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In vitro investigation of bovine monocyte-derived macrophages with previously calculated immune response EBVs towards Mycobacterium avium subsp. paratuberculosis

Edwards, Emily Maria

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In vitro investigation of bovine monocyte-derived macrophages with previously calculated immune response EBVs towards *Mycobacterium avium subsp. paratuberculosis*

Emily Maria Edwards

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Ethical Approval Certificate

The details provided in the ‘Ethics Approval Form’ by Emily Edwards was approved by the Research Committee before commencement of the research. The document is attached to this thesis.

This is to certify that the research undertaken and completed by the candidate and reported in this thesis has satisfied the requirements of the University of Coventry and Royal Agricultural University's Ethical Principles and Procedures for Teaching and Research and the Code on Good Research Practice.

Professor Meriel Moore-Colyer
Director of Research
Royal Agricultural University

Abstract

Johne's disease is of high economic and welfare importance with a cost of £13 million to the UK cattle industry per year (Bond and Guitian, 2015). In addition, it is of zoonotic importance with studies suggesting a causal effect between Johne's disease and Crohn's disease in humans (Botsaris *et al*, 2016). Johne's disease is caused by the *Mycobacterium avium subsp. paratuberculosis* (MAP) pathogen which results in a chronic enteric infection (Rue-Albrecht *et al*, 2014). There have been several management strategies applied to the control of Johne's disease including biosecurity measures (NADIS, 2016), herd management (SAC, 2003), vaccination (NADIS, 2016 b) and targeted breeding (Minozzi *et al*, 2012). This study investigates the use of the High Immune Response (HIR) technology (Mallard *et al*, 2011) to identify cattle which have estimated breeding values (EBVs) capable of promoting resistance to MAP pathogens. The researcher completed this research study *in vitro*, macrophages were isolated from the blood of cattle with HIR tested EBV types and measured for immune response indicators (nitric oxide (NO) release and pH) (Owen, Punt and Stanford, 2013). The results of the laboratory tests were analysed using GenStat 18th Edition. Using a confidence level of 0.05 it was found that there were no statistically significant differences between the immune EBV types for NO release, however as predicted the high cell mediated immune response (CMIR) and high antibody mediated immune response (AMIR) (HH) macrophages produced more NO than the low (CMIR) and low (AMIR) (LL) macrophages. The contrast level of pH release between HH and LL macrophages was 0.061 and although not statistically significant the results did show that there was a difference between the EBV groups. The difference between high AMIR, low CMIR (HL) and low AMIR, high CMIR (LH) cattle EBVs, was found to be statistically significant in relation to pH with cattle that had a high response for cell mediated immune response reducing pH to a lower level and therefore exhibiting a mechanism to destroy MAP pathogens *in vitro* more successfully than the other EBVs. The difference between HH and LL macrophage pH release was not found to be statistically significant. It is suggested that this study should be repeated with a larger cohort of cattle over a longer period as this study was limited by small group size.

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List of Abbreviations

ABRI	Agricultural Business Research Institute
CHAWG	Cattle Health and Welfare Group
µg	Microgram
µl	Microliter
AHDB	Agriculture and Horticulture Development Board
AIM-V	Lymphocyte-activation Medium
AMI	Antibody Mediated Immunity
CARD 15	Caspase Recruitment Domain-containing Protein 15
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CDN	Canadian Dairy Network
CIWF	Compassion in World Farming
CMI	Cell Mediated Immunity
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate-Buffered Saline
EBV	Estimated Breeding Value
EDTA	Ethylenediaminetetraacetic Acid
g	g-force
GWAS	Genome-Wide Association Study
HIR	High Immune Response
HH	High High (EBV)
HL	High Low (EBV)
IBD	Inflammatory Bowel Disease
IFN-y	Interferon gamma
LH	Low High (EBV)
LL	Low Low (EBV)
MAP	Mycobacterium Avium subsp. Paratuberculosis
MDA-5	Melanoma Differentiation-Associated protein 5
MHC	Major Histocompatibility Complex
ml	Millilitre
MØ	Macrophage
MPI	Moxi Population Index
NK	Natural Killer

NO	Nitric Oxide
NOD	Nucleotide-binding Oligomerization Domain receptors
PAMP	Pathogen-Associated Molecular Patterns
PPR's	Pattern Recognition Receptors
QTL	Quantatatative Trait Loci
RIG-1	Retinoic Acid-Inducible Gene 1
SAC	Scottish Agricultural College
SNP	Single Nucleotide Polymorphism
Th1	T helper type 1
Th2	T helper type 2
TLR	Toll Like Receptor

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1. Introduction

Johne's disease is a common endemic disease in the UK. It is a chronic enteritis which is caused by the *Mycobacterium avium subsp. paratuberculosis* (MAP) pathogen (Rue-Albrecht et al, 2014). Johne's disease has been estimated to cost £13 million to the UK dairy and beef industries per year (Bond and Guitian, 2015) with losses experienced directly from the culling out of infected cattle as well as indirectly through a loss of milk yield, veterinary costs, a reduction in fertility and increased labour and management (SAC, 2003). Johne's disease also causes problems for cattle welfare, with chronic wasting seen during the late phases of disease progression (Arsenault *et al*, 2014). In addition, MAP has been associated with human cases of Crohn's disease, making it of zoonotic importance (Botsaris *et al*, 2016).

Approaches to control Johne's disease include biosecurity measures which consist of reducing or stopping cattle movements and vaccination. A licence to vaccinate cattle can be applied for in the UK, however the current test interferes with the bovine tuberculosis (TB) skin test and can cause false negative reactions, meaning that TB infected cattle can go undetected (NADIS, 2016 b). Research is currently being undertaken to identify specific markers within the bovine genome which promote Johne's resistance, however it has been found that multiple loci contribute to resistance due to the complexity of the immune response caused by MAP pathogens (Minozzi *et al*, 2012). Research studies into bovine tuberculosis which is caused by a closely related pathogen, *Mycobacterium bovis* have indicated that TB resistance is heritable at 9% (AHDB, 2016 c) however, it is argued that breeding for individual traits can cause problems for broad based disease resistance due to negative genetic correlations (Thompson-Crispi, Miglior and Mallard, 2013).

The concept of broad based disease resistance is the underlying principle of the High Immune Response (HIR) technology. The HIR technology enables breeders to identify cattle as high (HIR), medium (MIR) or low (LIR) responders with research showing that HIR cattle have less cases of several diseases of both economic and welfare importance including mastitis, metritis, ketosis, retained placenta and displaced abomasum as well as preliminary evidence

showing that HIR cattle are less likely to test positive for Johne's disease (Mallard *et al*, 2014). The heritability of the HIR trait in cattle is ~30% (Semex, 2015).

The HIR test is based on a measurement of the adaptive immune response. Adaptive immune response in cattle works in two ways, through antibody mediated immune response (AMIR) which generally protects the body from extracellular pathogens such as mastitis (Thompson- Crispi et al, 2012) and cell mediated immune response (CMIR) which wards off intracellular pathogens (Nicholas, 1996) such as those presented in Johne's infection (Arsenault *et al*, 2014). Research has shown that these immune responses are both required at a high level in order to promote broad based disease resistance although in cattle are often genetically negatively correlated (Thompson-Crispi, Miglior and Mallard, 2013).

As stated above, Johne's disease is predominantly mediated by a CMIR although research indicates that if the CMIR is not strong enough or the pathogen is able to evade the hosts defence mechanisms, a non-protective AMIR will take over which can lead to dissemination of the pathogen (Magombedze, Eda and Ganusov, 2014).

This research study investigated if cattle with different immune response estimated breeding values (EBVs) as determined by the HIR technology were more or less likely to promote disease resistance to MAP pathogens. In order to measure immune response macrophages were isolated from blood and challenged *in vitro* with whole cell deactivated MAP and two recombinant proteins from MAP. Nitric Oxide (NO) and pH were measured, both indicators of an immune response towards MAP pathogens (Arsenault *et al*, 2014).

The researcher hypothesised that based on current HIR research, cattle with EBVs for high AMIR and high CMIR (HH) would produce higher NO concentrations than cattle with EBVs for low AMIR and low CMIR (LL). The production of a higher concentration of NO in cattle with HH EBVs was hypothesised as a result of the immune reaction to eliminate the pathogen as well as dropping the pH to a lower acidity, an indicator of phagolysosomal fusion and subsequent elimination of the pathogen (Rue-Albrecht et al, 2014).

It was also hypothesised that cattle with low AMIR and high CMIR (LH) would produce a stronger immune response than cattle with high AMIR and low CMIR (HL) due to the MAP pathogen being intracellular and requiring a high cellular response to destroy it. The null hypothesis stated that all EBV's would produce the same reaction.

The results of the experiment were analysed using Genstat 18th Edition. A confidence level of 0.05 was applied to an Analysis of Variance (ANOVA) test, with contrasts applied between the EBV groups. There were no statistically significant results found between the EBV groups for NO release, however as predicted the HH macrophages produced a higher concentration of NO than the LL macrophages. Contrast testing between the EBV groups for pH release found a statistically significant result between the HL and LH macrophages with the LH EBV (high CMIR) dropping the intracellular pH significantly and therefore exhibiting a mechanism to destroy MAP pathogens in vitro more successfully than the other EBVs. The difference between HH and LL macrophage pH release was not found to be statistically significant. The cohort of cattle tested was small and it is therefore concluded that further research into the area should be completed with larger cohorts before the alternate hypothesis is fully rejected.

2. Literature Review

2.1 UK Dairy industry

The size of the dairy industry in the United Kingdom has fallen dramatically over the past 20 years with a recorded 35,741 dairy farms in 1995 dropping to 13,815 by 2014 (Bate, 2016). However, despite a drop in dairy farming enterprises, herd numbers have recently been rising with a 5.9% increase in the UK average herd size between 2013 and 2014. In 1996, the average herd size stood at 78 cows, whereas statistics from 2014 show an average herd size of 136 (AHDB, Dairy, 2015a).

In addition to an increase in herd size, average milk yield rose by 373 litres per cow from 2013 to 2014 (AHDB Dairy, 2015b). This indicates that although the size of the dairy industry has contracted, the expectation for increased production from the dairy cow has increased with a recorded 93% growth in yield, per cow since 1975 (Bate, 2016).

An FAO (2015) report stated that world cow milk production stood at 636 million litres in 2013 with the largest producer being the United States of America. Production in the US has been predicted at 96.3 million tonnes for 2015.

2.2 Dairy industry trends

The UK is currently the third-largest milk producer in Europe and the tenth largest producer globally (Bate, 2016). Low milk prices however, have become an increasing issue globally; this is due to an uneven supply demand factor. Milk production has been growing 5% on average per year; however global demand for milk is only rising by 2% on average, leading to a rise in global stocks and therefore a reduction in pence per litre received by farmers (Parliament UK, 2015). In August 2015, farm-gate milk prices dropped to 23.3 pence per litre, the lowest recorded monthly figure since 2009 (Bate, 2016).

Despite negative economic trends in the dairy industry, the food industry as a whole is growing, with the world's population expected to increase to 9 billion by 2050 (World Bank, 2015). According to the World Bank (2015), cereal and

meat production will need to increase by 50% in order to feed the global population in 2050 and with this, dairy sales are also predicted to increase. In order for the dairy industry to meet future demands and to ensure economic stability it is important that on farm husbandry costs are reduced. One major cost implication to the dairy industry is the cost of disease. There are over 1.3 billion cattle globally and therefore the economics of disease incidence on a global scale is difficult to estimate (Meade, 2015) however, Casey et al (2015) indicate that the cost of infectious disease[1] breakdown in the US is between $200 million and $150 billion per year with cost rising accordingly with the increasing prevalence of disease.

2.3 Disease in dairy cattle

As previously mentioned, one factor which has a major implication for the economics of the dairy industry is disease. There are many dairy cattle diseases in the UK, with variations of severity in terms of cost as well as the differing impact on animal welfare and risk to human populations (AHDB Dairy, 2015c).

According to Compassion In World Farming (2010) the most common diseases in dairy cattle are mastitis, lameness (most commonly white line disease (AHDB Dairy, 2016)), metabolic diseases such as ketosis, acidosis hypocalcaemia (milk fever) and hypomagnesaemia (grass staggers) as well as fertility related diseases such as endometritis/ metritis.

In addition to the diseases above, the most common endemic infectious diseases to the UK include; bovine viral diarrhoea (BVD) which costs the UK farming industry £50- £75 million per year (XL Vets, 2011), infectious bovine rhinotracheitis (IBR)[2] and bovine tuberculosis which has cost the UK taxpayer ~£500 million between 2004 and 2014 and has an average on farm breakdown cost of £34,000 (TB Free England, 2014). Johne's disease also represents one of the most common endemic infectious diseases in the UK and is the focus of

[1] Infectious disease examples include; Johne's Disease, Neospora, Leptospirosis, Bovine Virus Diarrhoea, Infectious Bovine Rhinotracheitis (NADIS, 2015)

[2] An exact cost for IBR could not be found, however, calf pneumonia has an annual cost of £80 million to the UK farming economy. Calf pneumonia includes viruses such as IBR, RSV and Pi3 as well as bacteria pathogens such as mycoplasma bovis, pastuerella, mannheimia and haemophilus (XL Vets, 2015)

this research study. The diseases mentioned above all have serious welfare implications for the animal as well as having high economic consequences for the farmer (Gov.UK, 2012).

2.4 Johne's Disease

2.4.1 Overview and economics

Johne's disease, as previously mentioned, is a common endemic disease in the UK. It is a mycobacterial infection caused by the *Mycobacterium avium subspecies. paratuberculosis* (MAP) pathogen. Mycobacterium is a gram-positive genus of bacteria with over 120 species. Most mycobacteria species are non-pathogenic environmental bacteria; however a few sub-species have evolved highly effective intracellular mechanisms resulting in chronic infection and high morbidity and mortality rates. There are two major mycobacterium species that affect the dairy industry; these are MAP and *Mycobacterium bovis*, which causes bovine tuberculosis in cattle (Rue-Albrecht, et al 2014).

According to Bond and Guitian (2015) the cost of Johne's disease in the UK is £13 million annually (beef and dairy industry). The cost of the disease to individual farms in the UK dairy industry however, is highly variable and is dependent on management strategies and the testing regimes put in place by the famer and their vet. As a direct result of the disease animals will be culled out of the herd resulting in the loss of the infected animal. There are also a number of hidden costs associated with Johne's disease infection which include; production loss, a reduction in fertility, veterinary treatment, increased farm labour and management, replacement cost and the market value of the herd reduced (Scottish Agricultural College, 2003). The University of Reading (2015) have developed a financial model in order to determine the cost of a Johne's breakdown at farm level. Although this figure would vary farm to farm with different systems and environmental pressures, the calculation below can be used as a good indicator of a farm level scenario in the UK.

If a Johne's disease-free dairy herd with 150 cows in milk introduced 10 low-shedding carriers without using any test/control measures, the average cost in losses would equate to £17,000 per year over the following 10 years (£170,000 total loss). If management and control strategies were put in place, this cost

would reduce to £3100 per year on average (over 10 years, £31,000) (University of Reading, 2015).

According to Rue-Albrecht *et al* (2014), Johne's represents an estimated loss of $250 million to the US dairy industry annually with average herd prevalence in some US states (as well as some European countries) being greater than 50%.

2.4.2 Zoonotic importance

In addition to the cost of Johne's disease to the farming industry, the disease is also of potential zoonotic importance. A recent study carried out an analysis of viable MAP pathogens in pasteurised infant formulas, through the use of a phage-PCR assay it was found that of 32 samples, 4 samples contained viable MAP pathogens (12.5%) with 3 of the 4 samples being culture detected. Previous studies found that approximately 2% of pasteurised milk samples contained viable MAP pathogens (Botsaris *et al*, 2016). These findings are a matter of public health concern due to the associations between MAP and human Crohn's disease/ Inflammatory Bowel Disease (IBD). In 1913 the first associations where made between Johne's and Crohn's disease and although a causal link between the two has not yet been defined (Atreya *et al*, 2014) it has been shown that there is a positive association between the exposure of humans to MAP and Crohn's disease (Botsaris *et al*, 2016).

2.4.3 Johnes disease presentation

Johne's disease affects all ruminants with beef and dairy cattle being a major host. It is a chronic inflammatory disease of the gastrointestinal tract with four stages of disease progression: silent, subclinical, clinical and advanced infection. Tissue and regional lymph nodes of the gastrointestinal tract become inflamed following the hosts immune response mechanisms, this inflammation effectively results in reducing the host's ability to absorb nutrients and clinical disease presentation can therefore be seen with signs of wasting due to significant weight loss and diarrhoea (Rue-Albrecht *et al*, 2014). During systemic clinical infection the pathogen can also be detected in milk (Botsaris *et al*, 2016).

The disease is difficult to diagnose due to the silent infection phase coupled with a prolonged subclinical phase of around three to five years. Upon clinical disease presentation the disease can be detected through the use of a blood antibody test (ELISA), however this method isn't fully effective until the late stages of the disease, when an antibody mediated response becomes predominant (Animal Health and Welfare NI, 2014). MAP pathogens can also be detected through collection of faeces and subsequent laboratory culture; however this method is often ineffective in animals under the age of two due to insufficient bacterial load within the faeces. Due to the inefficiency of the two aforementioned detection methods disease classification is often not accurate until a post-mortem following death (SAC, 2003).

2.4.4 Modes of transmission

Johne's disease infection most commonly occurs in the neonatal period through the vertical transmission route (dam to offspring) (Arsenault *et al*, 2014). The pathogen has the ability to pass through the placenta into the foetus with one study concluding that 9% of calves from sub-clinically infected dams and 39% of calves from clinically infected dams were infected *in utero* with MAP (Behr and Collins, 2010). More commonly however, neonates are infected through the oral route following colostrum intake which has been shown to harbour a high pathogen load (Chai-Wei Wu et al, 2007). In addition to infection routes described above, calves can become infected through horizontal transmission. This can be through exposure to pathogens on dirty teats, which if the environmental pathogen burden is high may or may not be contracted directly from the dam (SAC, 2003). Other horizontal infection routes include oral intake of contaminated soil or through bodily fluids such as saliva (vertically through the dam or horizontally through other infected cattle/wildlife hosts), semen or uterine fluid on dirty bedding (Arsenault *et al*, 2014).

Pooled colostrum has been reported to increase disease transfer and is often advised against in Johne's control programmes. A study by Nielsen, Bjerre and Toft (2008) found that calves fed from a pooled colostrum tank had a higher probability of testing positive with an antibody test. In addition, the researchers found that pooled milk from cows with a high somatic cell count (which is deemed unfit for human consumption) also increased the likelihood of a

positive antibody test with the conclusion that pooled colostrum increased the chance of viable MAP pathogens being fed to calves.

Disease susceptibility reduces over the first year of a calf's life with the risk of transmission and subsequent infection being highest within the first few months of life. At one year of age a calf is expected to have the same level of disease tolerance as an adult cow with the level of MAP pathogens required to cause disease being much higher as well as a longer exposure period being required (Arsenault *et al*, 2014).

NADIS (2016), state that Johne's disease can be introduced to farms in a number of ways including the introduction of new stock which could be either clinically diseased or sub-clinically incubating the disease as well as through the introduction of animals that appear healthy but are in fact carriers. It is also possible that the pathogen could be introduced through the transportation of infected faeces from an infected farm to a clean farm via vehicles, equipment or on the clothing or footwear of people. It is therefore important that biosecurity measures are undertaken on farms in order to minimise the risk of cattle being exposed to the pathogen. Good biosecurity measures are based on four key principles and include the selection of all necessary purchased animals from herds which have a known herd health status, isolate animals following purchase and before introduction to the rest of the herd, control movements on and off of farm including vehicles, animals and people and finally facilitate good sanitation practices to ensure that all people and equipment entering the farm is properly disinfected.

2.5 Overview of the immune response system[3]

The immune system is responsible for recognising, resisting and eliminating health challenges which may be issued from a broad range of infectious organisms (Cooke, 2010). It is therefore important, in order to control infectious diseases such as Johne's disease in the dairy industry effectively that the immune response of dairy cattle is understood (Meade, 2015). It has been documented in the literature that disease prevalence on individual farms as well as within the national and global herd is dependent on immune response

[3] Cells of the immune system can be found in Appendix A

with a good herd health status being fundamental for optimal performance (Cooke, 2010).

The immune system of livestock species is generally based on two immune responses, innate and adaptive, although these systems are not independent of each other and there is a high proportion of cross over between the two response types (Owen, Punt and Stranford, 2013 pp.173-174) they will be discussed separately.

2.5.1 Innate Immunity

The innate immune response is immediate, non-specific and does not confer a long-lasting protection. It is activated upon host recognition of a foreign pathogen via the innate defence barriers including the epithelium (skin/feathers) and mucous membranes (Murphy, 2012 pp.37-40). The innate response also involves cells which are present in the adaptive immune system and includes phagocytic leukocytes, macrophages and Natural Killer (NK) cells, which act to destroy and remove invading pathogens (Owen, Punt and Stranford, 2013 pp. 168-169).

Invading pathogens will commonly be met by a macrophage (Brooks *et al*, 2013 p.125). Macrophages have microbial sensors which include toll-like receptors (TLRs), Nucleotide-binding Oligomerization Domain- like receptors (NLRs) and Retinoic acid-Inducible Gene 1 (RIG-1) like helicases and Melanoma Differentiation-Associated protein 5 (MDA-5). As described above, the key elements of the innate immune response include rapidity, non-specificity and short duration. These elements are all also features of the phagocytic process which the macrophage carries out (Murphy, 2012 pp.76-80). Phagocytosis is the process in which an invading pathogen is recognised by the macrophages microbial sensors and then upon confirmation that it is a foreign body engulfed by the immune cell. Once the pathogen is engulfed by a macrophage it is internalised into an endocytic vesicle called the phagosome, here it is subject to antimicrobial mechanisms designed to kill the pathogen, for example, the phagosome lyses with the cell lysosome in order to create the phagolysosome which has a highly acidic pH in order to destroy the invading pathogen. In addition, toxic reactive nitrogen and oxygen species

are produced and nitric oxide (NO) is formed which also helps to destroy pathogens (Owen, Punt and Stranford, 2013 pp. 147-151). In addition to the internal activities of macrophages, the cells also release cytokines and chemokines which initiate the process of inflammation. Phagocytosis and inflammation processes may also be activated by the complement system, a collection of proteins which work to activate a cascade of proteolytic reactions on invading cell surfaces. The complement system coats pathogens with fragments which are recognised by the phagocytic receptors on macrophages, offering an alternative pathway for pathogen destruction. The process of phagocytosis and the complement system are processes which cross over with the adaptive immune system and both function after the innate response has concluded (Janeway et al, 2005 pp.12-13).

If the pathogen challenge cannot be successfully removed by the innate immune response the adaptive response is activated (over a period of 4-7 days) (Owen, Punt and Stranford, 2013 p.12). This transition is primarily mediated by cells such as macrophages (and other white blood cells) as well as the complement system, which introduce the foreign agent to specific cells in the adaptive immune response as well as activating cells involved in the adaptive immune response (Cooke, 2010).

2.5.2 Adaptive Immunity

The adaptive immune response is highly specific, has memory and can therefore respond rapidly to a repeated antigen exposure. The adaptive immune response is further split into two types; antibody mediated immune response (AMIR) and cell mediated immune response (CMIR), although these responses are not independent of each other (Brooks *et al*, 2013 pp.127-128) research has shown that they can be negatively genetically correlated (Thompson- Crispi *et al*, 2012). An adaptive immune response is characterised by the production of antibodies from activated B cells and activities of T-cells (Cooke, 2010).

2.5.2.1 Antibody Mediated Immune Response

A protective antibody response is derived from lymphoid progenitor cells which have evolved into B cells, these cells can be found in the blood and are

developed in the bone marrow of cattle, as depicted in Figure 1 below. AMIR is activated by helper T-lymphoctes (Cluster of Differentiation 4 (CD4)) which recognise major histocompatibility complex class[4] (MHC) II antigen presentation on the surface of antigen presenting cells such as macrophages and then produce cytokines which activate B cells that express the antigen which matches the antigen being presented. Following the activation of B cells they undergo clonal proliferation in order to produce specific antibodies to combat the pathogen through a process of agglutination, precipitation and eventually cell death as well as creating memory B-cells[5] which are stored in the body ready to respond rapidly following a second exposure to the specific antigen (Brooks *et al*, 2013 pp.127-128).

Figure 1: Antibody Mediated Immune Response Illustration

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As mentioned in the description of the innate immune system in section 2.5.1, phagocytosis is also a part of the adaptive immune response. Pathogens which have been bound by antibody are delivered to phagocytic cells for ingestion, degradation and removal from the body. The complement system which also crosses over from the innate immune response, works alongside antibodies in the adaptive immune response in order to enhance bactericidal

[4] The Major Histocompatibility Complex (MHC) plays a major role in binding peptides from pathogens and presenting them to the cell surface as an antigen which is then recognised by receptor T cells. MHC in AMIR and CMIR are different, in antibody response MHC presentation of an antigen results in antibody production whereas in CMIR MHC antigen presentation results in the activation of cytotoxic T cells which are directly responsible for destroying pathogens (Owen, Punt and Stranford, 2013 pp.262-271)

[5] Memory B- cells make vaccination possible

actions of phagocytic cells such as macrophages. Both the phagocytic cells and the complement system are not antigen specific and rely on antibody molecules to mark the antigens as foreign.

Antibodies, also referred to as immunoglobulins (Ig) consist of two distinct regions as outlined in Figure 2 and are made up of four polypeptides. There are many different classes of antibodies (Johnson, 2015) (Figure 3) which are made up of multiples of the base unit shown in Figure 2.

Figure 2: Generalised antibody molecule structure. Image downloaded from http://www.biology.arizona.edu/immunology/tutorials/antibody/structure.html on 12 May 2016. Source: University of Arizona, 2000

Figure 3: Classes of antibodies. Image downloaded from https://www.labome.com/method/Mouse-Antibody.html on 22 February 2016. Source: Johnson, 2016

ANTIBODY CLASSIFICATION

The constant region takes one of four biochemically forms whereas the variable region can be composed of an apparent infinite number of forms[6] which enables the antibody to bind with an equally diverse number of antigens. As illustrated in Figure 2, each antibody molecule has a two-fold axis of symmetry and is composed of two identical heavy chains as well as two identical light chains. Antigen binding sites are composed of a heavy and a light chain, meaning that both chains contribute to binding specificity however it is the constant region that determines how the antigen will be destroyed. (Janeway *et al,* 2005 pp.17-18).

According to Thompson- Crispi *et al* (2012), extracellular pathogens such as extracellular bacteria, extracellular protozoan, parasites and helminthic worms are generally combated by an AMIR.

2.5.2.2 Cell Mediated Immune Response

Whist AMIR generally defends the host from extracellular pathogens, CMIR protects against intracellular pathogens such as viruses, intracellular bacteria and protozoa (Mogensen, 2009). Pathogens are only accessible to antibodies in the blood (extracellularly), however a range of pathogens including some bacterial and parasitic pathogens as well as all viruses replicate inside host cells where they cannot be seen by antibodies. T lympohcytes or T-cells are the cells responsible for CMIR (Janeway *et al,* 2005 p.26-27). The cell mediated response is activated through recognition of the antigen-MHC class II by helper (CD4+) T lymphocytes as well as the recognition of the antigen-MHC class I complex by cytotoxic (CD8+) T lymphocytes[7] on antigen presenting cells such as macrophages[8]. As with AMIR following activation, T cells produce cytokines and expand by clonal proliferation (Owen, Punt and Stranford, 2013 p.427-432). CD4+ cells stimulate B cells to produce antibodies which help with intracellular bacterial infection control from pathogens such as *Mycobacterium avium subspecies paratuberculosis* (Brooks *et al*, 2013 p.128) the pathogen of interest for this research study. CD8+ cells are primarily

[6] High variability is generated through random recombination of variable sector gene segments (DNA), this enables the production of antibodies that have the ability to recognise virtually any antigen

[7] CMIR can be measured through IFN-y release

[8] IFN-y release stimulates the macrophage to express the MAP antigen on its surface (Casey et al, 2015)

involved with the destruction of tumour cells, cells in tissue grafts or cells infected by viruses (Murphy, 2012 pp.369-374).

The adaptive immune response can be enhanced through breeding practices and forms the basis of the High Immune Response technology which will be discussed in Section 2.8.

2.5.3 Immunological mechanisms specific to Johne's Disease

Following ingestion of MAP, the ruminant digestive system activates the bacterial cell wall protein fibronectin attachment protein (FAP) in order to promote opsonisation by fibronectin (Arsenault *et al,* 2014). The intestinal epithelium is the largest surface area in the mammalian body and acts as a barrier against commensal and pathogenic bacteria, fibronectin links MAP to the luminal surface of intestinal microfold cells through fibronectin receptors. MAP pathogens must overcome mucus secretions, antimicrobial peptides, secretory IgA, tight junctions and the galcocalyx in order to establish themselves within the host (Lamont et al, 2012; Janeway et al, 2005 pp.40-41).

Following successful passage through the intestinal epithelium the secondary defence mechanisms of the host are activated. As described in section 2.4.5, the innate immune system is the body's first line of defence against invading MAP pathogens with the macrophage being the primary phagocytic cell to try and eliminate the MAP pathogen (Owen, Punt and Stanford, 2013, pp. 141-157).

Once MAP pathogens have been ingested they colonise the mucosa-associated lymphoid tissues of the upper gastrointestinal tract where they are endocytosed through the Peyers patch (Brooks *et al*, 2013). Macrophages specifically are able to see the invading pathogen through the interaction of mycobacterial pathogen-associated molecular patterns (PAMPs) which are displayed on the surface of MAP bacilli with pathogen recognition receptors (PPRs) such as toll like receptors (TLRs) which are displayed on the surface of host macrophages (Murphy, 2012, pp. 50-54; Casey *et al*, 2015). Sub-epithelial and intraepithelial intestinal macrophages work to phagocytose invading MAP pathogens following endocytosis through the Peyers patch. As

described in section 2.4.5 the phagosome[9] creates an antimicrobial environment which is designed to kill the invading pathogen so that it can be eliminated from the body (Janeway *et al,* 2005 pp.42-43).

Once the phagolysosome has been formed nitric oxide is recruited to kill the mycobacterium. A study that investigated mouse macrophages found that nitric oxide is directly related to the ability to kill a range of mycobacteria species including MAP, *Mycobacterium tuberculosis* and *Mycobacterium leprae*. In addition, it was found that the inhibition of nitric oxide enabled intracellular survival of *Mycobacterium tuberculosis* in the mouse model (Arsenault et al, 2014). Although it has been suggested that the quantitates of nitric oxide produced by bovine macrophages are insufficient to completely destroy MAP, the fact that MAP pathogens aim to inhibit nitric oxide release suggests that the immune mechanism presents a threat to the survival of MAP at an intracellular level (Davis *et al*, 2007).

Following the activation of macrophage PPRs, signalling pathways are activated leading to the production of cytokines and chemokines which lead to the activation of the adaptive immune response and promote CMIR (TH1) (Brooks et al, 2013). The ability of the host to mount a successful CMIR is highly dependent on the individual's immune system as well as the rate of exposure (Rue-Albrecht *et al*, 2014).

[9] Phagosome properties include; acidification, which drops the pH of the environment significantly (3.5- 4.0), toxicity, which generates toxic oxygen products, and antimicrobial peptides which are recruited to help eliminate the pathogen (Brooks et al, 2013).

Figure 4: Uptake route of MAP in cattle. Image downloaded from https://veterinaryresearch.biomedcentral.com/articles/10.1186/1297-9716-45-54 on 8 Februaury 2016. Source: Arsenault et al, 2014.

(Source: Arsenault et al, 2014)

According to Arsenault *et al* (2014), clinical disease only occurs in 10-15% of cattle exposed to MAP pathogens, indicating that most cattle (including calves) have the ability to successfully control the infection. An effective host immune system will result in the eradication of the pathogen; however the Mycobacteria family have evolved ways in which to effectively avoid the host's CMIR mechanisms which lead to the development of granulomas (Rue-Albrecht *et al*, 2014). Granulomas can be physically seen in infected culled animals and indicate clinical infection (SAC, 2003). The ability of the host to successfully control MAP infections relies on a range of factors including genetics, health and the level of infectious dose within the environment (Arsenault *et al*, 2014).

According to Rue-Albrecht *et al*, (2012) MAP has the ability to evade the CMIR of the host effectively by preventing phagolysosomal fusion. This inhibits macrophage apoptosis and suppresses the antigen presentation signalling mechanisms of the macrophage. In addition to inducing cytokine induced necrosis which is essential for the control of the infection (Coussens, 2004), T-cell interactions[10] are also prevented. Clinical infection develops in the 10-15% (Arsenault *et al*, 2014) of cattle that cannot clear the infection with a shift from a CMIR to a non-protective AMIR being seen during the late sub-clinical phase. CMIR and AMIR can therefore exist simultaneously and it is possible for MAP infection to latently infect the host if the animal becomes immune-compromised due to its ability to reside in macrophages after the CMIR has changed to an AMIR (Casey *et al*, 2015) which is characterised by the production of immunolglobin G1 (IgG1) antibodies (Coussens, 2004). Upon clinical infection, immunopathology enables the infection to be disseminated within the host, eventually leading to bacterial shedding from the animal (Casey *et al*, 2015; Rue-Albrecht *et al*, 2012; Lamont *et al*, 2012; Kuehnel *et al*, 2001).

It is due to this long incubation stage and the ability of MAP to reside in macrophages that enables a high infection rate.

2.5.4 Johne's Disease Immunological Research

Due to the economic and welfare issues outlined earlier in this study, research has been completed in order to find solutions to the problem of MAP infection and Johne's disease. One of the key attributes of the adaptive immune system is that it can be manipulated in order to promote a response which will provide long lasting protection. In 1796, Edward Jenner developed the first commercial vaccination against smallpox. The concept of vaccination at this time was to infect the a human patient with a small amount of dried pathogen from a bovine host in order to stimulate the immune system to produce antibodies for future infectious challenge (Janeway et al, 2005 p.642-657).

[10] T-cell reactions are characterised by a release of pro inflammatory cytokines such as IFN-Y, interleukin-1α, interleukin-6 and the production of interleukin-2 (Coussens, 2004)

A vaccine against Johne's disease has been developed for cattle. The vaccine is inactivated and permitted for use in the UK under licence. It should be administered to calves under one month old (ideally less than a week of age) in the brisket area. Use of the vaccine has demonstrated fewer clinical cases and losses however, it does not have the ability to completely reduce Johne's disease with faecal shedding and subsequent cases in the herd still occurring. A significant problem with vaccinating against Johne's disease in the UK is that it interferes with the current test for bovine tuberculosis (TB) which is compulsory under Government policy. The TB test measures immune response to *Mycobacterium* antigens in order to determine if the animal has been exposed to *Mycobacterium bovis*, the test currently uses the MAP antigen to measure immune response and therefore if the animal has previously been vaccinated against Johne's disease it is likely that a false negative result will occur. This will result in cattle that have been infected with TB going undetected, posing a threat to the TB eradication strategy in the UK (NADIS, 2016 b).

In addition to the vaccination control method, researchers have also investigated individual resistance to Johne's disease in order to identify cattle that are less susceptible to Johne's disease. Several studies have identified genes for MAP resistance and therefore marker assisted breeding for natural immunity towards Johne's disease has been a popular concept amongst researchers (Hinger, Brandt and Erhardt, 2008). Despite gene identification the heritability of MAP resistance is very low. An early study in 2000 found that MAP resistance was heritable at 0.08 (Koets et al, 2000), this has been improved by increased sire daughter records with Mortensen *et al* (2004) and Gonda *et al* (2006) both reporting heritability at 0.10. Hinger, Brandt and Erhardt (2008) reported that heritability ranged between 0.05 and 0.13 depending on daughter data from sires. It is noted that even with the provision of daughter data, the heritability rate for MAP resistance as a single trait is very low and therefore both vaccine use and genetic enhancement of specific MAP resistance are considered to be unfavourable forms of disease control.

2.6 Breeding

2.6.1 A historical perspective

The domestication of cattle began 8000- 10,000 years ago with breeding techniques based on cattle characteristics such as; lack of aggression, size and the ability to consume unconventional feed. Cattle breeding took off in the industrial revolution with an increased demand for meat and milk. Breeding became systematic and traits such as milk production led to dairy cattle being distinguished from beef cattle. Between 1750 and 1880 there was a period of agricultural revolution which led to a rapid increase in the development of cattle breeds with new technologies such as milking machines increasing the viability of certain traits in dairy cattle. Through new technologies, traits began to be bred for more selectively with teat size and ability for milking as well as the size of the cow to fit into milking parlours being routinely selected. Breeding was further helped by the development of artificial insemination methods in 1899 which ensured that cattle breeders could select specific traits from bulls across the country in order to gain the highest potential from their offspring. The 20th Century brought the classification of the Holstein-Friesian which was selectively bred for size in order to reduce labour requirements and therefore increase the profit of dairy farmers (Phillips, 2010, pp.50-56).

2.6.2 Genetics

The structure of Deoxyribonucleic Acid (DNA) was discovered in 1953 by James Watson and Francis Crick. Not only did this lead to key developments in medicine but it also enabled scientists to understand the body systems in a range of species, including cattle. This fundamental finding led to the ability to breed cattle based on their genetic potential and specific traits can therefore be bred for as opposed to the phenotypic observations that farmers would have made in the past (Phillips, 2010, pp.55-59).

A key advance in genetic improvement was the introduction of estimated breeding values (EBVs) which enabled farmers and breeders to predict the offspring performance of animals they wanted to selectively breed from. An animal's breeding value is essentially its genetic merit, half of which will be passed to its progeny (Agricultural Business Research Institute, 2016). EBVs

incorporate the individual's performance records as well as their parent's data which can then be benchmarked against breed performance data (Weigel, 2010). The accuracy of EBVs can vary and are dependent on the available data for the trait being measured, for example, bulls with a high number of daughters have higher EBV accuracy than those bulls with fewer proven daughters (ABRI, 2016). It is important to remember that EBVs can only be predicted and are based solely on genetic potential. The environment and management system that the offspring is reared in will have a significant impact on the true performance of the animal.

In addition to the accuracy of EBV traits, the heritability of traits can also be determined and is the term used to describe how certain characteristics are transferred from the sire/dam to their progeny (The Beef Site, 2011). Heritability can be described as the proportion of variation observed in a trait which is due to genetics (Mallard *et al*, 2014). The strength of heritability varies with each trait, for example, daughter fertility is heritable at 4-7% and longevity at 8-10% (Semex, 2012), the higher the percentage value of heritability the faster genetic improvement will take place and the positive benefits observed in the herd.

Predicted Transmitting Ability (PTA) is another method which is used to improve breeding and measures the relative production potential which will be transmitted to the offspring with sires generally selected on the basis of having a PTA greater than 70% in order to gain genetic improvement within the herd (Andrews, 2014 p.60).

In addition to PTA, the dairy industry also uses the Profitable Lifetime Index (£PLI). £PLI is a within breed genetic index and was developed by AHDB Dairy in order to fit with its vision in the UK to breed for dairy cows which have the ability to thrive in a number of different farming systems in the country as well as giving farmers the opportunity to improve their herd traits (Cattle Health And Welfare Group, 2014).

£PLI value indicates the additional profit a bull with high £PLI is expected to return from each of its daughters over her milking lifetime in comparison to a bull ranked as an average £0 PLI. Bulls can be selected from AHDB Dairy's

database, where they are ranked in terms of their traits and can therefore be selected in order for the farmer to gain economic potential. Cows and heifers also have £PLI indices and it is important that for genetic gain to be experienced, the £PLI of the bull is always greater than the £PLI of the dam. As mentioned above, the index encompasses traits which have been a focus of the national breeding strategy for the past 10 years and include cow health, welfare and longevity. The current national breeding goal is 'fitness', a trait which currently outweighs production in a ratio of 32:68. AHDB Dairy have recently moved away from the economical/profit focused traits such as yield that £PLI initially worked towards and are instead encouraging the index to be used to promote the health and welfare of cattle which in turn will improve the productivity of the national herd (AHDB Dairy, 2016b; CHAWG, 2014). In addition to 'fitness', specific production traits included in £PLI rankings include: locomotion, udder composition, fertility and Somatic Cell Count (SCC).

The introduction of genomics into the dairy industry has enabled breeders to measure individual animal DNA in order to determine the form, production and health that the specific animal will achieve within its life (given that the correct management and environment is provided). In 2006, the Bovine Genome Sequencing Project was completed (Canadian Dairy Network, 2009) which allowed individual genomic analysis of animals through the examination of single nucleotide polymorphisms (SNPs) which use makers to detect mutations of single base changes (A,T,C and G) in the DNA structure. The sequencing of the bovine genome alongside the ability to detect SNPs has enabled livestock scientists to identify important traits such as milk yield (Womack, 2009). A high throughput tool has been developed in order to track genetic differences on the chromosomes using SNP's (Winters, 2014). It is due to the development of this latest technology that health traits can be observed through the identification of quantitative trait loci (QTL) (EC, 2003).

Current technology includes a new SNP-chip assay which has the ability to identify more than 800,000 SNP's in dairy cattle and therefore cattle can now be genotyped in an efficient automated manner leading to increased accuracy in breeding as well as a reduction in the generation interval as animals can be tested at birth for genetic merit as opposed to previous methods which took

five years for bulls to provide sufficient EBV data based on their progeny (DairyCo, 2010b).

With regards to breeding for health, genomic processes have enabled a greater understanding of host response to infection and differing response types to infection and pathogenic load which has in turn enabled scientists to understand individual genetic variation towards disease resistance (Bishop *et al*, 2010, pp.6-8).

2.7 Breeding for disease resistance

As discussed above it is possible for breeders to advance production and profitability in dairy systems through the use of EBVs, PTAs, £PLIs and more recently SNP technology through the selection of traits which are desirable to the specific management system. According to Bishop *et al* (2010, pp.4-5), there is evidence for host genetic variation for more than fifty diseases throughout the major livestock species. This covers a range of parasites and pathogens as well as the genetic resistance of the host ranging from single major genes to polygenic gene collaboration (Bishop et al, 2010, pp.4-5). Despite the possibility to breed selectively against disease, some genes associated with resistance to a specific disease, such as the Johne's disease resistance gene outlined in section 2.4.7 only provide low heritability estimates, meaning that genetic gain could only be achieved over many generations (Hinger, Brandt and Erhardt, 2008).

2.7.1 Genetic variation to disease

As previously discussed the advancements in breeding technologies coupled with a better understanding of individual cattle genomics have made it possible to breed for disease resistance to specific problematic diseases affecting the dairy industry. The causative agent of bovine tuberculosis, *Mycobacterium bovis* a pathogen from the same family as MAP has been shown to have a genetic basis to host susceptibility (Allen *et al*, 2010) with a range of studies indicating that heritability for TB disease resistance is possible. Bermingham *et al* (2014) carried out a genome-wide association study (GWAS) on female Holstein-Freisian cattle concluding that SNP markers for TB resistance occur

and therefore genetic variation to bovine tuberculosis occurs in cattle. The potential to select for resistance genes towards TB in cattle breeding programmes therefore exists.

Another disease which has been researched due to its impact on loss of productivity is mastitis. Mastitis resistance is considered as a functional trait with a combination of somatic score, udder depth, fore udder attachment, recorded mastitis and body condition score contributing to positive resistance. However, despite several years of investigation the heritability of mastitis resistance remains at only 4% (CDN, 2013).

2.7.2 Potential to breed for Johne's resistance

Research studies to determine if genetic markers exist in order to predict Johne's disease susceptibility have been completed. However, genetic susceptibility to Johne's disease is complex. For example, in a recent GWAS completed by Minozzi *et al* (2012) it was found that multiple loci (11 different chromosomes) are associated with MAP infections in cattle. This is furthered complicated by breed factors with a study completed by Ruiz-Larranaga *et al* (2010) finding that in Braham-Angus crossbred cattle, biomarkers for MAP infection were found in the caspase associated recruitment domain 15 (CARD15). The study revealed however, that the gene is not found in Holstein cattle and is therefore unrepresentative of many dairy cattle.

Specific immunological genes can be targeted, for example the Solute Carrier Family 11 Member 1 (SLC11A1) gene has been identified to exhibit polymorphisms that are associated with MAP susceptibility in cattle. The SLC11A1 gene is an iron transporter protein which is primarily expressed in phagosomes (Purdie *et al*, 2011). An early study completed by Arias *et al* (1997) found that the protein exhibits pleiotropic effects on the early innate macrophage in the mouse model, leading to regulation of inducible nitric oxide synthase in order to control intracellular bacterial growth.

As outlined in Section 2.4.5, the MHC is involved in antigen presentation to T-cells resulting in the secretion of IFN-gamma. IFN-gamma leads to macrophage activation as well as NO production. MHC genes are therefore key targets for genetic association studies towards MAP. Human studies have

identified that susceptibility has been associated with mutations in IFN-gamma receptor genes (Vosse, Dissel and Ottenhoff, 2009) in animal studies, research has shown that in Merino flocks of sheep, an association of MHC polymorphisms to MAP infection susceptibility has also been found (Reddacliff et al, 2005). These associations show that there is genetic potential to breed for MAP resistance with SNP technology ensuring that specific genes can be identified for breeding programmes. The heritability of these traits however, have not been investigated.

2.7.3 Problems with breeding for disease resistance

Despite there being an opportunity to breed for specific disease resistance, there are problems associated with breeding for specific traits. In addition to low heritability, breeding for single traits can have negative effects on other desirable traits. For example, milk production traits which, in the past have commonly been selected by breeders have shown to have negative consequences for mastitis incidence in dairy cattle. An unfavourable genetic correlation between milk yield and mastitis resistance, as well as mastitis resistance being of low heritability has meant that a reduction of mastitis through traditional breeding has been unsuccessful (Kadri *et al*, 2015).

Another problem in breeding for disease resistance is that AMIR and CMIR have a negative genetic correlation[11] (Thompson-Crispi, Miglior and Mallard, 2013). This means that in breeding for a reduction of one specific disease such as mastitis, which is predominantly met by AMIR the breeding could inadvertently be breeding out a CMIR response which defends against intracellular pathogens such as *Mycobacterium* therefore reducing the animal's ability to defend against a broad range of infectious organisms (Animal Genetics and Breeding Unit, 2014).

[11] In the diseases studied: mastitis, retained placenta and ketosis

2.8 High Immune Response Technology

The literature has demonstrated that disease prevalence in dairy systems can be problematic. It poses a risk to farm economics, risks the welfare of cattle and in diseases such as Johne's disease there is also the potential for zoonotic threats (Botsaris et al, 2016). In addition, it has been discussed how disease resistance is increasingly being bred for through the development of breeding technologies which can mark genes in order to encourage certain health traits. However, despite the advancements in technology, breeding for disease resistance is often based on a specific disease such as mastitis and at present does not cover a multitude of diseases in the UK. This has been shown in practice recently with the 'TB Advantage' index being published by the Dairy division of the Agriculture and Horticulture Development Board on the 19th January 2016. This index is designed to help farmers breed for enhanced resistance to bovine tuberculosis in the UK, however, with a heritability of 9% and a focus on a single trait it does not cover a range of diseases although bulls in the index do rank as high health performers (AHDB, 2016c).

According to Thompson-Crispi, Miglior and Mallard (2013): 'A robust and balanced immune system of the dairy cow is vital for the protection against economically important diseases'. This is the basis of an immune technology in disease prevention, patented as the High Immune Response (HIR) technology. This technology was developed through an investigation of the negative correlation between AMIR and CMIR and uses a test system in order to identify cattle which have high and balanced AMIR and CMIR and in turn has been proven to reduce disease such as mastitis, metritis, retained placenta, displaced abomasum (Mallard et al, 2011) and pneumonia (Semex, 2015) as well as increasing farm profits, improving milk quality and assuring animal welfare is at a high standard (Mallard *et al*, 2011). As well as promoting a good adaptive immune response through a balanced and high AMIR CMIR ratio, the HIR technology also increases the efficiency of the animal's innate immune response (Mallard *et al*, 2014).

The benefits that the HIR technology provides include:

- Lower disease occurrence and severity

- Reduced veterinary intervention and cost
- Better vaccination response
- Colostrum quality improvement
- Testing can be from 2 months old
- Lifelong test
- Cost analysis= significant savings in HIR identification (Thompson-Crispi and Mallard, 2011)

2.8.1 Genetics and HIR

Research carried out by Mallard et al (2011), has found that selective breeding for high (HIR), average (AIR) and low responders (LIR) to disease is possible in rodents, poultry, pigs and cattle. In addition to the evidence supporting the breeding of genetic resistance to disease into these livestock species, research has also shown that the heritability of HIR is sufficient enough to allow genetic improvements within the herd, especially when combined with *in vitro* fertilisation and embryo transfer methods. According to Semex (2015), the heritability of immune response (IR) in cattle is 30% which shows a significant genetic component in IR traits and is at a similar rate of production traits at 25-35% such as milk yield which is commonly bred for in the dairy industry. This compares to other traits which are routinely bred for such as fertility which exists at a 4-7% heritability rate, longevity 8-10% and calving ease 6-7%. As previously outlined in the literature, heritability for resistance to specific diseases are even lower than these commonly used breeding traits and are generally lower than 10% (as referred to previously with the 'TB Advantage'). Daughters of HIR sires have been shown to have 44% less mastitis and 25% less calf pneumonia (Mallard *et al*, 2014). The potential to breed HIR genetics in to dairy herds therefore has promising outcomes (Thompson-Crispi and Mallard, 2010).

2.8.2 HIR test system

The HIR test system allows dairy managers to identify cattle as low, average or high responders with results enabling a selection of cattle to breed from in

order to gain the genetic merit of immune response as well as identifying cattle with low immune response either for culling out or applying tailored management such as enhanced vaccination programs or through nutrition techniques to ensure good welfare standards are met.

The HIR test is carried out over 15 days with 3 farm visits (see Figure 5). Firstly cows are immunized with the patent test antigen system in order to measure their ability to mount an immune response towards a challenge. Blood and or milk samples are then collected in order to measure the animals AMIR. CMIR is measured through a skin fold test and the two results correlated. It is critical that for HIR both tests provide a sufficient balanced immune response.

Figure 5: HIR Test procedure

Some materials have been removed due to 3rd party copyright. The unabridged version can be viewed in Lancester Library - Coventry University.

Finally, cattle are ranked according to their ability to pass on the genetic immune response based on EBVs (Thompson-Crispi and Mallard, 2010).

2.8.3 Application

As discussed previously in the literature genomics have enabled breeding practices to revolutionise, especially through the use of SNP chip technology. Genetic gain can now be achieved at a rapid rate through a reduction of the genetic interval and accuracy of selection. The HIR technology combined with genomics can therefore increase immune response objectives in breeding programs and accelerate the time in which it takes to gain a herd with HIR status. In addition, the HIR technology does not require genetic manipulation and can therefore be bred for naturally, thus avoiding the controversies surrounding genetically modified organisms (GMO's) (Mallard et al, 2014).

Through research in Canada it has been found that around 200 SNP markers are associated with HIR (Thomson-Crispi and Mallard, 2010) although this is expected to have increased over time with further research in the field.

In practice, the patent of the HIR technology is owned by Semex who have launched a line of Immunity+ sires (Semex, 2015). These sires are currently being used in dairy breeding and are therefore passing on the heritability of immune response to their daughters. Previous research found that high AMIR is correlated with lower milk yields which initially makes the test system and use of HIR in breeding practices unfavourable, however it has been found that high CMIR is correlated with high milk yield and therefore when both traits are selected for, ultimately there are no adverse effects on milk yield (Watger *et al*, 2003). A strong response has been found towards the technology in the US and Canada (Mallard *et al*, 2011) however the technology is not widely recognised in the UK and therefore research into how HIR could aid disease control of Johne's disease has had little acknowledgement.

2.8.4 Potential for HIR in the UK

The literature has outlined the implications that Johne's disease has for the welfare of cattle (Gov.UK, 2012) as well as the economic losses experienced by farmers with Johne's disease infected herds (Guitian, 2015). Potential exists in breeding programmes with the identification of gene markers which contribute to disease resistance (Minozzi *et al*, 2012) (Purdie *et al*, 2011), however these genes are heritable at low rates as well as posing a risk to other desirable traits (Hinger, Brandt and Erhardt, 2008) (Kadri *et al*, 2015). It is therefore believed that the HIR technology, which focuses on equipping cattle with broad based disease resistance (Thompson-Crispi, Miglior and Mallard, 2013) and has shown in preliminary studies that HIR cattle are less likely to test positive for Johne's disease (Semex, 2015), is a breeding tool which can be used to reduce Johne's disease in the UK.

2.9 Hypothesis Formation

The literature has indicated that breeding for health is not only viable but also recognised by the industry as a key performance trait. Through an analysis of the literature it is hypothesised that cattle with high immune response traits will be better equipped immunologically to defend against pathogens such as MAP due to having a better innate response as well as high CMIR which defends against intracellular pathogens. Preliminary studies also indicate that cattle with HIR EBVs are less likely to be test positive for Johne's disease.

The researcher investigated macrophage response towards whole cell deactivated MAP and two proteins from MAP, from cattle with the following EBVs: H (AMIR) H (CMIR), L (AMIR) L (CMIR), H (AMIR) L (CMIR) and L (AMIR) H (CMIR). In order to measure the immune response, Nitric Oxide (NO) and pH of the phagosome was measured.

The researcher has made the following alternate hypotheses (H1):

- HH macrophages will have higher production of NO
- HH macrophages will have a lower drop in pH
- LL macrophages pH will not drop as significantly- NO will also be produced at lower rates
- LH macrophages will produce slightly more NO and drop pH to a lower level than HL macrophages, as they have a higher CMIR. However, as this research will be recording an innate response it is likely that there won't be a significant difference between the two

The null hypothesis (H0) is that macrophages from each cow, regardless of their EBV will produce the same results.

3. Methodology

3.1 Aims and objectives of the research

The aim of this research project was to complete an *in-vitro* investigation in order to determine if macrophages from cattle with high immune response traits were more capable of mounting a successful immune response towards MAP pathogens (deactivated) and recombinant proteins from MAP[12] than cattle with a low immune response. In order to measure immune response the researcher recorded NO release as well as the pH of the phagosome, both indicators of immune response as defined in the literature review.

The key research objectives behind this methodology are:

- To investigate if HH macrophages produce higher NO than LL macrophages, and if HH drop pH lower than LL
- To establish if there are any differences between HL and LH macrophage types in terms of immune response
- To establish whether HH EBV cattle be used to breed for increased resistance to Johne's disease
- To investigate if the UK dairy industry could benefit from adopting the HIR test system at a commercial scale

3.2 *In-vitro* Experiment

In total 16 Holstein cows (mid-lactation with no clinical sign of disease and negative for Johne's disease) were sampled from the University of Guelph's cattle research station in Elora, Ontario. Although 16 samples were run, two samples were lost due to macrophage infection in-vitro. The researcher therefore only used three cow samples per cohort (12 cows, in total). The cattle had been tested for immune response previously with the following HIR EBVs:

[12] Referred to as Protein 1 and Protein 2

Table 1: HIR EBV's of cattle selected for research study

Cow Number	AMIR	CMIR	EBV
4221	1.13	-1.41	HL
4322	1.21	-1.47	HL
4200	0.91	-1.00	HL
4215	0.94	-1.58	HL
4095	-1.79	1.13	LH
4314	-1.04	1.19	LH
4323	-2.16	1.11	LH
4349	-2.14	1.95	LH
4209	1.83	1.13	HH
4345	1.33	1.84	HH
4056	0.98	2.06	HH
4238	0.88	1.49	HH
4192	-1.29	-1.89	LL
4212	-1.40	-1.46	LL
4233	-1.14	-1.41	LL
4294	-1.75	-1.25	LL

The cattle were then further split into four cohorts:

- Low AMIR, Low CMIR (**LL**)
- High AMIR, High CMIR (**HH**)
- Low AMIR, High CMIR (**LH**)
- High AMIR, Low CMIR (**HL**)

Table 2: EBV cohorts

LL	**HH**
4214, 4192, 4294, 4233	4056, 4345, 4209, 4238
LH	**HL**
4349, 4095, 4314, 4323	4221, 4322, 4200, 4215

In order to minimise bias the researcher ran a LL sample at the same time as an HH sample and a LH sample at the same time as an HL sample. It was decided that only 2 samples could be run per day due to the complexity and time consumption of the procedure, this ensured that blood was not standing for pro-longed periods of time which was found to have a negative effect on macrophage viability and yield. The preliminary research phase also highlighted that macrophage yield was highly dependent on the blood collection method and that the inversion of EDTA vacutainer tubes was essential in the ultimate viability of the macrophages.

The procedure followed was derived from Okudolo and Cash (2015) and optimised by Emam (2015). The procedure is outlined below:

Day 0: Culturing Blood Mononuclear Cells

1. Collect 10 EDTA coated vacutainer tubes of blood from each research cow (tube to be inverted several times following collection)
2. Prepare cell culture hood using 70% ethanol
3. Centrifuge for 20 minutes at 1200g at room temperature (brake on)
4. Remove the buffy coat layer from all tubes from one animal with a transfer pipette and put into a 50ml conical tube, dilute up to 15ml with DPBS
5. Add 15ml of histopaque into a SepMate-50 tube through the central hole
6. Keeping the SepMate-50 tube vertical, add the diluted sample of buffy coat by pipetting down the side of the tube, slowly.
7. Centrifuge for 12 minutes at 1200g at room temperature (brake off)
8. Pour off the top layer (which contains mononuclear cells) into a 50ml tube. Do not hold the SepMate-50 tube in the inverted position for longer than 2 seconds. Mix with a transfer pipette and top up to 45ml with DPBS
9. Centrifuge for 10 minutes at 100g at room temperature (brake on)

10. Remove the supernatant using a 25ml pipette. Use a transfer pipette to take the last 2ml of supernatant very slowly (pellet will be loose)
11. Add 45ml DPBS and re-suspend the pellet using a transfer pipette.
12. Centrifuge for 5 minutes at 400g (brake on)
13. Pour off the supernatant into the waste bucket (invert tube slowly and only once)
14. Add ~1ml of autoclaved distilled water to the tube with the pellet to lyse and red blood cells left in the pellet. Re-suspend by pipetting up and down with a transfer pipette. IMMEDIATELY add DPBS to 50ml (this should be done in less than 5 seconds)
15. Centrifuge for 5 minutes at 400g at room temperature (brake on)
16. Pour off supernatant being sure not to dislodge the pellet and add 10ml of DPBS to re-suspend
17. Add 50µl of cell suspension into a 0.6 micro-centrifuge tube and then add 450µl of DPBS (1:20 dilution). Pipette up and down to mix.
18. Use a Moxi-Z cell counter[13] (S type cassette) to determine the cell concentration which should be ~10e6 with a moxi population index (MPI) of greater than 0.90 (Multiply the cell count by the dilution factor 10 to get actual cell concentration of the sample)
19. Calculate the amount of cell media to be transferred to a 75ml culture flask by dividing cell concentration by 7.5e7 (see Figure 6)

[13] A Moxi Z Cell Counter performs cell count and size measurements for particle sizes of 3 – 20 microns (Type S cassette). It also provides an assessment of mammalian culture viability using a proprietary software algorithm to report the standardized Moxi Population Index (MPI) (Orflo, 2012)

Figure 6: Calculation example for 75ml flask

Sample: 4314 (LH)

Dilution factor 1:10

MPI- 1.00

1.01e6 cells/ml = 1,010,000

1,010,000 x 10 = 10,100,000

75,000,000 ÷ 10,100,000 = 7.42 ml of cell media to transfer

20. Transfer calculated amount of cell media to the culture flask along with 15ml of room temperate monocyte attachment media[14]
21. Incubate for 2 hours at 37ºC at 5%CO^2
22. Remove the flask from the incubator and wash twice using room temperature DPBS to remove all non-adherent cells
23. Add 20ml of room temperature AIM-V media and return to the incubator (37ºC at 5%CO^2)

Day 2: Change Media

24. Add 50% of the total volume of fresh AIM-V media (10ml)
25. Return flask to incubator (37ºC at 5%CO^2)

Day 5: Replace Media

26. Take out 50% of the media (15ml) and replace with 15ml of fresh AIM-V
27. Return flask to incubator (37ºC at 5%CO^2)

[14] Monocyte Attachment Medium allows efficient adherence selection of Monocytes from freshly isolated Mononuclear Cells. It also maintains optimal cell health. Large numbers of 80-90% pure monocytes can be obtained through the use of monocyte attachment medium (PromoCell, 2013)

Day 7: Harvest Macrophages

28. Take flask out of the incubator and wash twice using room temperature DPBS
29. Add 10ml of ice cold DPBS containing 5mM EDTA
30. Place the flask on ice for 15 minutes, move the flask over the ice for 30 seconds and tap the flask on the edge of a bench every 5 minutes, when the flask is returned to the ice keep it moving for the initial 30 seconds
31. Transfer the detached cells into a 50ml conical tube
32. Add 10ml of pre-warmed 1X TrypLE® to the flask and incubate for 15 minutes (37ºC at 5%CO^2)
33. Take the flask out of the incubator and into the hood, load the media into the pipette and flush over the surface of the flask several times
34. Return the flask to the incubator (37ºC at 5%CO^2) for 5 minutes
35. Repeat step 33 and then transfer the cell solution into the conical tube from step 31
36. Centrifuge the concial tube containing the cell solution for 10 minutes at 600g (brake on, room temperature)
37. Discard the supernatant and re-suspend the pellet in 2ml of room temperature AIM-V
38. Make a 1:10 dilution of cell suspension in DPBS by diluting 10µl of cell suspension with 90µl of AIM-V
39. Use the Moxi cell counter (M type cassette) to count the cells and then multiply the cell count by the dilution factor to get the actual cell count
40. Cells to be added to a 90 well flat bottomed plate and to a phago-assay clear bottomed plate at 50k cells/well, in order to determine the amount of cell media to add to each well follow the C1 x V1 = C2 x V2 formula, see Figure 7:

Figure 7: Calculation example for plating

Sample: 4314 (LH)

Dilution factor 1:10

9e4 cells/ ml = 90,000 x 10 (dilution factor) = 900,000 cells/ ml

C1	x	V1
900,000 (cells/ml)		2.1ml (amount of cell stock)

C2	x	V2
500,000 (cells to be added/well)		?

C1 x V1 = 1,890,000 ÷ C2 500,000 = 3.78ml

41. Return the plated cells to the incubator for 24 hours (37ºC at 5%CO^2)

Day 8: Macrophage Challenge

The macrophages from each sample were challenged on day 8 with recombinant proteins from MAP, Protein 1 (P1) and Protein 2 (P2) as well as whole cell heat deactivated MAP. In order to optimise the experiment 4 cattle samples were run by the researcher prior to the 16 cow sample so that the most effective challenge dose could be determined. It was found that the most effective challenges were protein 1 at a dose of 0.1 µg/ ml (C1) and protein 2 at a dose of 1 µg/ml (C2). The most effective for the whole cell deactivated MAP was found to be at a ratio of 10:1 (MAP: MØ)

Tables 3 and 4 show the plate layout and pathogen challenge used for all 16 samples, in order to measure pH as well as nitric oxide separate plates were used. Replicates ensure standardisation and reduce error.

Table 3: pH Experiment Plate Layout

	1	2
A	P1C1 (0.1µg/ml)	P1C1 (0.1µg/ml)
B	P1C2 (1µg/ml)	P1C2 (1µg/ml)
C	P2C1 (0.1µg/ml)	P2C1 (0.1µg/ml)
D	P2C2 (1µg/ml)	P2C2 (1µg/ml)
E	Control	Control

Note: Whole cell deactivated MAP was not used in this experiment due to its lack of availability. The whole cell MAP was used in the pilot however, did not yield successful results and therefore a decision was made to use the proteins only.

Table 4: Nitric Oxide Experiment Plate Layout

	1	2
A	MAP (10:1)	MAP (10:1)
B	P1C1 (0.1µg/ml)	P1C1 (0.1µg/ml)
C	P2C2 (1µg/ml)	P2C2 (1µg/ml)
D	Control	Control

Following the pathogen challenge both plates were returned to the incubator (37ºC at 5%CO^2).

Day 8: pH Experiment: Phago Assay

The phago assay plate was incubated for three hours following the pathogen challenge. It was then removed from the incubator and the supernatant discarded from all wells. The wells were washed with 100µl of DPBS/ well

before phago assay e-coli solution being added to each well at 100µl/ well. The plate was then returned to the incubator for a further 3 hours. This method was used as a suitable pH indicator had not been conjugated to MAP or MAP recombinant proteins, as the protein challenges had been introduced 24 hours previously the macrophages would have begun to phagocytose the pathogen and therefore using the conjugated e-coli served as a method of reading the internal pH of the phagosome without interfering with the results of the MAP recombinant proteins significantly.

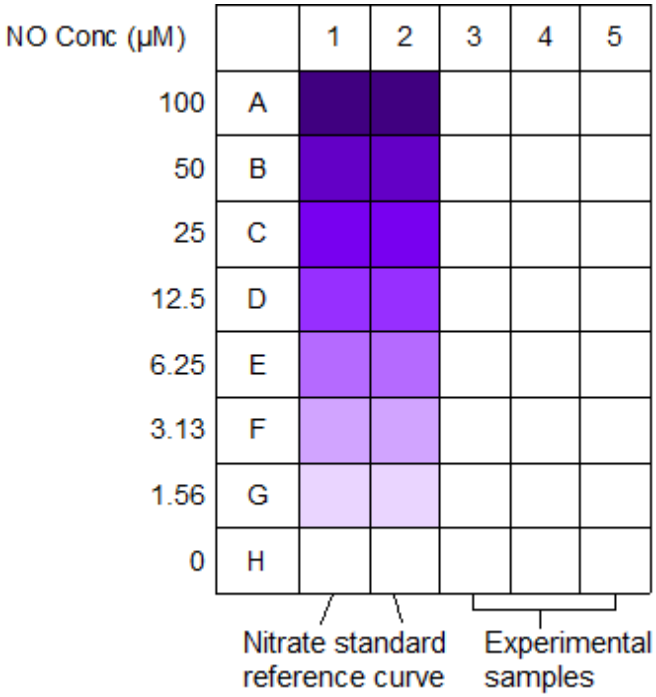
Following 3 hours of incubation the plate was removed from the incubator, the contents from each well were removed and each well was washed with 150µl of DPBS. The plate was then read using a pH reader in the laboratory and results recorded.

Day 10: Nitric Oxide (NO) Concentration Experiment

The NO experiment was carried out following 48 hours of macrophage and pathogen challenge co-culture. The experiment followed the Greiss Reagent System, a protocol from Promega and is outlined below:

1. Make a 1:10 dilution of nitrate solution with AIM-V, vortex well to mix
2. Use a new 96-well flat-bottom enzymatic assay plate. Add 50µl of AIM-V to first two columns starting at B1 and B2 (leave A1 and A2 empty)
3. Add 100µl of the nitrate solution to the top two wells (A1 and A2)
4. Take 50µl from the top two wells to the second two wells (B1 and B2) and mix 10 times with a pipette
5. Repeat process until the two wells before the final two wells are reached- take 50µl out and dispense (the final 2 wells will remain as AIM-V only and act as the control) this will create the nitrate standard reference curve (see Figure 8 for visual description)

Figure 8: Visual Representation of Steps 3 to 5 of the NO Experiment



6. Add 50µl of the supernatant from each well from the co-cultured macrophage and challenge plate to each well in the enzymatic assay plate
7. Add 50µl of sulfanilamide solution to each well, cover and leave for 10 minutes in the dark
8. Add 50µl of N-1-napthylethylenediamine dihydrochloride (NED) solution to each well, return to the dark for a further 10 minutes
9. Read the plate using a reader with a 520–550nm filter

(Promega, 2009)

3.3 Data Collection and Statistical Analysis

All data that was collected through the experiment (NO release, pH read out) was entered into Excel spreadsheets. The data was then analysed through GenStat fifteenth edition, using an analysis of variance (ANOVA) test. Histograms were also produced in order to visually explore the data.

This form of research is quantitative in nature as it based on numerical elements using figures, numbers and mathematical equations to help analyse data (Dransfield et al 2004:621). This will enable the researcher to produce reliable data from a sample group which can then be applied to a larger population.

3 Results

Experimental data can be found in Appendix B. A full GenStat report can be found in Appendix C.

3.1 Nitric Oxide Release Experiments

An analysis of the data through GenStat using the two-way analysis of variance (ANOVA) method showed that there were no statistically significant differences between the EBV groups for NO release (probability score of <0.05 required to show statistical significance).

Please note, the control for each experiment was 0, the control was not used within the statistical analysis in order to ensure the results weren't skewed. This applies to figures 9, 10, 13 and 14.

Figure 9: Genstat two-way ANOVA results for NO release

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
EBV	3	247.83	82.61	7.35	0.001
Treatment	2	70.36	35.18	3.13	0.062
EBV.Treatment	6	31.07	5.18	0.46	0.830
Residual	24	269.80	11.24		
Total	35	619.05			

Source of variation explained in footnote[15]

The researcher also ran contrast testing to measure HH against LL and HL against LH to determine statistical difference. Although the HH EBV produced a higher concentration of NO throughout the experiments, the difference between the two cohorts was not statistically significant.

15

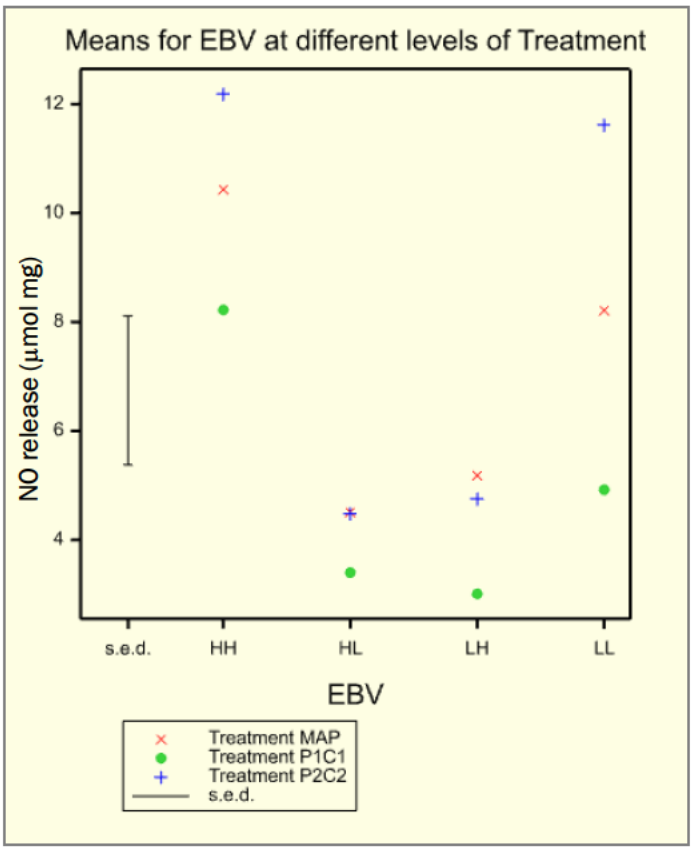
d.f	degrees of freedom in the source
s.s	the sum of squares due to the source
m.s	the mean sum of squares due to the source
v.r	variance ratio
F-Pr	the P-value- Probability

Figure 10: Genstat two-way ANOVA contrast test between HH and LL EBV groups

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
EBV	3	247.83	82.61	7.35	0.001
Contrast 1	1	18.55	18.55	1.65	0.211
Treatment	2	70.36	35.18	3.13	0.062
EBV.Treatment	6	31.07	5.18	0.46	0.830
Contrast 1.Treatment	2	5.70	2.85	0.25	0.778
Residual	24	269.80	11.24		
Total	35	619.05			

Statistically, the largest difference was observed between the HH EBV cattle and the HL EBV cattle. This is represented visually in the plot graph below.

Figure 11: Plot graph of NO release by EBV group



Note: Controls were taken at a value of 0 and are not represented on this graph.

s.e.d : Standard error of differences

Figure 12: Bar graph to show average NO release by EBV group

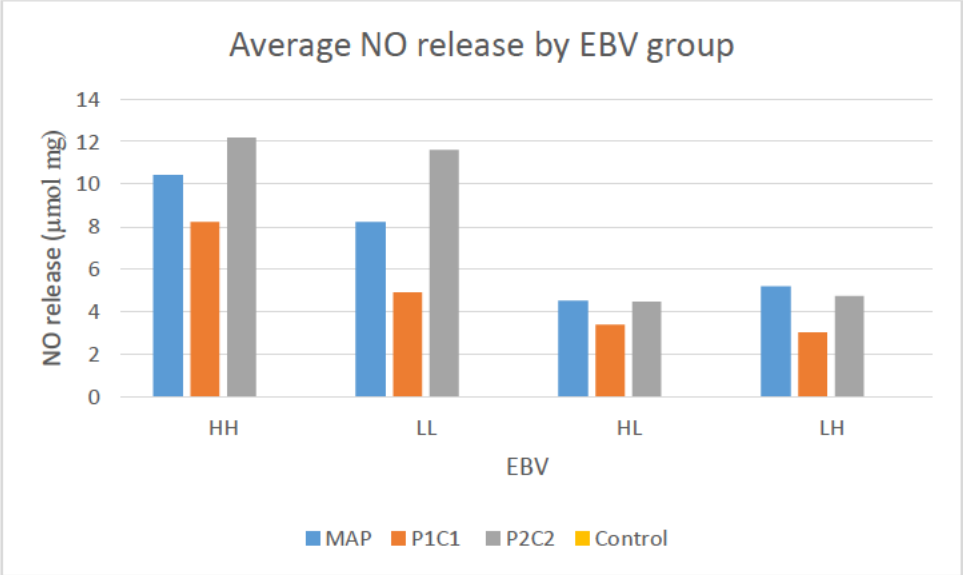


Figure 12 shows average NO production by EBV group. The graph visually represents that the HH macrophages produced a higher amount of NO than the other EBV groups. The data also shows that the macrophages were able to recognise and react to the recombinant protein 2 more effectively than recombinant protein 1. The HH and LL EBV's were run on the same day and the HL and LH on a separate day, it is therefore recommended that the HH and LL are compared separately to the HL, LH macrophages. This will be discussed further in the analysis.

3.2 pH Experiments

The researcher also ran the pH data through a two-way ANOVA in order to test EBV against treatment.

Despite the HH EBV dropping pH to a lower level than the LL EBV (see Figure 13, the data did not prove it to be statistically significant (<0.001)

Figure 13: Genstat two-way ANOVA results for pH release

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
EBV	3	3032514.	1010838.	3.67	0.063
Treatment	1	2856.	2856.	0.01	0.921
EBV.Treatment	3	64559.	21520.	0.08	0.970
Residual	8	2202676.	275334.		
Total	15	5302606.			

ANOVA two-way contrast testing was performed between the EBVs to investigate any statistically significant differences. A statistically significant results was found between the HL and LH EBVs. This is outlined in Figure 14 below and visually represented in Figure 15.

Figure 14: Genstat two-way ANOVA contrast test between HL and LH EBV groups

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
EBV	3	3032514.	1010838.	3.67	0.063
Contrast 1	1	2916667.	2916667.	10.59	0.012
Treatment	1	2856.	2856.	0.01	0.921
EBV.Treatment	3	64559.	21520.	0.08	0.970
Contrast 1.Treatment	1	55331.	55331.	0.20	0.666
Residual	8	2202676.	275334.		
Total	15	5302606.			

Figure 15: pH recording by EBV group

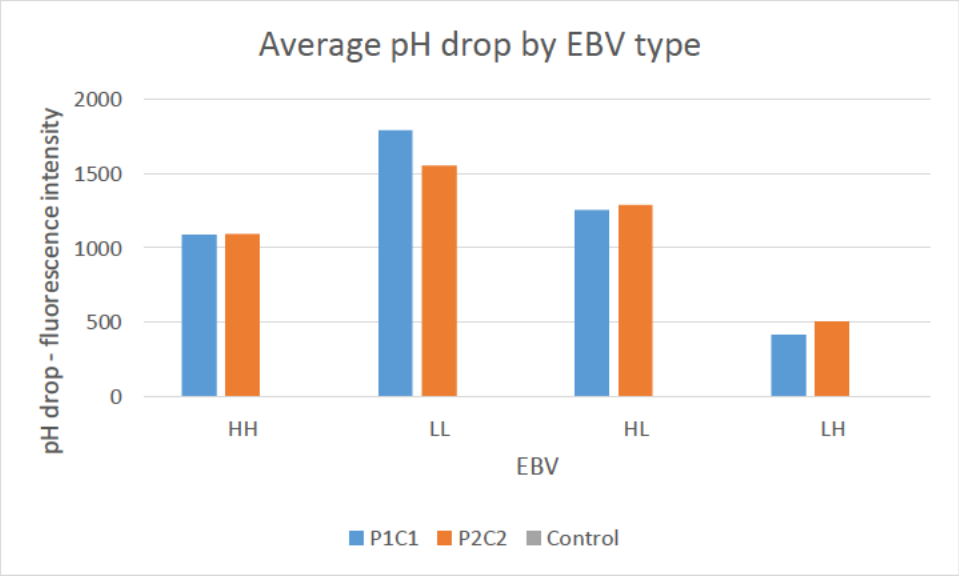


Figure 15 shows average pH drop by EBV. The pH is measured by fluorescence intensity, the lower the intensity the further the drop in pH. The graph visually represents that HH macrophage pH was lower than LL macrophage pH. Additionally, the LH EBV shows a much lower drop than the HL EBV. Despite GenStat analysis providing statistically insignificant results for HH and LL contrasts, the visual representation above fits with the alternate

hypothesis that the HH cohort would produce drop the pH to a lower level than the LL cohort.

4 Discussion

This research study has investigated the immune response of cattle towards MAP pathogens, *in vitro*. Previous research studies have found that once MAP pathogens enter the host they are primarily met by macrophage cells as part of the innate immune response (Rue-Albrecht et al, 2014). The production of nitric oxide and an internal drop in the pH of the macrophage, are both mechanisms of immune response (Janeway et al, 2005) and work to eliminate invading pathogens.

The HIR technology has been shown to reduce a range of diseases including mastitis, metritis and displaced abomasum as well as preliminary research showing that cattle are less likely to test positive for Johne's disease. HIR cattle have been shown to have a stronger innate immune response than LIR cattle (Mallard *et al,* 2014). The HIR technology works by measuring the two components of the adaptive immune response, AMIR and CMIR. If both AMIR and CMIR are high, cattle have a good broad based immune response and can tackle a range of intracellular and extracellular pathogens. As MAP is an intracellular pathogen it is important that CMIR is high, but as identified in the literature review, AMIR and CMIR can be negatively genetically correlated (Thompson-Crispi, Miglior and Mallard, 2010) and therefore by breeding for resistance to one specific disease such as with TB breeding strategies, it can have implications for broad based disease resistance.

The researcher measured NO production and pH drop of four immune response EBV's, HH, LL, HL and LH. The null hypothesis was that all cohorts would produce approximately the same amount of NO as well as pH being equal across the EBV groups. The alternative hypothesis however, stated that HH macrophages would have higher NO production than LL macrophages as well as dropping pH to a lower level. It was also hypothesised that LH macrophages would have higher NO production and drop pH further than HL macrophages.

4.1 Nitric Oxide Production

As outlined in the results section, NO release from HH macrophages was higher than LL macrophages. However, the data collected in this research study did not show any statistically significant differences. A two-way analysis of variance (ANOVA) test examines the influence of two independent variables on one continuous dependable variable (VSNi, 2016). In this research study, the two independent variables were treatment (whole cell deactivated MAP, recombinant protein 1 and recombinant protein 2) and EBV (HH, LL, HL, LH) the continuous dependable variable was NO release.

The alternate hypothesis stated that cattle with a HH EBV, would produce a higher concentration of NO than cattle with a LL EBV, based on HH cattle having a better innate and adaptive immune response (Mallard *et al*, 2014). Whilst the alternate hypothesis was proved correct with HH EBV cattle having a mean production of 10.28 compared with LL EBV cattle which produced a mean of 8.25, in terms of statistics no significance was found, even when ANOVA contrast testing between the two EBVs was employed. This means that the alternate hypothesis should be rejected statistically. Despite the alternate hypothesis being rejected in this study, it should be noted that the sample size was small and therefore a larger group of animals should be tested before the alternate hypothesis is fully rejected.

The HL and LH EBV cattle exhibited very little difference in terms of NO production with HL and LH cattle producing means of 4.13 and 4.32 retrospectively. The alternate hypothesis stated that LH macrophages would produce a slightly higher concentration of NO, and although marginally higher the alternate hypothesis is rejected due to there being no statistical significance.

The researcher expected that cattle with LH EBVs would produce a higher concentration of NO than those with HL EBVs due to MAP being an intracellular pathogen and therefore requiring a strong CMIR to eliminate it. The macrophages were challenged on day 8 of the experiment with pH being recorded on day 8 and NO being recorded on day 10. In a live host, this stage of infection would be met by an innate response which is non-specific and

therefore does not confer adaptive AMIR and CMIR responses. It is therefore assumed that this is why HL and LH EBV cattle didn't produce significantly different immune responses at this stage within an *in vitro* experiment. The nature of the experiment being *in vitro* also limits the complex reactions between the immune response cells such as cytokine release and the complement system which would occur in an *in vivo* experiment.

ANOVA testing also enabled the researcher to compare treatments. It was found that the recombinant protein 2 produced the highest NO mean at 8.26 across all EBVs (MAP whole cell, 7.09 and recombinant protein 1, 4.89). The researcher expected the proteins to produce a higher reaction than the MAP whole cell as they would bind directly to the macrophage cell surface. Whole cell pathogens on the other hand were predicted to cause less of a reaction due to cell surface reactors having to pass over each other before the macrophage recognised the MAP pathogen as a foreign body. It should be noted that the MAP pathogen was deactivated and therefore if live MAP pathogens were used a stronger reaction may have been observed.

4.2 pH drop

The pH experiment enabled the researcher to determine if macrophages had the ability to drop the intracellular compartment to an acidity capable of promoting phagolysosomal fusion and therefore eliminating the pathogen. As stated in the literature, MAP pathogens have the ability to evade the host immune system by blocking phagolysosomal fusion and therefore have the ability to reside in host macrophages (Arsenault *et al*, 2014). The researcher was therefore interested to investigate if HH macrophages were better equipped to create an acidic pH than LL macrophages. The researcher hypothesised that the HH EBV macrophages would drop pH to a lower level than LL macrophages and the LH macrophages would drop pH to a lower level than HL macrophages. The null hypothesis was that no significant difference would be found between groups.

Genstat analysis, using a two-way ANOVA found that there were no statistically significant differences between EBV groups. Contrast testing found that although not statistically significant a probability score of 0.061 existed

between HH and LL macrophages, indicating that difference did occur and that further investigation with a larger cohort of subjects should be undertaken. The researcher also measured the difference between the LL and LH EBV groups which had a probability of 0.012 making it a statistically significant result.

The researcher notes that the EBV group with the largest pH drop was the LH group. Whilst this indicates that this EBV had the most successful immune response towards the MAP challenge *in vitro* and could therefore work successfully to promote Johne's disease resistance, breeding for CMIR specifically has the potential to inadvertently breed out AMIR due to the negative genetic correlation between the two types of adaptive immunity, leaving the host more susceptible to extracellular diseases such as mastitis (Thompson-Crispi, Miglior and Mallard, 2013). In addition, this experiment predominantly measured the innate response and therefore the adaptive immune response which would be activated several days after the challenge may have produced an even stronger CMIR response making the LH EBV a desirable cohort for breeding specific resistance to Johne's disease, however as previously mentioned it is recommended that broad based disease resistance is bred for and not single traits such as CMIR specifically (Thompson-Crispi, Miglior and Mallard, 2013).

The researcher used the two recombinant proteins to measure pH due to the complexity of conjugating whole MAP to the florescent dye used to measure pH. The ANOVA test showed that recombinant protein 2 promoted a stronger reaction than protein 1. Reports in the literature investigated the use of vaccination as a method to reduce Johne's disease susceptibility and prevalence within herds (NADIS, 2016). It is suggested that recombinant protein 2 could be trialled as a vaccination component as it would not result in infection but successfully enable the host to identify the pathogen and therefore result in protective immunity based on an initial innate, followed by a CMIR response.

This research was completed using recombinant proteins from MAP, as opposed to the live whole cell. It is known that mycobacteria species use a range of mechanisms in order to evade the host immune response (Arsenault

et al, 2014) including phagolysosomal blocking with sulphide reactions and the secretion of liquid phosphate (SapM). Brumell and Scidmore (2007) found that deactivated MAP did not produce SapM which effectively prevents phagolysosomal fusion, whereas whole cell active MAP did. It should therefore be noted that if the researcher were to have used whole cell live MAP, the macrophages may not have been as successful in blocking the reaction. On the other hand, a greater variation could have been seen between the genetic cohorts as shown in research by Pais and Appelberg (2000) which found that only 10-15% of cattle exposed to MAP became infected which was influenced by family and genetics.

4.3 Application of HIR in the UK

This research has shown that there is potential to use the HIR technology to reduce diseases such as Johne's disease. The literature review has indicated that preliminary research has shown that the HIR technology can reduce susceptibility to Johne's disease (Mallard *et al*, 2014). The research carried out as part of this study supports this assumption, although a considerable amount of further laboratory research as well as *in vivo* experimentation is required to fully understand the interactions at a higher level. The researcher also acknowledges that this research study measured immune response at an innate stage and therefore the adaptive response was not be measured.

4.4.1 Breeding

As described in the literature, HIR traits are heritable at 30% (Semex, 2015). Heritability of immune response could therefore be achieved over a relatively short period of time in UK dairy herds, especially if breeding technologies such as *in vitro* fertilisation and embryo transfer are employed (ABRI, 2016). Additionally, the heritability of single traits are generally lower than HIR. For example, TB resistance is heritable at 9% (AHDB, 2016 c) it can be argued that the causative agent of TB is closely related to that of Johne's disease, a similar heritability rate could be found although as described in the literature due to the multiple loci associated with Johne's resistance disease, heritability is difficult to predict.

It is argued that breeding for HIR is beneficial in terms of promoting broad based disease resistance and as stated throughout this research study is recommended in order to defend against a range of intracellular and extracellular pathogens (Thompson-Crispi, Miglior and Mallard, 2013) (Mallard et al, 2014).

4.4.2 Potential problems

The researcher has identified that potential problems exist in breeding for resistance to specific diseases due to the negative correlation between AMIR and CMIR. In addition, the problems associated with breeding cattle with a LH EBV have also been discussed (Section 4.3).

This research study investigated the difference between immune response EBVs at an innate level, based on the evidence that HIR cattle have a stronger innate response that LIR cattle (Mallard *et al*, 2014). HIR cattle also promote a better adaptive immune response which is key to management of MAP within the host. As identified in the literature, MAP is predominantly met by CMIR (characterised by the production of IFN-gamma) which activates macrophages to kill the bacteria, due to its intracellular nature. A successful CMIR response will result in elimination of the pathogen however, if the pathogen is able to evade the host's immune response and reside within macrophages the immune response will shift to one that is mediated by AMIR (Arsenault *et al*, 2014). It is at this stage in live animals that the Johne's disease test will identify infected animals.

AMIR is activated once production of IFN-gamma is reduced and is characterised by the production of antibody. Whilst it is known that a HH EBV will promote broad based disease resistance, the researcher acknowledges that further research needs to be completed in order to determine whether a strong AMIR could lead to the response switching from a protective CMIR to an AMIR at an earlier stage. Research studies have so far not been able to define whether MAP specific antibodies play a significant role in controlling the rate of bacterial shedding or conversely increase the rate of uptake of bacteria by macrophages (Mundo et al, 2008). According to De Silva *et al* (2013), a strong CMIR resulted in delayed shedding in a sheep model, whereas Koets

et al (2002), found that both CMIR and AMIR were equally impaired during the later infection stages of Johne's disease. Magombedze, Eda and Ganusov (2014) suggested that one factor that influenced the switch between a CMIR and AMIR was the degree of competition between the two arms of response. This fits with the assumption made by the researcher above and is in relation to the principles of the HIR technology. With this assumption in mind, HL cattle could be less resistant to MAP than LH cattle, this was also represented in the data analysis. As previously mentioned however, breeding specifically for LH cattle would result in the host being more susceptible to intracellular infections as well as the AMIR response in Johne's disease potentially helping to disseminate infection (Magombedze, Eda and Ganusov, 2014).

5 Conclusion

This research project has shown that statistically significant differences did not exist between the four EBV groups of cattle (HH, LL, HL, LH) when macrophages were challenged *in vitro* with whole cell deactivated MAP and 2 recombinant MAP proteins for NO release, however there was a statistically significant result between the HL and LH groups for pH. The researcher measured two immune responses which indicate susceptibility/ resistance. Although the data analysis did not provide a statistical difference, it was observed that HH macrophages produced more NO and dropped pH to a lower level than LL macrophages, as predicted in the alternate hypothesis.

In addition, it was found that LH macrophages produced slightly more NO than HL and LL macrophages as well as dropping pH considerably lower than HL macrophages and statistically significantly lower than LH macrophages for pH. The literature indicates that MAP infection is primarily met by an innate immune response, this is followed by an adaptive immune response which is predominantly cell mediated (Arsenault et al, 2014). If the CMIR is unable to clear the pathogen, AMIR takes over and is argued to contribute to pathogen dissemination (Mundo et al, 2008) (Magombedze, Eda and Ganusov, 2014). Although the reason for the switch between CMIR and AMIR is poorly understood it is suggested that it could be due to competition between the two arms of response (Magombedze, Eda and Ganusov, 2014). Based on this assumption and the results of this research study suggest that the ideal EBV for breeding resistance to Johne's disease is the LH EBV. However, breeding for specific disease resistance has a number of problems due to the negative correlation between the two arms of immune response (Thomson-Crispi, Miglior and Mallard, 2013) which in the case of LH cattle, could lead to the animal being more susceptible to extracellular pathogens.

The aim of this study was to investigate whether differences in immune response towards MAP existed between cattle EBV groups. Although no statistical significance was identified, clear differences were observed between groups and therefore it is advised that further in depth study is undertaken *in*

vitro in order to determine if HIR cattle are more successful at promoting an immune response toward MAP.

6 Limitations and Recommendations

This study has been limited in a number of ways. The key limitation was researcher experience with cell culture and macrophage isolation. The process of macrophage isolation from blood is complex and therefore the researcher needed to become confident in the research procedure before running actual samples which reduced the available time for data collection. In addition to researcher experience, the methodology also needed to be optimised which also reduced the available time to collect data and therefore reduced the number of animals in the experiments.

The research phase was carried out over a period of three months with each experiment being run over two weeks. Although the process of macrophage isolation had already been optimized by Emam (2015), a number of issues arose during further optimization and experimentation which need to be taken into account when analysing the results. One of the key issues observed was the method of blood collection. For each animal, EDTA vacutainer tubes of blood were taken, EDTA tubes help to prevent clotting, however in order to ensure this, it is important that the tube is inverted during and after blood collection in order to mix the anticoagulant. It was identified that due to the time constraints of the research facility, the tubes were not being inverted fully and therefore the viability of the macrophages were implicated due to clotting. The researcher observed that within a cohort of four cows (HH, LL, HL and LH) the blood collection process had an effect on how viable the macrophages were. For example, on the 12th October 2015 a HH and a LL cow were identified for experimentation. The first cow to have blood taken was the LL cow, the subject was calm, remained lying down and inversion of the tube was carried out throughout the process of drawing blood. The HH cow however became restless after the first tube of blood was drawn and began to move away from the researcher, due to this the researcher found it hard to invert the tube correctly. It was identified in the laboratory when observing the blood that the HH blood was clotted and when centrifuged a very poor buffy coat layer was formed. The researcher continued the experiment and measured macrophage

health on day 8. As expected the LL macrophages were healthy, whereas the HH macrophages had very poor viability.

Figure 16: Example of poor EDTA blood tube collection and subsequent effect on experimental results. NO measurements in μmol mg.

Sample 14 (4294) LL			
NO Experiment			
			Average
MAP 40:1	19.881	22.553	**21.217**
P1C1	20.211	17.98	**19.0955**
P2C2	22.838	19.076	**20.957**

Sample 13 (4209) HH			
NO Experiment			
			Average
MAP 40:1	2.009	1.758	**1.8835**
P1C1	1.943	1.877	**1.91**
P2C2	2.273	2.339	**2.306**

Figure 16 shows the LL and HH experiment results, the LL macrophages produced a significantly higher concentration of NO than the HH macrophages. This experiment was therefore removed from the analysis due to an error in blood collection. This example indicates that caution should be exercised when analysing the results as the health of the macrophage is likely to impact immune response measurements (NO, pH). It should therefore be noted that all macrophages should be tested to ensure that they have equal viability so that the reaction is based on EBV. Due to issues with blood collection the number of cattle in each cohort was reduced, this meant that whilst statistical analysis was possible the results were not as reliable as if a larger cohort of cattle were researched.

In addition to blood collection issues, it is possible that an infection could have arisen within the cell culture. In order to ensure that the EBVs can be measured equally, it is recommended that this experiment is run again by a researcher who has trained in cell culture and macrophage isolation for six months or more. This will help to ensure that the macrophage health is equal across all EBVs providing more reliable data.

This experiment was run *in vitro* and therefore the complex interactions of a live host immune system were not observed, this experiment should therefore

also be subject to live animal testing in order to test if the outcome is the same in and *in vivo* scenario.

Due to MAP pathogens being closely related to *Mycobacterium bovis* pathogens, it is recommended that a similar study is set up in order to determine if the HIR technology can defend cattle against both Johne's disease and TB. This research could have a significant impact in reducing mycobacterial diseases in the UK whilst reducing the cost involved in disease breakdown for the farmer and taxpayer as well as reducing zoonotic disease threat.

7 Appendices

7.1 Appendix 1: Cells of the Immune System

Image downloaded from http://textbookofbacteriology.net/adaptive_2.html on the 15 June 2016. Source: Textbook of Bacteriology, 2012

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7.2 Appendix 2: Laboratory Experiment Data

HH Laboratory Data Set

(NO release measured in μmol mg)

All laboratory data sets include a control of 0 which is not represented in the tables below:

EBV - 4056 HH					
	NO Experiment				**Average**
MAP 40:1	11.604	11.15			**11.377**
P1C1	4.882	4.475	4.347	4.544	**4.562**
P2C2	7.836	6.94			**7.388**

EBV - 4345 HH				
				Average
		NO Experiment		
MAP 40:1	8.175	8.304		**8.2395**
P1C1	8.526	8.128		**8.327**
P2C2	10.515	9.392		**9.9535**
		pH Experiment		
P1C1	1585	1592	1614	**1597**
P2C2	1915.4	1699.55	1772.63	**1795.86**

EBV - 4238 HH				
				Average
		NO Experiment		
MAP 40:1	10.819	12.568		**11.6935**
P1C1	13.185	10.39		**11.7875**
P2C2	18.089	20.369		**19.229**
		pH Experiment		
P1C1	542.33	578.009	590	**570.113**
P2C2	358.734	409.307	400.786	**389.609**

LL Laboratory Data Set

EBV - 4212 LL				
				Average
		NO Experiment		
MAP 40:1	9.557	9.197		**9.377**
P1C1	3.161	3.207		**3.184**
P2C2	5.614	5.138		**5.376**

EBV - 4192 LL				
				Average
		NO Experiment		
MAP 40:1	8.07	7.075		**7.5725**
P1C1	7.216	5.625		**6.4205**
P2C2	12.574	10.924		**11.749**
		pH Experiment		
P1C1	2022.19	1701.33	1861.75	**1861.76**
P2C2	2094.11	2087.21	2089.32	**2090.21**

EBV - 4233 LL				
				Average
		NO Experiment		
MAP 40:1		7.699	6.824	7.699
P1C1		5.161	4.252	5.161
P2C2		17.746	20.678	17.746
		pH Experiment		
P1C1	1673.39	1736.67	1733.96	1714.67
P2C2	1032.19	987.11	997.12	1005.47

HL Laboratory Data Set

EBV - 4322 HL				
				Average
		NO Experiment		
MAP 40:1	7.391	7.005		7.198
P1C1	6.198	6.28		6.239
P2C2	9.041	8.889		8.965
		pH Experiment		
P1C1	702.307	921.016		811.662
P2C2	1030.08	1050.6		1040.34

EBV - 4200 HL				
				Average
		NO Experiment		
MAP 40:1	4.266	4.213		4.2395
P1C1	2.563	2.721		2.642
P2C2	3.659	1.996		2.8275
		pH Experiment		
P1C1	1268.44	1280.99		1274.72
P2C2	1402.75	1513.98		1458.37

EBV - 4215 HL				
				Average
		NO Experiment		
MAP 40:1	1.886	2.298		2.092
P1C1	1.08	1.56		1.32
P2C2	1.612	1.697		1.6545
		pH Experiment		
P1C1	1167.65	1288.46		1228.06
P2C2	1650.21	566.709		1108.46

LH Laboratory Data Set

EBV - 4349 LH					
		NO Experiment			Average
MAP 40:1	7.153	6.921			7.037
P1C1	2.344	2.305	1.777	1.97	2.099
P2C2	5.825	9.01			7.4175

EBV- 4314 LH			Average
	NO Experiment		
MAP 40:1	4.411	4.226	4.3185
P1C1	3.302	2.655	2.9785
P2C2	2.959	2.919	2.939
	pH Experiment		
P1C1	379.155	381.92	380.538
P2C2	608	619.19	613.595

EBV- 4323 LH			Average
	NO Experiment		
MAP 40:1	4.84	3.56	4.2
P1C1	3.59	4.31	3.95
P2C2	3.76	4.05	3.905
	pH Experiment		
P1C1	512.287	383.86	448.074
P2C2	442.869	356.198	399.534

7.3 Appendix 3: GenStat Analysis

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Genstat 64-bit Release 18.1
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                    Genstat Eighteenth Edition
                    Genstat Procedure Library Release PL26.1
                    ________________________________________

                        Nitric Oxide Release Experiment

    Two-way Analysis of Variance
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9 Glossary

Antibody	A blood protein produced in response to and counteracting a specific antigen. Antibodies combine chemically with substances which the body recognizes as alien, such as bacteria, viruses, and foreign substances in the blood
Antigen	A toxin or other foreign substance which induces an immune response in the body, especially the production of antibodies.
Autoclaved	To heat (something) in an autoclave
Bacilli	A rod-shaped bacterium
Bovine Tuberculosis	Bovine tuberculosis (TB) is a chronic disease of animals caused by a bacteria called Mycobacterium bovis, (M.bovis) which is closely related to the bacteria that cause human and avian tuberculosis. This disease can affect practically all mammals, causing a general state of illness, coughing and eventual death.
Buffy coat	The buffy coat is the fraction of an anticoagulated blood sample that contains most of the white blood cells and platelets following density gradient centrifugation of the blood.
Cohort	A group with a shared characteristic
Enteric	Relating to or occurring in the intestines
Histopaque	A solution containing polysucrose and sodium diatrizoate, adjusted to a density of 1.077 g/mL. This medium facilitates the recovery of large numbers of viable mononuclear cells
in vitro	(Of a process) performed or taking place in a test tube, culture dish, or elsewhere outside a living organism.
Lymphoid	Relating to or denoting the tissue responsible for producing lymphocytes and antibodies. This tissue occurs in the lymph

	nodes, thymus, tonsils, and spleen, and dispersed elsewhere in the body.
Moxi-Z cell counter	Moxi Z is the only automated cell counter that combines the Coulter Principle typically used in high-end cell counters with a patented thin-film sensor technology to allow for highly accurate (> 95%) and repeatable particle counting and sizing for a broad range of cell types
Pathogen	A bacterium, virus, or other microorganism that can cause disease
Phagocytic	A cell, such as a white blood cell, that engulfs and absorbs waste material, harmful microorganisms, or other foreign bodies in the bloodstream and tissues.
Proliferation	Rapid reproduction of a cell, part, or organism.
Vaccination	Vaccination is the administration of antigenic material (a vaccine) to stimulate an individual's immune system to develop adaptive immunity to a pathogen. Vaccines can prevent or ameliorate morbidity from infection.
Vacutainer	A Vacutainer blood collection tube is a sterile glass or plastic tube with a closure that is evacuated to create a vacuum inside the tube facilitating the draw of a predetermined volume of liquid.
Zoonotic	A zoonotic disease is a disease that can be spread between animals and humans. Zoonotic diseases can be caused by viruses, bacteria, parasites, and fungi. These diseases are very common.