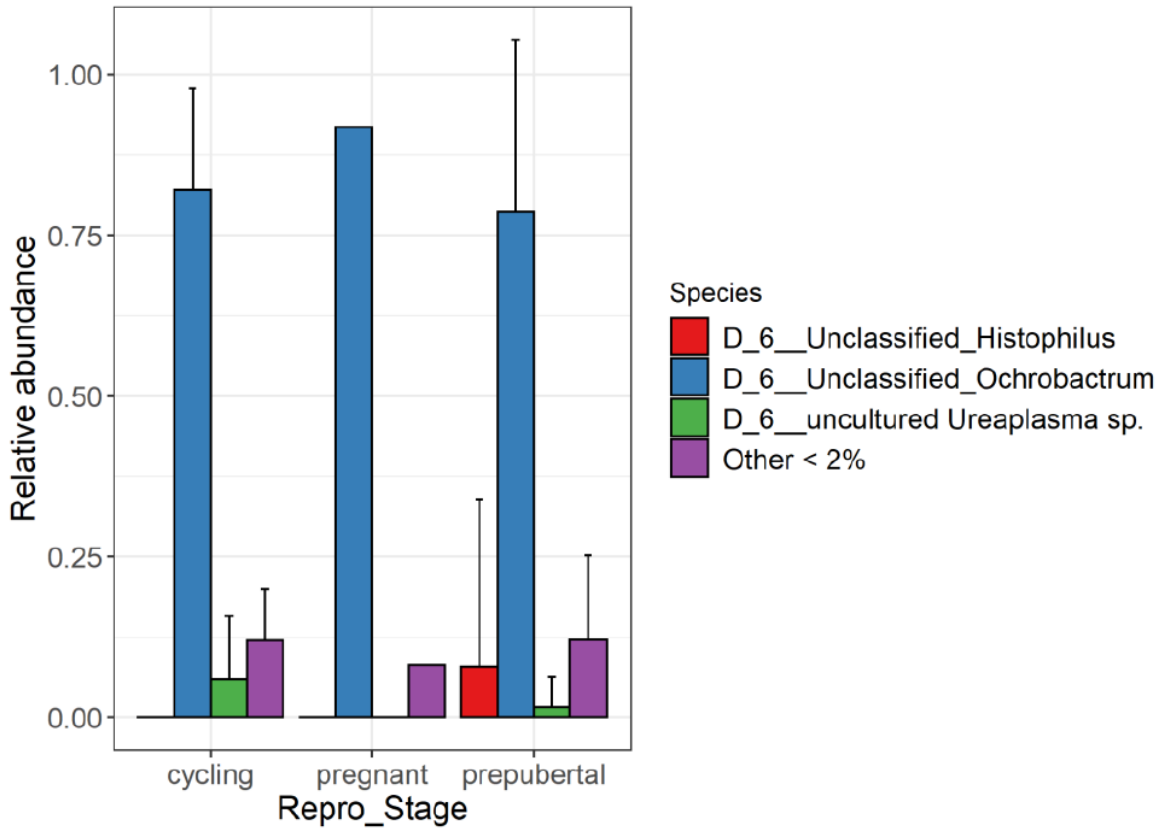


Figure 31. Species with more than 2% abundance in the bovine female microbiomes from herd NG034.

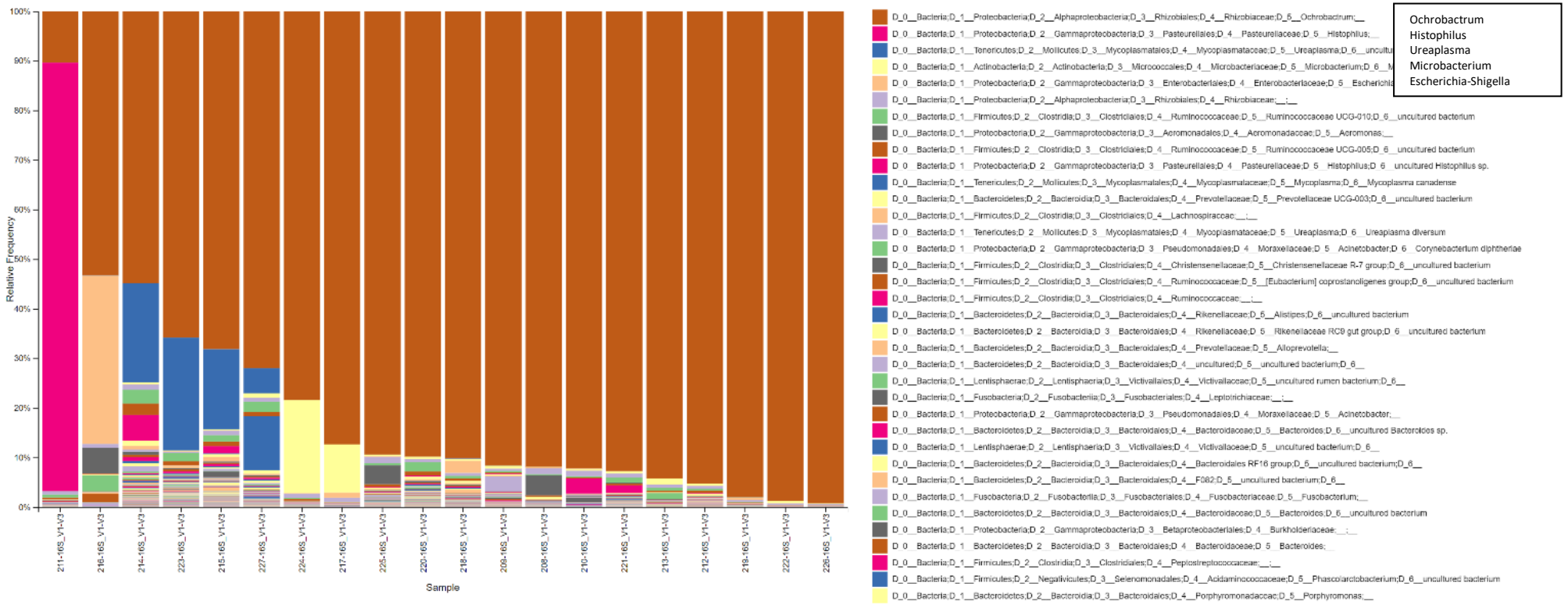


Figure 32. Most abundant bacterial species showing relative frequency percentages of the vaginal microbiome from each cow (x axis) from herd NG034 including 'uncultured' and 'unclassified' species. The list of bacteria on the right from highest abundance down to lowest (top to bottom) showing all levels of phyla to species level where relevant for each species. The top 5 abundant populations have been added in the text box in larger font.

Table 17. The most abundant species and their mean abundances from microbiome analyses of NG034 cows without including the 'uncultured' and 'unclassified' species from Figure 32.

No.	Species	Percent abundance	Significance/Source	Reference
1	*Microbacterium oxydans*	1.76%	Environmental	Kim et al., 2013
2	*Mycoplasma canadense*	0.553%	Mastitis	Tamiozzo et al., 2014
3	*Corynebacterium diphtheriae*	0.240%	Mastitis and skin infections	
4	*Ureaplasma diversum*	0.177%	Infertility and abortion	Macedo et al., 2018
5	*Mycoplasma bovigenitalium*	0.0212%	Infertility and abortion	Macedo et al., 2018
6	*Fusobacterium massiliense*	0.0200%	Putative oral/gut pathogen	Mailhe et al., 2016
7	*Rhodococcus sp.* 2G	0.0162%	Environmental	Zhao et al., 2019
8	*Micrococcus sp.* G2DM-40	0.0143%	Environmental	
9	*Porphyromonadaceae bacterium* FC4	0.0128%	Human urine	
10	*Porphyromonas sp.* canine oral taxon 361	0.0114%	Unknown	

4.5.3 Mining the microbiomes for *Campylobacter* sequences

The relative abundancies of *Campylobacter* species in the metagenomics data were specifically compared using the same groups of bulls and cows described above for NG036 and NG037 (Section 4.5.1), See Figure 33. In the bulls (NG036), the abundancies of different species inverted between 2018 and 2021 from high to low and from low to high. Notably, *C. fetus* abundance increased, note that the bulls from both herds had VibroVax® vaccination histories. When the female cattle are cycling they appeared to have high abundancies of many species of *Campylobacter*. However, if they were prepubertal they had only a limited number of *Campylobacter* species. The mature but not pregnant (Cycling) females appeared to have the greatest diversity with respect to *Campylobacter* for both the Droughtmaster (NG037) and Composite breed females (NG036). This could reflect variation in cycle stage but could also be influenced by other factors including potential dysbiosis in the vagina, due to disease or recent mating, affecting the *Campylobacter* composition. In comparison, the pregnant animals, which are hormonally more stable due to the presence of high levels of progesterone from the *corpus luteum* of pregnancy, had a different composition and less diversity. The postpartum group also showed less variation, which could be attributed to postpartum anoestrus; hence cycling and mating are not occurring. Most of the detected *Campylobacter* species are present in the gastrointestinal tracts of cattle. Some gastrointestinal *Campylobacter* spp. can cause enteritis and abortion, including *C. jejuni*, *C. fetus fetus*, *C. hyointestinalis* subsp *hyointestinalis*, and *C. sputorum* (abortions in sheep). Notably, *C. fetus* has a high presence overall in the younger Droughtmaster heifers, which could affect fertility in some herds (Table 3) and is not listed as abundant in the older female composite and Droughtmaster cows (both pregnant and postpartum). It can be suggested from this that the cycling heifers recovered from the initial vaginal infection.

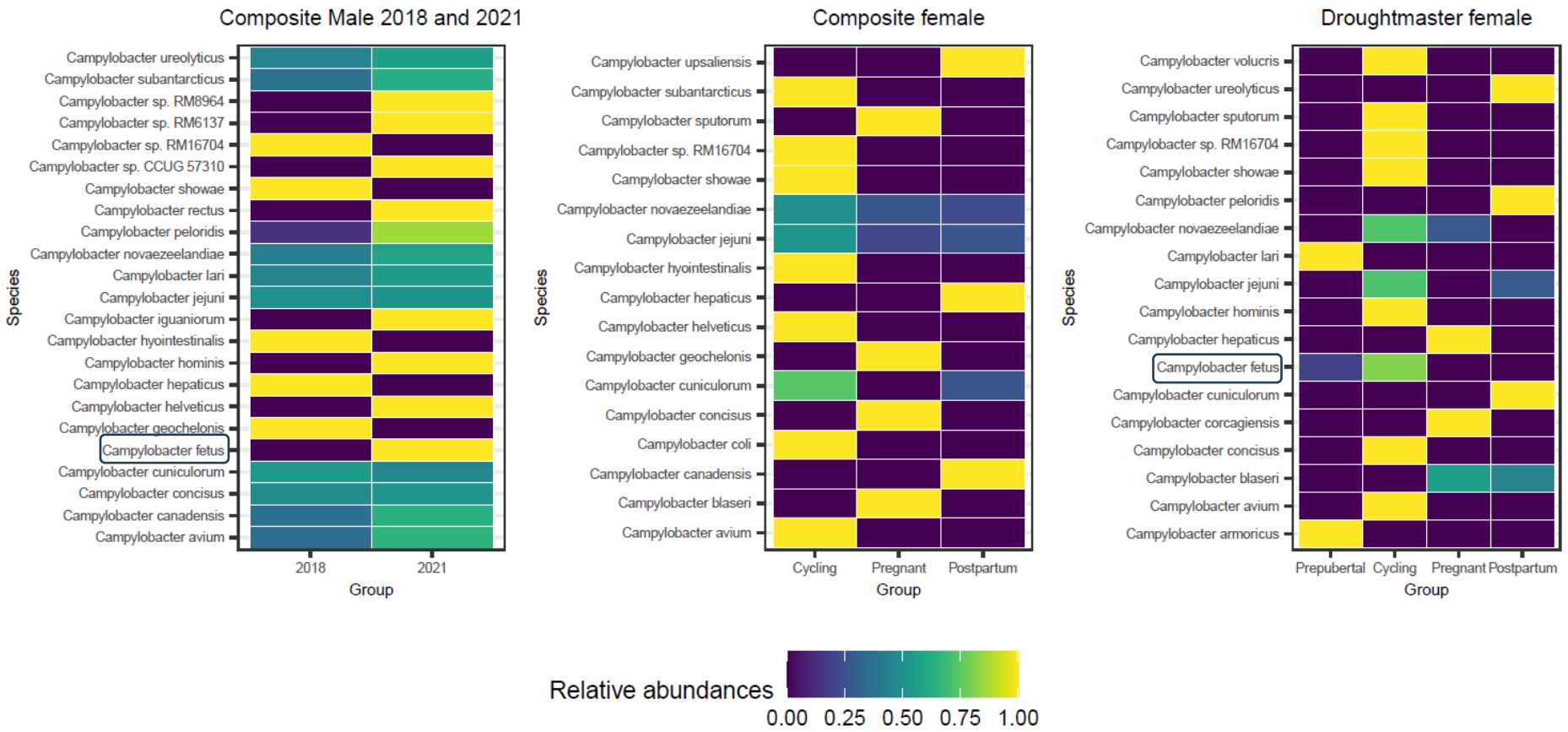


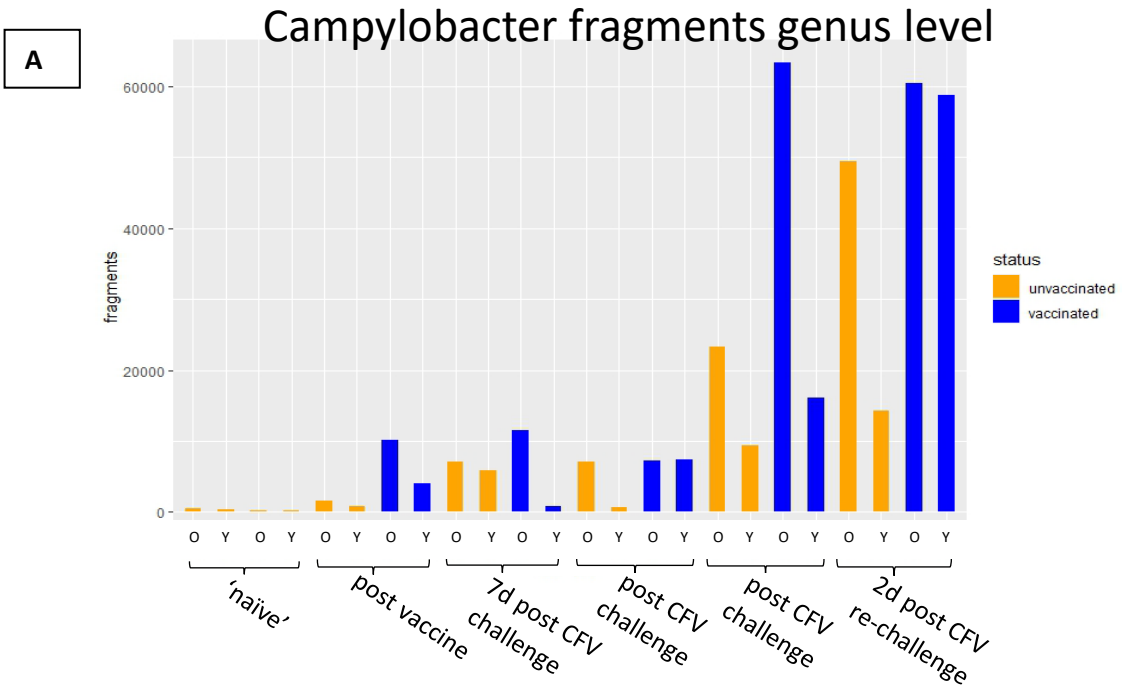
Figure 33. Comparison of the relative abundances of Genus *Campylobacter* between (A) preputial samples collected from Composite breed bulls in 2018 and 2021 (NG036); (B) Vaginal samples collected from Composite breed cows (NG036) which were mature but not pregnant (Cycling), pregnant and postpartum during sampling and (C) Vaginal samples collected from Droughtmaster breed cows (NG037) which were prepubertal, mature but not pregnant (Cycling), pregnant and postpartum during sampling. *Campylobacter fetus* highlighted in rectangles.

For NG034 analysed using 16S amplicons (not deep metagenomic sequencing applied to NG036 and NG037 herds above), the most abundant *Campylobacter* species was an unclassified group (mean abundance of 0.0329%) followed by *C. corcagiensis* (0.00136%). *Campylobacter fetus* was present with a mean abundance of 0.000196% found in only 2 out of the 20 female cattle. Reads are assigned to this species, but it is among the rare species and therefore it was not listed in the barplot above (Figure 32) and would not appear in a barplot for the top 10 species with a mean abundance of at least 0.0114% (Table 17). Alternatively, Table 15 above demonstrates long read detection of *C. fetus venerealis* in one of NG034 cattle which may have been also detected if metagenomics was applied instead of the 16S microbiome here. Section 4.5.1 demonstrated a much deeper analysis of the metagenomes in regard to *Campylobacter* species within Herd NG037 showing presence of *C. fetus* specifically. Notably NG037 and NG034 may have had BGC outbreaks unlike NG036 which had good pregnancy rates. This suggests that low levels of potential pathogens in metagenomic/microbiome studies may be significant.

4.6 Determined whether BGC immune biomarkers (post infection or vaccination) can be identified for assay development

4.6.1. Metagenomic biomarkers

Metagenomic analyses of pooled samples from the bull study was undertaken to identify biomarkers associated with vaccination and infection. When investigating *Campylobacter* species fragments there is a marked rise after *C. fetus venerealis* challenge with the highest level 1 day post re-challenge (Figure 34A). The analysis at *C. fetus* species level is most informative for comparing vaccinated and unvaccinated bulls (Figure 34B). Interestingly, the vaccine does not have the expected effect, with vaccinated animals consistently having higher levels of *C. fetus* across all samples, a trend replicated in the genus level analysis. Young animals have markedly less *C. fetus* fragments than old animals, indicating faster clearing of infection. This indicates that old bulls are at risk of *C. fetus venerealis* transmission whether vaccinated or not. Note the samples used here were pools and thus it is uncertain if different groups had outliers that may be influencing these outputs. There was not enough remaining DNA from the samples to undertake average analyses from individual samples.



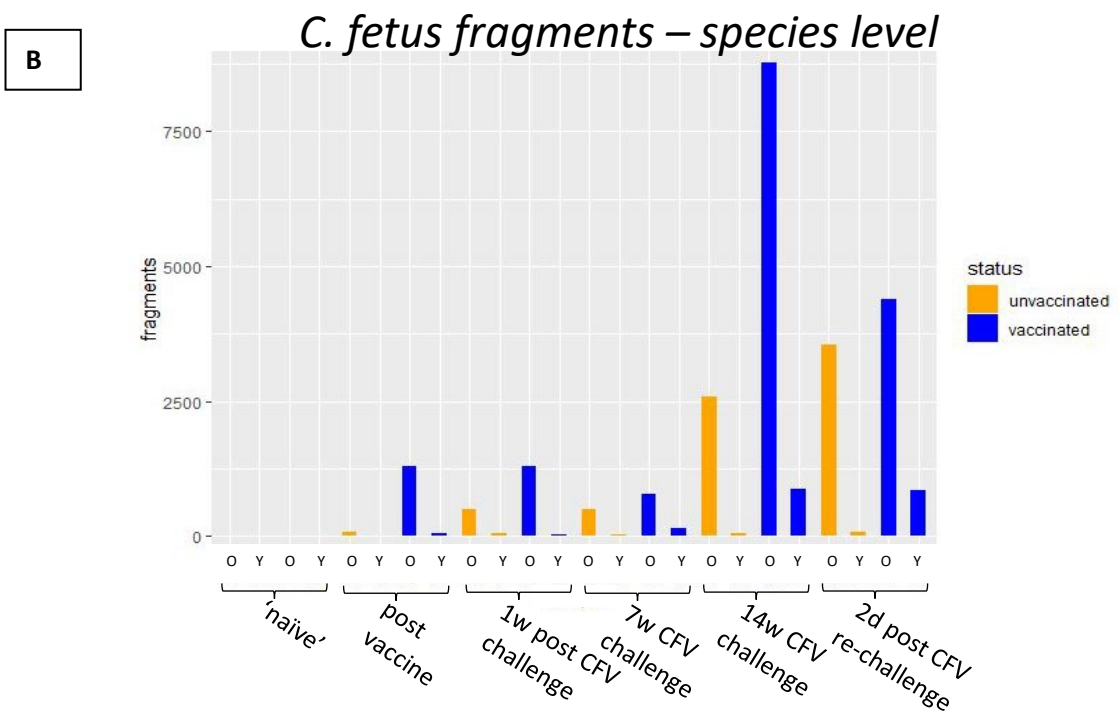


Figure 34. Comparison of *Campylobacter* fragments in vaccinated (blue) and unvaccinated (orange) bulls at each challenge point from the bull study, assigned to the **A**. genus (Campylobacter) level, **B**. species level (*C. fetus*). Y=young, O=old

There is no clear difference in the genera present between vaccinated and unvaccinated bulls, indicating that vaccination does not affect the microbiome (Figure 35). However, there is a shift in the genera composition following live challenge in all bulls. Of note is the reduction in *Pseudomonas* in post challenge groups and the increase in *Histophilus*. Note that samples at 14 weeks post challenge 1 and 2 days post re-challenge had higher proportions of unclassified sequences, resulting in the low totals in these groups (Figure 35).

The metagenome data was also presented as phyla (Figure 36) to compare the data to the bull microbiome data (Figure 8). *Proteobacteria* were the most abundant, followed by *Actinobacteria*, and similar levels of *Fusobacteria*, *Firmicutes* (both detected in the microbiome) and *Bacteriodetes* (not detected in the microbiome Figure 8). There was a spike of *Cyanobacteria* in sample 4 of the naïve cattle not detected in the microbiome data.

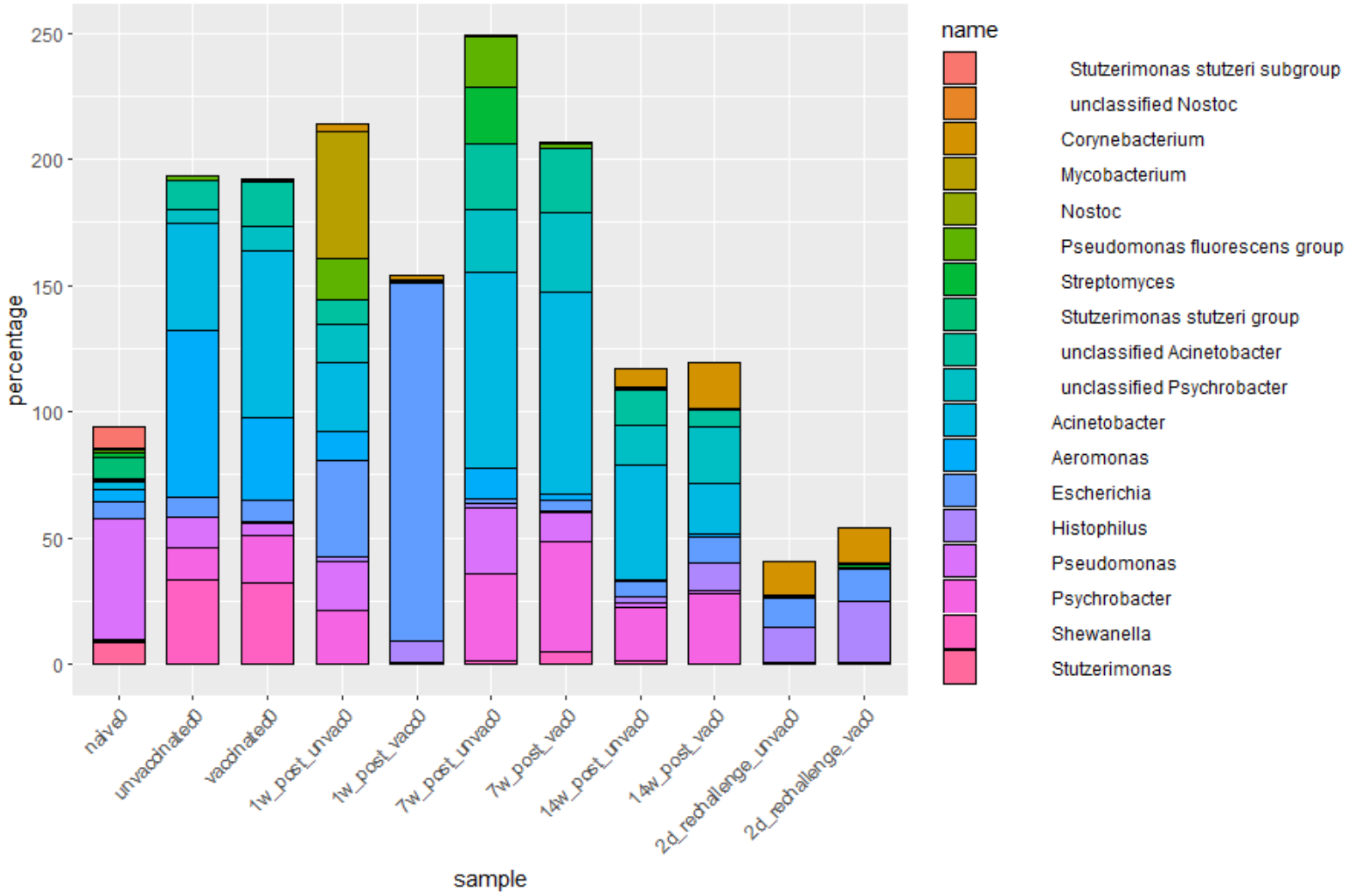


Figure 35. Genera present in each pooled sample set from the bull study with more than 5% of fragments in at least one sample. Legend: Unvaccinated and vaccinated abbreviated to 'unvac' and 'vac' respectively; 1w_post= 1 week post challenge; 7w_post= 7 weeks post challenge; 14w_post=14 weeks post challenge; 2d_rechallenge=2 days after re-challenge.

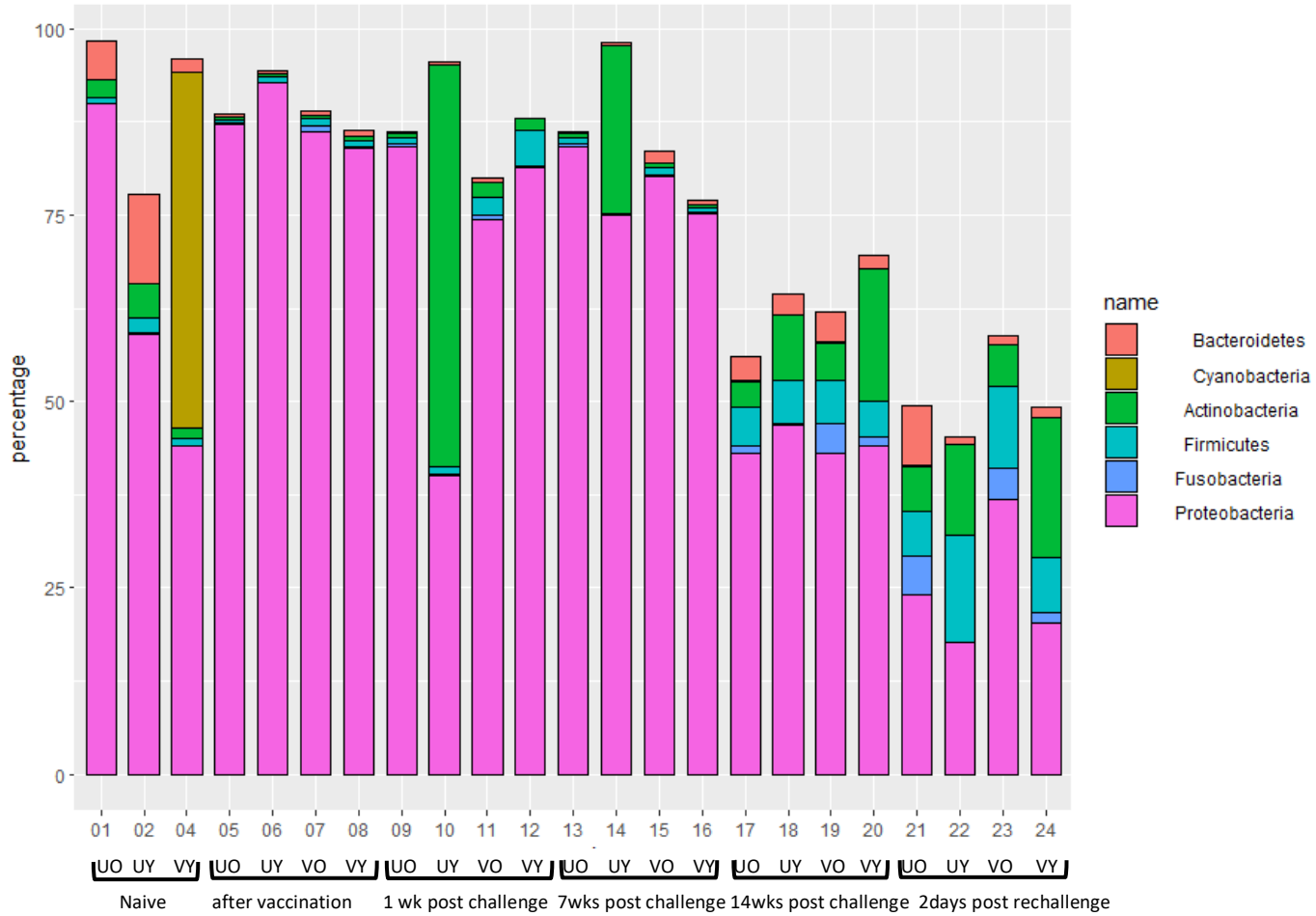


Figure 36. Phyla present in the metagenome data from the bull study to enable comparison to microbiome data from the same trial (Figure 8). Legend: U=unvaccinated; V=vaccinated; Y=young <3; O=old ≥3

Figure 37 shows a matrix of the genera found in the bull metagenomic data using Manhattan distance showing clustering mainly at each time point. The 'naïve' group forms a distinct clade in the matrix, separate to all vaccinated and post-challenge groups. This is marked by the higher levels of *Stutzerimonas* and *Pseudomonas* in these naïve groups compared to other timepoints. Interestingly, the groups from 7 days post *C. fetus venerealis* challenge and 2 days post re-challenge cluster in two closely related clades. Again, clustering by vaccination status is not observed and live challenge influences the microbiome as described above (Figure 35). Group 09 (unvaccinated, old) and Group 14 (unvaccinated, young) are outliers to the clustering patterns observed (Figure 37).

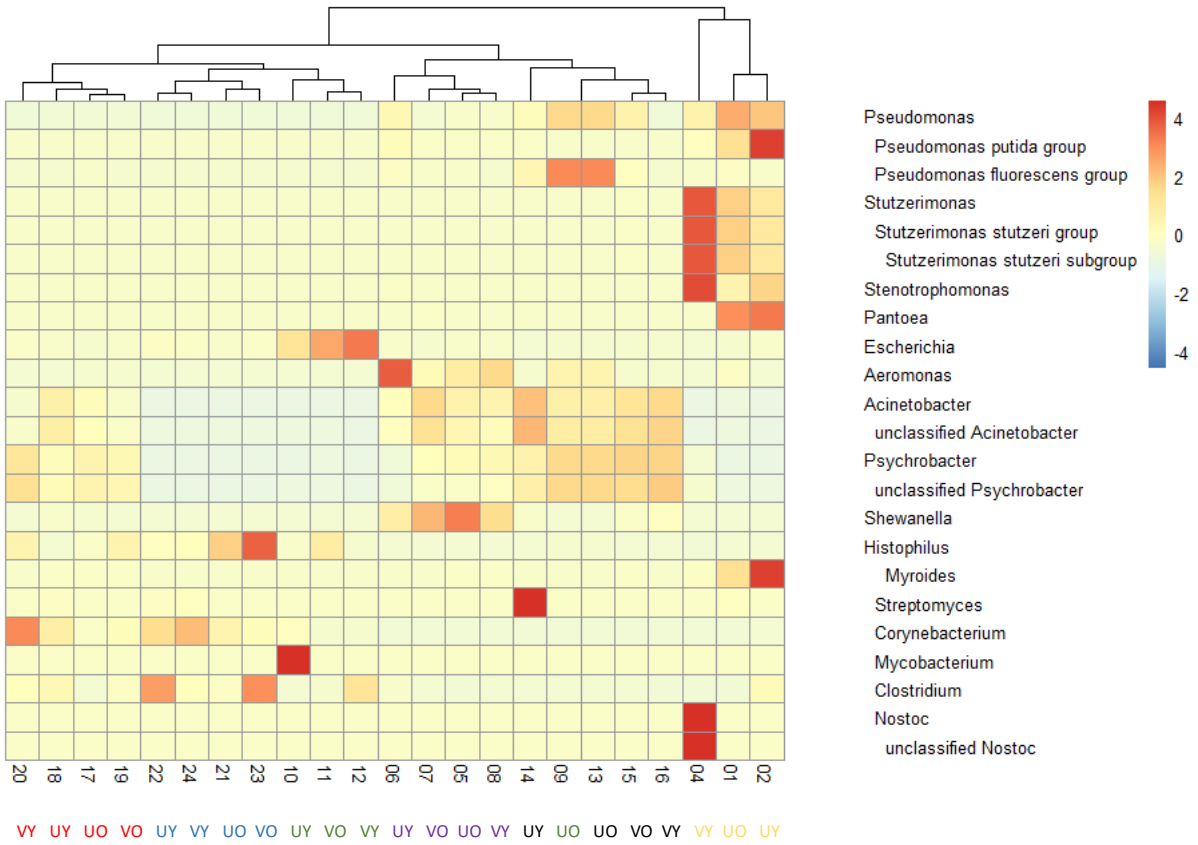


Figure 37. Matrix of genera clustered by group using Manhattan distance in bulls. Legend: U=unvaccinated, V=vaccinated, Y=young <3, O=old ≥3. Colour code: 14 weeks post challenge (17-20); 2nd day after re-challenge 21-24); 1 week after challenge (9-12); after vaccination (5-8); 7 weeks after challenge (13-16); naïve (01,02,04)

4.6.2 Proteomic biomarkers

SWATH-MS was used to measure the serum proteome in vaccinated vs un-vaccinated bulls before and after challenge with *C. fetus venerealis*. The designated time points for assessing immune biomarkers in bulls are listed in Table 18.

Table 18. Comparative time points used to measure serum proteomes of vaccinated and un-vaccinated bulls.

Time points	Day	Vaccinated	Unvaccinated
Pre-vaccination	Day-0	V0 (n=10)	NV0 (n=10)
Post-vaccination	Day-49	V1 (n=10)	NV1 (n=10)
Post-challenge	Day-63	V2 (n=10)	NV2 (n=10)
Post-recovery	Day-145	V3 (n=10)	NV3 (n=10)

In SWATH-MS analysis, numbers of significantly differentially abundant proteins (DAPs) (p-value <0.05; log2Fold Change >0.2) were comparatively higher in the post-vaccination (V1vsNV1 = 233) compared to pre-vaccination (V0vsNV0 = 216) and post-challenge (V2vsNV2 = 204) comparisons. The lowest number of DAPs was found at post-recovery (V3vsNV3 = 178) (Figure 38). The number of proteins with increased abundance in vaccinated bulls was greater in the post-vaccination (n=102) followed by post-challenge (n=97) and pre-vaccination (n=94) compared to post-recovery (n=69) (Figure 38).

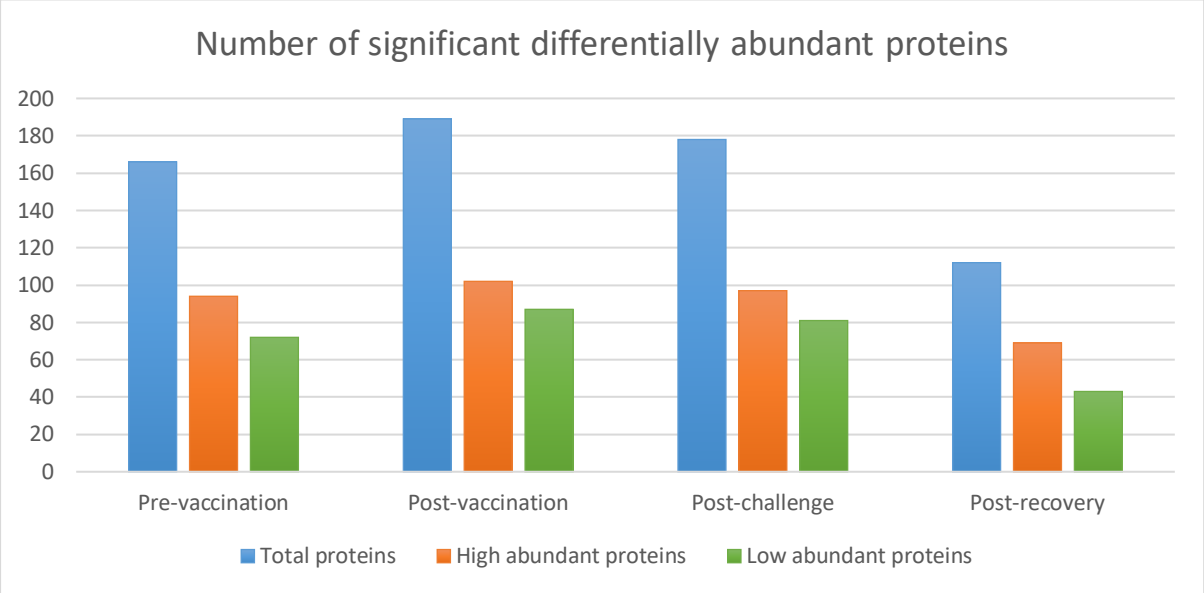


Figure 38. Bar graph presenting the total number of significantly differentially abundant proteins (DAPs) in pre-vaccination (V0vsNV0), post-vaccination (V1vsNV1), post-challenge (V2vsNV2) and post-recovery (V3vsNV3) time points between two groups of bulls with p-value $<10^{-5}$ and log2FC > 0.2. Orange colour bars demonstrate higher abundant proteins found in vaccinated bulls at different time points.

Table 19 outlines the top five most abundant proteins in vaccinated (proteins with positive logFC value) and unvaccinated (proteins with negative logFC value) bulls at the four distinct timepoints. The abundance of **vitamin D binding protein** (DBP) was significantly higher in the vaccinated group after vaccination (log2FC = 2.27; adj.p-value = $1E^{-19}$), indicating high abundance. In the unvaccinated group, abundance of DBP was increased after challenge (log2FC = -2.62; adj.p-value = $1E^{-19}$) and after recovery (log2FC = -1.98; adj.p-value = $1E^{-19}$) (Table 19, Figure 39). **Haptoglobin** (HP) another important protein showed lower abundance in vaccinated group at post-challenge (log2FC = -0.33; *p-value* = $9.3E^{-09}$) and post-recovery (log2FC = -2.99; *p-value* = $1E^{-15}$) timepoints while it was absent in pre-vaccination and post-vaccination group (Figure 39). Vitamin D binding protein (DBP), also known as Group-specific Component (GC) protein, serves as the primary carrier for vitamin D and its metabolites (Daiger et al., 1975). Its vital role involves the activation of macrophages, contributing to immune responses, and enhancing the chemotactic functions of C5a (Speeckaert et al., 2006), which later enhances complement activation. Notably, the abundance of DBP (GC) protein was exhibited as a highly abundant protein in post-vaccination group (log2FC = 2.27). This elevation may be attributed to the influence of the vaccine antigen (Vibrovax®). Conversely, in the post-challenge and post-recovery stages, DBP's abundance also increased. In the context of human studies, DBP has emerged as a potential target for researchers due to its considerable impact on reproductive health and pregnancy outcomes, including conditions like endometriosis (Fernando et al., 2020). Cho et al (2012) identified DBP as a significant urinary biomarker for endometriosis. Beyond this, Vitamin D binding protein is associated with various human diseases, such as cancer, tuberculosis, coronary artery diseases (as reviewed by Rozmus et al., 2020), diabetes (Jorde, 2019), and kidney diseases (Denburg and Bhan, 2015).

Table 19. 5 top differentially abundant proteins in vaccinated (green) and unvaccinated group (red). With their log2fold change and abjusted p-values.

Pre-vaccination

Protein (V0 (+)vsNV0(-)	Protein names	Gene Names	log2FC	adj.pvalue
G3N3D3	Chromosome 3 C1orf68 homolog	C3H1orf68	3.2	1.10E-07
E1BLU4	Caspase 8 associated protein 2	CASP8AP2	2.0	5.88E-12
E1BI56	Triggering receptor expressed on myeloid cells like 1	TREML1	1.8	1.00E-17
A0A3Q1LFA6	Ig-like domain-containing protein	Ig_A0A3Q1LFA6	1.7	1.00E-19
P13753	BOLA class I histocompatibility antigen	P13753	1.7	1.00E-16
A0A3Q1LL87	Ig-like domain-containing protein	Ig_A0A3Q1LL87	-2.2	1.00E-19
A0A3Q1NCQ6	Cyclin B3	CCNB3	-2.5	3.85E-13
F1MG35	Class II major histocompatibility complex transactivator	CIITA	-2.9	3.06E-05
A0A6B9SCR1	Ig heavy chain variable region	Ig_A0A6B9SCR1	-4.0	2.41E-13
A5PK52	MUC12 protein	MUC12	-7.1	8.97E-15

Post-vaccination

Protein (V1 (+)vsNV1(-)	Protein names	Gene Names	log2FC	adj.pvalue
A0A173FD75	MHC class I antigen	BoLA	4.32	2.14E-06
P13753	BOLA class I histocompatibility antigen	P13753	3.46	1.00E-15
A0A3Q1LRN8	RAN binding protein 17	RANBP17	2.90	3.80E-05
A0A3Q1MS75	Ig-like domain-containing protein	Ig_A0A3Q1MS75	2.55	1.00E-18
A0A0K1L4Y9	MHC class I antigen 6*04001	A0A0K1L4Y9_MHC	2.40	7.58E-14
A0A3Q1LQ02	Vitamin D-binding protein (Gc-globulin)	GC	2.27	1.00E-19
P05786	Keratin, type II cytoskeletal 8 (Cytokeratin-8) (CK-8) (C)	KRT8	-1.95	8.85E-11
G3N172	Cyclin K	CCNK	-2.00	3.08E-12
A0A3Q1LN98	Cartilage acidic protein 1	CRTAC1	-2.08	3.47E-07
M0QW09	Cytochrome P450 2J2	LOC107132327	-2.13	1.07E-15
P62935	Peptidyl-prolyl cis-trans isomerase A (PPIase A) (EC 5.2	PPIA	-2.20	3.96E-06

Post-challenge

Protein (V2 (+)vsNV2(-)	Protein names	Gene Names	log2FC	adj.pvalue
G3X696	non-specific serine/threonine protein kinase (EC 2.7.11.1	SLK	9.58	1.39E-11
A0A3Q1N785	Golgi associated RAB2 interactor 5A	GARIN5A	3.28	1.00E-18
K7QF89	MHC class I antigen	BoLA	2.42	1.78E-09
A6QNM8	Threonine--tRNA ligase 2, cytoplasmic (EC 6.1.1.3) (Thr	TARS3 TARSL2	2.38	8.81E-06
P13753	BOLA class I histocompatibility antigen, alpha chain BL3-7		1.78	1.08E-13
F1MEW8	Protein Wnt	WNT10A	-1.27	6.79E-10
E1BE11	Hemicentin 1	HMCN1	-1.41	1.00E-16
Q2KI62	Pentatricopeptide repeat domain-containing protein 3, mit	PTCD3 MRPS39	-1.43	1.00E-15
A0A3Q1LFA6	Ig-like domain-containing protein	Ig_A0A3Q1LFA6	-2.14	1.00E-19
A0A3Q1LQ02	Vitamin D-binding protein (Gc-globulin) (Group-specific	GC	-2.62	1.00E-19

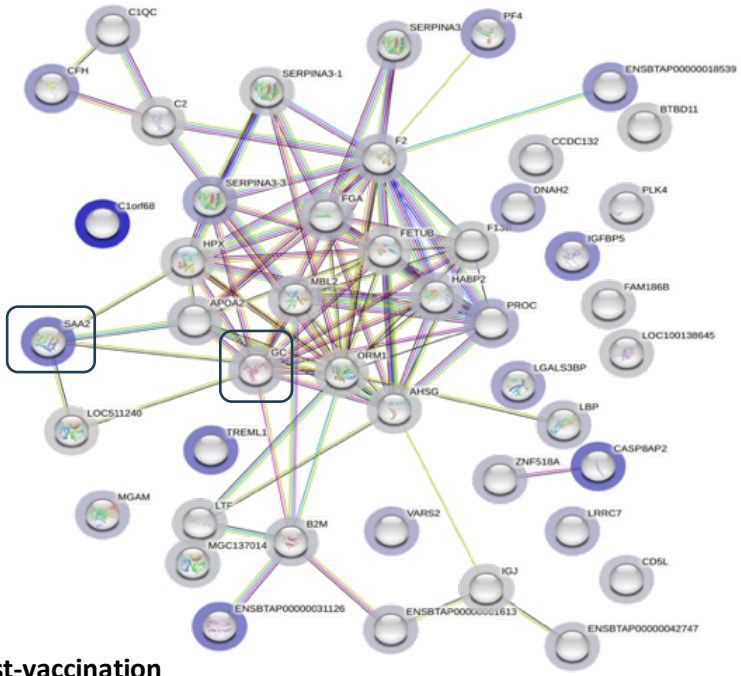
Post-recovery

Protein (V3 (+)vsNV3(-)	Protein names	Gene Names	log2FC	adj.pvalue
E1BE11	Hemicentin 1	HMCN1	4.14	2.05E-08
A6QNM8	Threonine--tRNA ligase 2, cytoplasmic (EC 6.1.1.3) (Thr	TARS3 TARSL2	3.63	1.34E-07
A0A0K1L4Y9	MHC class I antigen 6*04001	A0A0K1L4Y9	2.12	1.00E-19
F6R1G6	tRNA:m(4)X modification enzyme TRM13 (EC 2.1.1.22	TRMT13	1.51	1.05E-05
F1MAV4	BRCA1 associated RING domain 1	BARD1	1.50	1.00E-17
A0A6B9SCR1	Ig heavy chain variable region	Ig_A0A6B9SCR1	-1.57	1.12E-08
A0A6B9SE63	Ig lamda chain variable region	Ig_A0A6B9SE63	-1.79	1.00E-18
A2VDZ4	Serine/threonine-protein kinase PLK4 (EC 2.7.11.21) (Pc	PLK4 SAK	-1.84	1.00E-18
A0A3Q1LQ02	Vitamin D-binding protein (Gc-globulin) (Group-specific	GC	-1.98	1.00E-19
Q2TBU0	Haptoglobin [Cleaved into: Haptoglobin alpha chain; Ha	HP	-2.99	1.00E-15

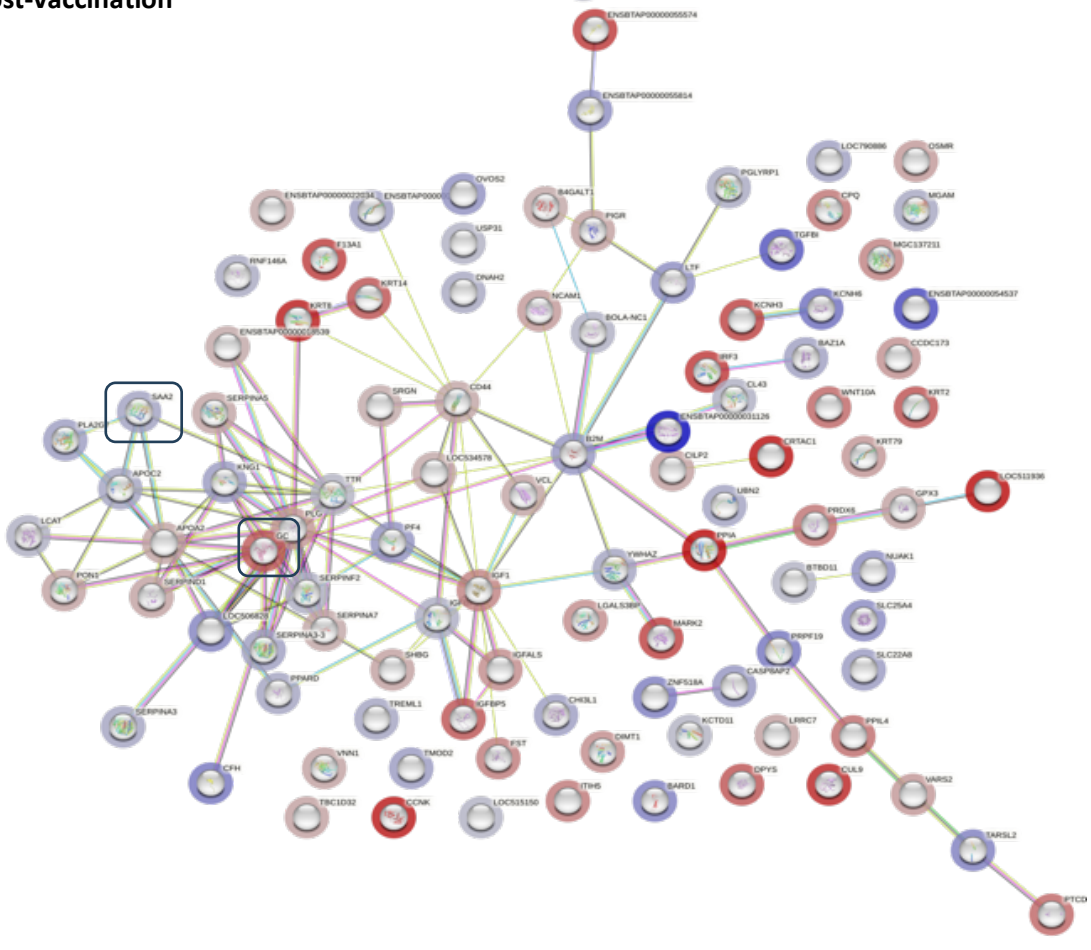
Haptoglobin (HP) and **serum amyloid A 4 (SAA4)** are crucial acute-phase response proteins and are important indicators of reproductive health and inflammation (Bazzano et al., 2022; Sack 2018). These proteins showed higher abundance in the unvaccinated group post-challenge phase and post-recovery stage (Figure 39). Researchers have identified HP and SAA proteins as significant diagnostic and prognostic biomarkers for endometritis (Kaya et al., 2016; Pereira et al., 2020; Zhang et al., 2018), as well as subclinical endometritis (Bogado Pascottini and LeBlanc, 2020) in cows. These findings highlight the importance of these proteins in understanding and diagnosing reproductive health issues in cattle. Increased levels of HP and SAA are associated with the severity of endometritis in cow (Pereira et al., 2020, Kaya et al., 2016). Menta et al (2023) found higher

concentration of HP in bovine metritis. Note that SAA2 showed a lower abundance post challenge – the functional differences between these two SAA types in bovines is currently unknown.

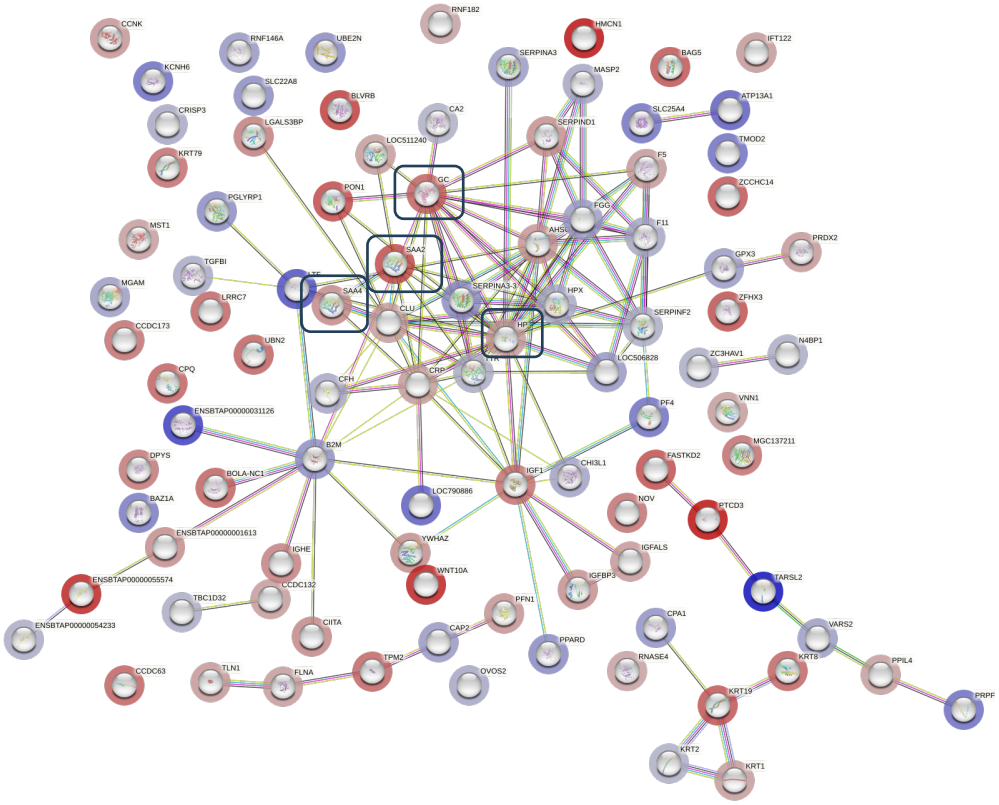
A. Pre-vaccination



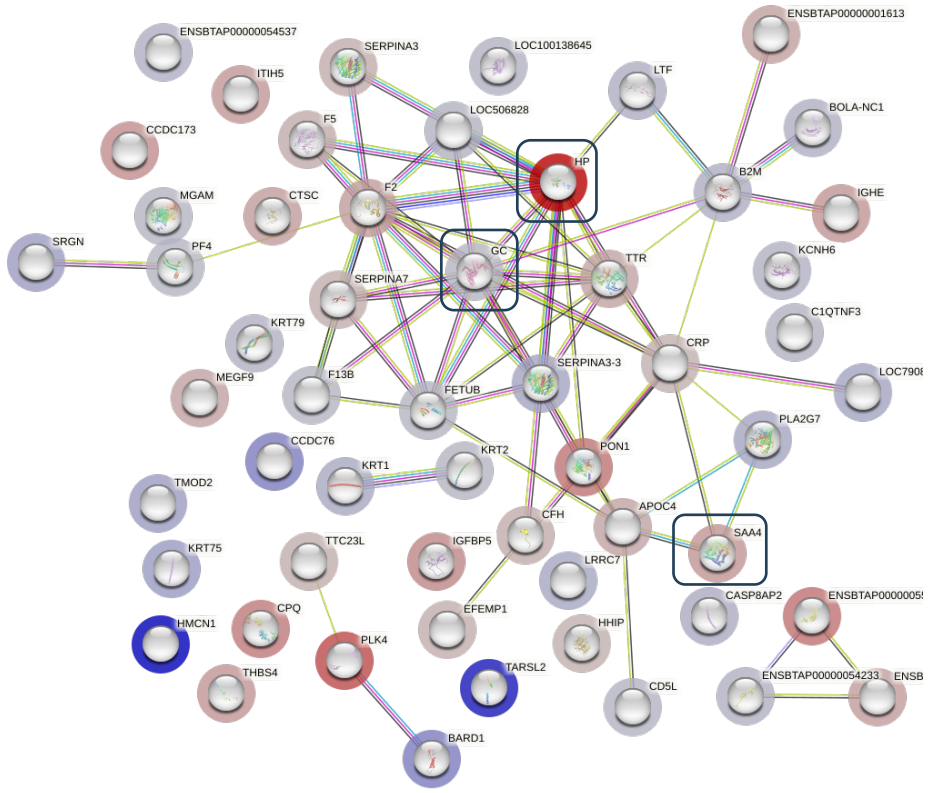
B. Post-vaccination



C. Post-challenge



D. Post-recovery



Halo colour: based on log2FC

Figure 39. String analysis representing the Protein-protein interaction

between differentially abundant significant proteins. Higher abundant proteins with positive log2FC presented in blue colour and lower abundant proteins with negative log2FC in red colour. The intensity of colour increased with increased log2FC value. A. Pre-vaccination, B. Post-vaccination, B. Post-challenge, D. Post-recovery. Vitamin D binding protein (GC), Haptoglobin (HP) and Serum Amyloid A proteins 2 and 4 (SAA2, SAA4) are highlighted by rectangle boxes.

The most significant proteins from Table 19 and Figure 39 are also presented in Figure 40.

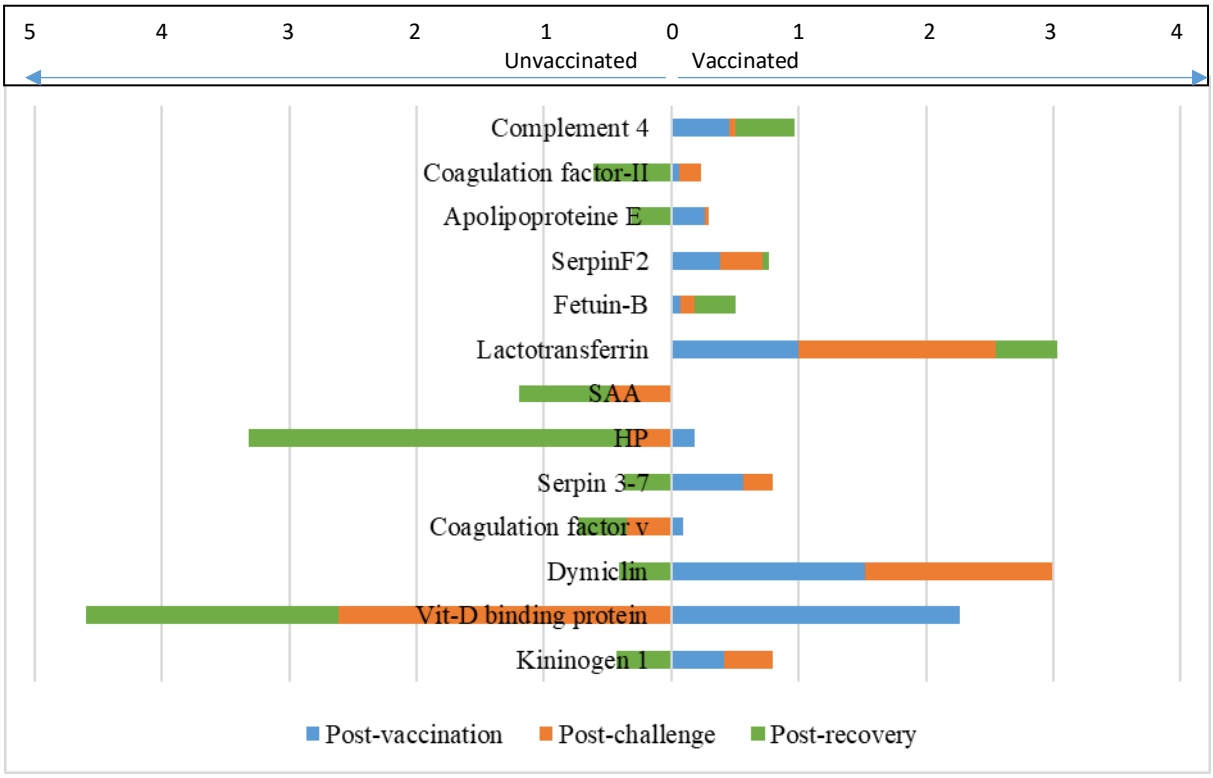


Figure 40. The pattern of abundance for some of the important proteins in vaccinated (to the right) and unvaccinated (to the left) group pre- and post-challenge summarised from Figure 39 and Table 19.

The **enrichment analysis** (statistical test to identify significant differences in the frequency of Gene Ontology (GO) terms associated with modified proteins relative to their frequency in the genome) of significant proteins (p-value<10-5; lof2FC>0.2) involved with pre-vaccination (V0vsNV0), post-vaccination (V1vsNV1), post-challenge (V2vsNV2), and post recovery (V3vsNV3) comparisons are presented in Figure 41. To simplify the presentation of the results, we chose the top 11 pathways involved with biological functions. In the string analysis, we found HP, SAA, inter alpha-trypsin inhibitor (ITIH4) and SERPINF2 proteins were involved with acute phase responses (GO:0006953) and acute inflammatory responses (GO:0002526) and mostly present in the unvaccinated group except for SERPINF2. HP and SAA are the acute phase response proteins mainly produced in response to inflammation or tissue damage and also act as pro-inflammatory mediators. Inter Alpha-Trypsin Inhibitor Heavy Chain (ITIH4) modulates inflammatory responses by regulating protease activity during inflammation. SERINF2 is a serine protease inhibitor involved in inhibiting the activity of plasmin, which is crucial for fibrinolysis. Results suggest that these proteins were associated with the initial immune response to infection after challenge.

Proteins found in vaccinated groups were involved with the negative regulation of proteolysis (GO:0030162), endopeptidase activity (GO:0010951), protein metabolic process (GO:0051248) and

hydrolase activity (GO:0051336) (Complement 3, Serpin A3-3, Lactotransferrin, Fetuin B). Complement 3 or C3 is a component of complement system that enhances phagocytosis and is involved with the negative regulation of proteolysis and endopeptidase activity. Lactotransferrin is an iron-binding glycoprotein with various functions including antimicrobial activity. By limiting the availability of iron, lactotransferrin acts as an antimicrobial agent, hindering the ability of pathogens to thrive. Fetuin B is a glycoprotein associated with immune response modulation. While its specific role in immunity is not as well-defined as some other proteins, it could potentially regulate immune reactions by influencing various signalling pathways involved in immune cell activation and function.

Humoral immune responses (GO:0006959) involve the production of antibodies by B cells and their release into the bloodstream. This response was only present in the post-vaccination group, suggesting that it's a specific immune reaction that occurred after vaccination.

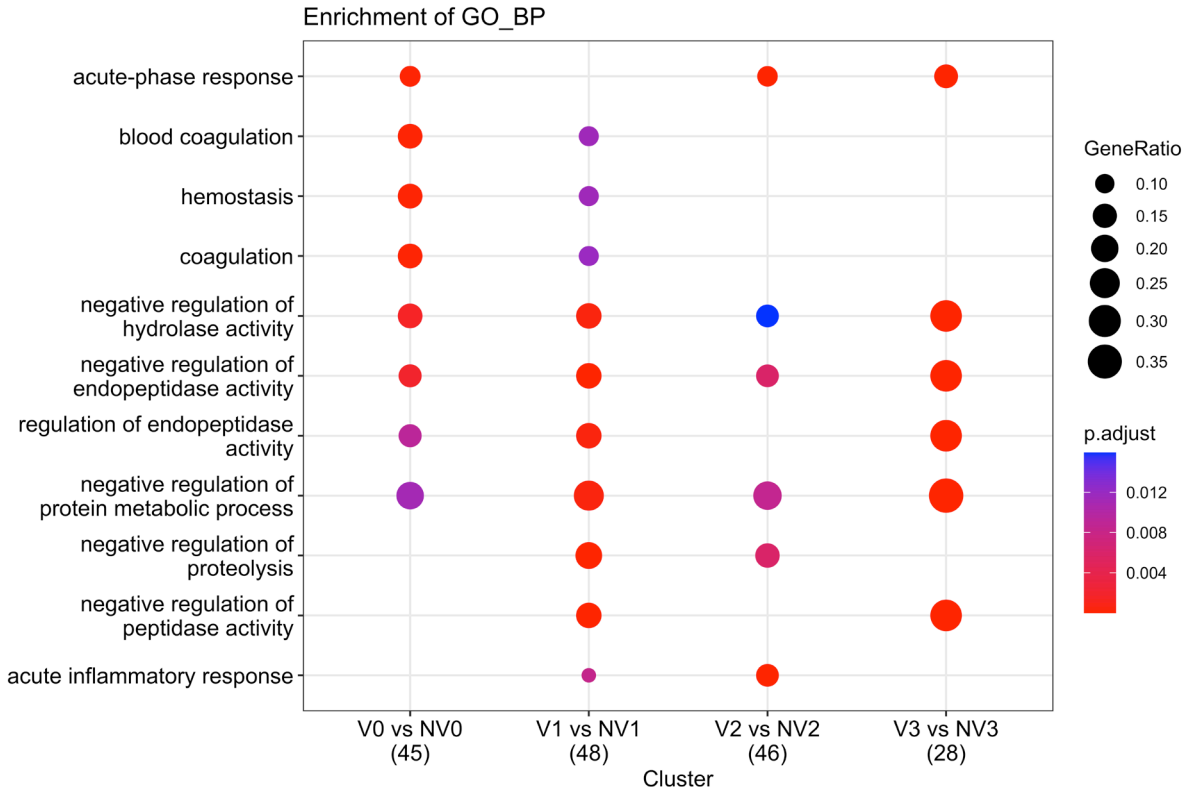


Figure 41. Enrichment analysis of gene ontology (GO) analysis. Size of the circle indicates the number of genes involved with the biological process and colour indicates the level of significance. (P=value<10^{-5}). V=vaccinated, NV=not vaccinated, 0=naïve, 1=post vaccination, 2=post challenge, 3=14 weeks post challenge (recovery phase)

4.7 Piloted the development of genomic markers that can be used in breeding programs to select for resistant genotypes.

As described in section 4.3 to few *C. fetus venerealis* positive cattle were found to undertake genotyping towards developing GEBVs for BGC resistance in heifers. We thus investigated the relationship between metagenome and genotype which suggests that the host genome may play some role in influencing the microbial genome. This data was established using 28 cattle from two herds.

We attempted to genotype 67 cattle from the Roma herd however many of the empty cows and qPCR positive bulls had been culled and were no longer available for genotyping. Essentially not enough samples with both genomic and metagenomic data could be collated to demonstrate this potential genomic influence.

This project has had challenges in identifying adequate *C. fetus venerealis* positive cattle to enable any correlations with host genomics. We thus had far too many healthy herds (9 unvaccinated herds) and only a minority of unhealthy herds (only 2 unvaccinated herds with qPCR positives) which were insufficient to draw conclusions at the genomic marker scale.

5. Conclusion

This project was able to develop a new specific molecular diagnostic method (mraY) for BGC and was subsequently screened in controlled experimental herds as well as field samples. However, long read genomic sequencing methods through metagenomics or targeted direct sequencing were found to be much more sensitive than qPCR for *C. fetus venerealis* detection. Through this research, it was found that qPCR positivity for BGC is transient thus may yield false negatives. Genomic sequencing is comparatively expensive however, barcoding, and multiplexing methods may assist to make this a cost-effective screening method which could be accessible to the red meat industry for BGC screening. This finding has highlighted the need for subsequent research to develop methods whereby samples could be screened for BGC (*C. fetus venerealis*), trichomoniasis (*Tritrichomonas foetus*) (and potentially *Histophilus somni*) and bovine genotypes using long read sequencing tools rather than three different laboratories to achieve the same.

We also identified 2-3 proteins which could be exploited as ELISA tests (commercially available kits) as biomarkers for BGC. This is a simpler alternative to pathogen detection by genome sequencing. However, alternative rapid antibody tests could be developed as kits for yard side screening rather than submission to diagnostic laboratories.

5.1 Key findings

- The reproductive microbiome is a product of the environment and future studies to identify unhealthy microbiomes can only be undertaken at the herd level.
- Bacterial genomics guided the identification of gene (mraY) which was exploited a SNP to develop a *C. fetus venerealis* specific assay.
- The mraY qPCR assay had only transient positivity in clinical samples (controlled bull and heifer vaccination and challenge trials)
- Controlled bull and heifer VibroVax® vaccination and challenge trials demonstrated that *C. fetus venerealis* is still detected in vaccinated and unvaccinated animals after challenge.

- Long read sequencing methods are much more sensitive and specific in detecting *C. fetus venerealis* in clinical samples compared to qPCR when the bacterial load is low
- *Histophilus somni* bacteria co-infect with *C. fetus venerealis* as confirmed by metagenomic studies and also during *C. fetus venerealis* artificial challenge- it is not known if this pathogen is contributing to infertility
- Metagenomics is a useful tool to identify sporadic/opportunistic pathogens associated with abortion
- Vitamin D binding protein, Haptoglobin and serum amyloid A were identified as biomarkers for *C. fetus venerealis* infections (all 3 biomarkers) and BGC vaccination (DBP only).

5.2 Benefits to industry

Currently there is only a herd IgA ELISA diagnostic test for vibriosis/BGC offered by Veterinary diagnostic laboratories in Australia, which retains long term positivity following initial exposure to the disease. This test cannot be used to confirm the present of the causative pathogen *C. fetus venerealis*.

We have developed tools which can provide advice for red meat producers in terms of:

- *C. fetus venerealis* detection
- BGC biomarker detection

If an entity could be identified to provide genomic sequencing screening as a service, the tools developed here will ultimately enable red meat producers to manage vibriosis/BGC and other reproductive diseases.

Our research also demonstrates and confirms that to manage Vibriosis/BGC red meat producers should:

1. Effectively apply (annual boosting) VibroVax® in bulls and the maiden heifers
2. Only mate *C. fetus venerealis* negative bulls

6. Future research and recommendations

During this research program which commenced in 2018, the development of rapid antigen tests as a standard COVID19 test was established. We had not planned to develop tools like this; however, the technology is now available, and our biomarkers could be delivered as crush antibody tests for vibriosis/BGC if production of these tests is cheap to produce.

Through our thorough investigations including controlled experiments in bulls and heifers, as well as field sampling, we found that qPCR for vibriosis/BGC is far too transient and can yield false negative results. Advances in genomic sequencing are such that we could deliver a methodology which detects both BGC and trichomoniasis in mucus samples as well as cattle genotyping records if required. The detection *Histophilus somni* would also be added to this sequencing screening simultaneously. QAAFI researchers need to partner with a commercial entity who may be able to develop these technologies into a service which will benefit red meat producers in Australia.

For immediate adoption, the qPCR assay could be used however biomarker ELISA assays require further evaluations. In the long term, a service provider who can apply novel long read sequencing diagnostic data would be ideal for the industry.

Ultimately, the effective use of VibroVax® including annual boosters negates the need for these tests.

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8. Appendix

8.1. Appendix 8.1. Database of samples collected for 'pathobiome' analysis

8.2. Appendix 8.2. VibroVax® un-vaccinated herds, diagnostic samples and Campylobacter fetus venerealis qPCR data

8.3. Appendix 8.3. Ong et al 2023 Publication draft

8.4. Appendix 8.4. Ong et al 2021

8.5. Appendix 8.5. Ong et al 2022a

8.6. Appendix 8.6. Ong et al 2022b

8.7. Appendix 8.7. Ong et al 2022c

8.8. Appendix 8.8 Supplementary files from Appendix 7 Ong et al 2022c

Supplementary 8.8.1. Breed makeup and *Bos indicus* content (%) of the herds Station A and Station B

Supplementary 8.8.2. Thirty-seven samples collected from Station A and Station B

Supplementary 8.8.3. Sequence data generated for each sample using Oxford Nanopore Technologies long-read adaptive sequencing

Supplementary 8.8.4. The proportions of sequence data belong to cattle host and metagenome

Supplementary 8.8.5. Sequence data after quality filtering

Supplementary 8.8.6. Multidimensional scaling analysis of the dissimilarity based on the leading log2 fold change of the functional annotations of the reproductive metagenomes between (A) female from Station A and Station B and between (B) male and female from Station A.

8.9 Appendix 8.9. Raw qPCR data from the heifer trial

8.10 Appendix 8.10. Proteomics data from field evaluation for biomarker detection