Investigation into Mycobacterium Avium Complex and its Presence in Australian Commercially Available Pasteurised Milk

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1. INTRODUCTION

1.1 MYCOBACTERIUM SPECIES

Mycobcterium species were first identified in 1882 and since then, some strains have been found to be pathogenic to humans (Chapman 1977, Field 2004). Some species are known to be infectious to cattle, sheep, ruminants, birds and other wildlife (Bercovier 2001, Schmitte 2002). The bacteria are normally found in soil, water and animal feed (Prim 2004). The route of infection is from host to host, normally airborne or in some cases via ingestion (Langer 2014).

Mycobacterium bovis has been known to cause tuberculosis in humans, especially in developing countries (Drewe, Kaneene 2014). The transmission occurs from host to host normally originated from an animal source due to consumption of unpasteurised milk, poorly cooked meat or contact with infected animals (Ayele 2004). The bacteria are commonly found in domesticated cattle and other wildlife (Drewe, Kaneene 2014). Just like M. tuberculosis, the route of infection is airborne, causing pulmonary disease and by ingestion, causing extra pulmonary infections (LoBue 2004).

Australia is one of the developed countries that have managed to eradicate tuberculosis (Bercovier 2001). In December 1997, Australia was declared free from bovine tuberculosis after a 20 years program that started in 1970 (Australian Government 2012). In 2002 the last detected tuberculosis specie was eradicated due to surveillance programs that are still effective today (Australian Government 2012). Australia is currently the only major live stock exporter that has been able to achieve tuberculosis eradication (Australian Government 2012).

1.2 NONTUBERCULOSIS MYCOBACTERIA (NTM)

NTM are all *Mycobacterium* strains excluding *Mycobacterium tuberculosis complex* and *Mycobacterium leprae* that do not belong to the tuberculosis group but share genetic similarities (Yaeger 2011). They were first referred as "atypical" mycobacterium; but as further studies were conducted between the different types of bacteria and their relation as causation agents for disease, the classification was renamed (Falkinham 1996).

There are over 125 different NTM species (Yaeger 2011). Timpe and Runyon classified NTM by their growth rate and pigmentation. Type 1, 2 and 3 took more than 7 days to grow and type 4 took less than 7 days to grow (Runyon 1959) (See Table 1). NTMs are generally resistant to high temperatures and a low pH (Brodmer 2000). They are not susceptible to Gram stains, so flourochrome technique is generally the staining technique used (Ray 2004).

TABLE 1`

NTM GROWTH CLASIFICATION

NTM			
> 7 DAYS SLOW GROWTH			< 7 DAYS RAPID GROWTH
TYPE 1	**TYPE 2**	**TYPE 3**	**TYPE 4**
M. kansasii	*M. scrofulaceum*	*M. avium complex*	*M. fortuitum*
M. marinum	*M. szulgae*	*M. xenopi*	
M. asiaticum	*M. gordonae*	*M. ulcerans*	*M. abcsesus*
M. simiae	*M. flavescens*	*M. terrae*	*M. chelonae*
		M. haemophilum	

(Ray 2004)

NTM are known to cause a range of diseases including pulmonary diseases, skin disease and cervical lymphadenitis, being *M. kansaii, M. avium* and *M. gordonae*, the most common pathogenic species (Falkinham 1996) (see Table 2).

TABLE 2

NTM AND DISEASES ACCORDING TO THEIR GEOGRAPHY AND MORPHOLOGIC FEATURES

FEATURES OF THE COMMON SPECIES			
DISEASE	**SPECIES**	**GEOGRAPHY**	**MORPHOLOGIC FEATURES**
Pulmonary disease	*M. avium complex*	Worldwide	Slow growth usually no pigment
	M. kansasii	USA, coal mining regions, Europe	Pigmented, slow growth
	M. xenopi	Canada and Europe	Slow growth, pigmented
	M. abcsesus	Worldwide, USA	Rapid growth, not pigmented
	M. malmoense	UK, North Europe	Slow growth, not pigmented
Cervical Lymphadenitis	*M. avium complex*	Worldwide	Slow growth usually no pigment
	M. scrofulaceum	Worldwide	Slow growth, pigmented
	M. malmoense	UK, North Europe (specially Scandinavia)	Slow growth, not pigmented
Skin disease	*M. marinum*	Worldwide	Slow growth, pigmented
	M. fortuitum	Worldwide, USA	Rapid growth, not pigmented
	M. chelonae		
	M. abcsesus		

	M. ulcerans	Australia, tropics, Africa, Southeast Asia	Slow growth, pigmented
Disseminated disease	*M. avium complex*	Worldwide	Slow growth usually no pigmented
	M. kansasii	USA	Pigmented, slow growth
	M. chelonae	USA	Rapid growth, not pigmented
	M. haemophilum	USA, Australia	Slow growth, not pigmented

(Schlossberg 2011)

For diagnosis purposes, it is necessary to combine clinical, bacteriological and radiological features in order to find the species of the bacteria (Griffith 2007). In case of *M. avium complex, M. kansasii* and *M. gordonae*, DNA fragments or liquid chromatography, are sometimes used for detection (Griffith 2007).

1.3 MYCOBACTERIA AVIUM COMPLEX (MAC)

One of the most common NTM is *Mycobacteria avium complex* (MAC) (Goodfellow and Magee 1998) The first characterization of the bacteria was published by Thorel (1990) isolating three subspecies: *M. avium, M. paratuberculosis* and *M. silvaticum.* However, now, due to DNA testing, MAC strains are *M. avium avium, M. avium paratuberculosis* (MAP), *M. avium hominissuis* and *M. intracellulare*; they

have all been isolated from human hosts and are genetically similar (Turenne 2007).

MAC is found in the environment, mainly in soil and water (Paradise, Friedman, Bendinelli, 2002).

1.4 MAC AND DISEASE

MAC bacteria have different virulent and drug response characteristics depending on the species (Wolinsky 1995; Paradise, Friedman, Bendinelli, 2002).

In the 1980's the AIDS epidemic occurred and between 20% and 50% of patients contracted a nontuberculosis bacteria and the most common strain found was MAC (Falkinham 1996). There was a direct relation between AIDS patients and pulmonary infection with a high mortality rate (Jacobson 1991; Nightingale 1992). With the introduction of Highly Active Antiretroviral Therapy for AIDS patients there has been a decrease in mortality rates, however it is still a concern for patients who are not able to acquire the drug and for patients who have shown resistance to the therapy (Wu 2009). It is important to mention that each strain of MAC can have different infection effects on patients (Wu 2009).

1.4.1 CERVICAL LYMPHADENITIS

Cervical lymphadenitis was first described in the 1950s and is mainly caused by nontuberculosis mycobacterium (Wolinsky 1979).

Children who get cervical lymphadenitis are normally between 1 to 5 years of age. In some cases it correlates to the time when their teeth start to erupt due to the oral accumulation of bacteria (Bayazit 2004). Common symptoms of infection include draining sinus, fever and the inflammation of the lymph nodes (Thegerstrom 2008) although in many cases the child can be asymptomatic (Chadwick 2003). The inflammation of the nodes can range from 1 to 6 cm (Penn 2011). Treatment can include antibiotics and in some cases surgical removal of the affected nodes, however antibiotic resistance is a concern due to the fact that reoccurrences can happen up to 7 years (Wolinsky 1995, Panesar 2003). In Australia, a study reported an incidence of 0.88 cases per 100,000 of lymphodenitis in children, due to NTM, most caused by MAC; and a recurrence in 23% of the cases after therapy (Blyth 2009).

1.4.2 PUMONARY INFECTIONS

MAC can cause a range of pulmonary illnesses (Johnson 2014). Since the main mechanism of transmission is though aerosol droplets, shower heads have been one of the major sources of exposure of NTM (Falkinham 2008) There has also been a correlation between water heater temperatures in households and NTM colonisations; resulting in a higher probability of colonization in houses with water heaters under 50 °C than water heaters over 55° C (Falkinham 2003).

Upper lobe fibrocavitary disease has been constant in elderly males and particularly males with a history of pulmonary problems like chronic obstruction, histoplasmosis or tuberculosis (Yeager 2011, Johnson 2014). Unlike tuberculosis, MAC lung infected patients are not contagious (Field 2004).

Prince (1989) first described adults without any previous lung disease, getting respiratory infection leading to failure and even death due to MAC. Patients were predominantly female and presented with bronchiectasis (Prince 1989, Scout 2011). It is also referred to as the Lady Windermere Syndrome, and patients tend to voluntarily suppress the cough, damaging the right lobe (Scout 2011). Early in the infection, the patient may be asymptomatic, and it is through chest radiographies that it may be detected (Field 2004).

Hypersensitivity pneumonitis (HP) has also been associated with MAC infection, most commonly from hot tubs (Cappelluti 2003). Patients normally present fever and acute pulmonary symptoms with chest radiography discrepancies (Johnson 2014).

Other lifestyle factors like cigarette smoking and alcohol consumption, have showed an effect on a higher incidence of pulmonary infection caused by MAC (Field 2004)

Pulmonary infection treatment has been controversial due to the complexity of the bacteria (Johnson 2014). Antibiotic treatment has shown to have positive results when administered with different types of antibiotics, but showed resistance when administered individually (Nash 2001). In addition, gastrointestinal side effects that have been a concern and in some cases a reason for unfinished treatment

(Roussel, 1998, Field 2004). Treatment is suggested to continue for at least one more year after the result for MAC has turned from positive to negative due to its high resistance (Griffith 2007). Furthermore, after ending treatment, some patients have resulted with MAC regrowth (Hasegawa 2009).

Surgical treatment for pulmonary infection due to NTM, has not been studied extensively (Johnson 2014). However, a study performed in Denver showed a low mortality rate and post surgery bronchial complications (Mitchell 2008). Another study revealed that 22% of the patients relapsed with NTMs pulmonary infections post surgery (Yu 2011).

1.4.3 CUTANEOUS INFECTION AND DISSEMINATED DISEASE

Cutaneous infections are rare and normally associated with disseminated disease, and more common in immune compromised patients (Kayal 2002). Cutaneous infection can be caused by, sub cutaneous nodules or skin ulcers inoculations, through cervical lymphadenitis that extends to skin as abscess or as multiple ulcers that are present in disseminated disease (Ichiki 1997). Noguchi then reported that solitary or multiple subcutaneous nodules are the most common skin infection of MAC (Noguchi 2000).

1.4.4 CROHN'S DISEASE

Inflammatory bowel disease has two forms, Crohn's Disease and ulcerative colitis, both affect the intestinal tract and can have severe nutritional outcomes (Naser 2004). Crohn's disease is an inflammation of the gastrointestinal tract, normally involving the distal ileum and colon (Beyer 2004). Symptoms include abdominal pain, diarrhoea, vomiting, anal abscesses, fistula and weight loss (Beyer 2004). As the inflammation, abscesses, fistulas and ulcerations evolve, fibrosis, gut wall thickening, and scarring take place, which then results in partial or complete obstruction of the intestinal lumen (Sutherland 2003). Gastrointestinal inflammation can cause changes in the digestive system and nutrient absorption resulting in a decrease of fat absorption in severe inflammation episodes and enteric leakage of protein, blood, minerals and electrolytes resulting in weight loss and malnutrition and in some cases growth impairment (Shils, Shike, Ross and Caballero, 2006). Patients with long term Crohn's disease are at higher risk of colorectal cancer (Kiesslich 2012).

In the past 25 years, Crohn's disease has been showing a rise in prevalence around the world (Economou and Pappas 2007). Epidemiological studies have shown a higher incidence and prevalence of Crohn's disease in westernized countries like North America, Europe and some developing countries (Marrollo, Armuzzi, Zannoni 2010). In North America it is estimated that the incidence and prevalence rates range from 3.1 -14.6 per 100,000 and 26 – 199 per 100,000 cases per year of Crohn's disease (Loftus 2004). Historically, Australia has reported a low incidence of Crohn's disease, however in recent years, there has been a considerate increase

reaching up to 29.3 per 100,000, together with, Canada and northern Europe, reaching 20.2 and 10.6 respectively (Kalla, Ventham and Satsangi 2014). Asia on the other hand has low incidence of the disease, however some studies have shown a substantial increase in Crohn's disease incidence in India, China and Japan; probably due to industrialisation (Zheng 2005, Desai 2005, Vatn 2013). It is important to consider that India and China have a high population, which would increase the ratio of actual patients to those in other countries (Economou and Pappas 2007). Finally, migration of people from low incidence countries to high incidence countries, have shown a higher prevalence of Crohn's disease especially in first-generation children (Vatn 2013).

Research has demonstrated Crohn's disease to be a result of multiple factors including genetics, environmental factors and lifestyle factors (Marrollo, Armuzzi, Zannoni 2010).

There is evidence that suggests onset of Crohn's can be due to genetic susceptibility, specifically the NOD2/CARD15 genes (Shananhan 2002). These genes cause a predisposition to an immune response in the presence of a certain bacteria (Loftus 2004). However, only 25% of genetic susceptibility for Crohn's disease has been accounted for, suggesting there are other influential risk factors that cause the disease (Frank 2013).

Environmental factors also have a direct influence in the prevalence of Crohn's disease (Baumgart and Carding, 2007). The most substantial environmental factor that affects Crohn's disease is a microbacterial one (Vatn 2013). There has been

evidence that suggests that Mycbacterium avium subspecies paratuberculosis (MAP) is related to Crohn's disease as a causative agent (Hermon-Taylor 2000). However it is still debatable given the fact that other studies have shown no direct correlation (Ricaneck 2010). In addition, MAP has been extracted from Crohn's disease patients but evidence supports that it is not the sole cause of the disease or that it is present in every patient (Chamberlin, Graham, Hulten 2001).

Another environmental factor has been seasonal change there has been an incidence increase during autumn and winter (Baumgart and Carding, 2007).

Lifestyle factors like smoking, drugs, no breast-feeding and diet, have also been reported to affect the prevalence of the disease; since they are factors that lower immune system response (Naser 2004).

Smoking has been known to be a risk factor for Crohn's disease, and in some cases it has been shown to affect treatment response or cause surgery complications (Solber 2007).

Diet has been shown to be one of the main factors that influence the risk of Crohn's disease, however the availability of data is sometimes hard to acquire do to the general gastrointestinal symptoms of the disease itself (Loftus, 2004, Vatn 2013).

Numerous case studies have shown that a high intake of sugars may increase the risk of Crohn's disease (Shils, Shike, Ross and Caballero, 2006). Sugar was first implicated as a risk factor 25 years ago and the conclusion has been confirmed through studies around the world including in Germany, Uk, Japan, Italy and Israel (Vatn 2013). Other studies have shown monounsaturated and polyunsaturated fats and red meats to be risk factors for Crohn's disease and on the contrary,

dietary fiber, fruits and vegetables have been found to be protective (Geerling 2000, Maconi 2010). In a recent study, it has been reported that breast-feeding works as a protective factor against Crohn's disease as well (Mikhailov 2009).

In addition, oral contraceptives have also shown to be a risk factor in women (Zappata 2010).

Treatment for Crohn's disease has been difficult since antituberculous antibiotics are ineffective, even after long periods of time (Hulten 2000)

The symptoms of the disease are treated with antibiotics and anti-inflammatory drugs and steroids, however in some cases treatment has failed, supporting the idea that Crohn's disease is a multifactorial disease (Sutherland 2003, Kalla 2014). Surgery has also become a common treatment method resulting in approximately 50% of patients undergoing surgery and about 30% is done during the first five years after diagnosis (Bernstein 2012).

Furthermore, treatment for Crohn's disease continues to be a problem since long-term studies have shown that between 80 and 90 % of the patients relapse (Solberg 2007).

Australia has one of the highest incidences of Crohn's disease in the world since 28 thousand have the disease (Australian Government 2015). It can take up to 8 months to diagnose and 3 out of 20 people who have the disease, also have one relative with the same disease (Australian Government 2015).

The annual cost of inflammatory bowel disease in Australia is of 2.7 billion. In addition, 70% of people with Cohn's disease stay home during the episodes (Australian Government 2015).

1.5 EVIDENCE LINKING CROHN'S DISEASE AND MYCOBACTERIA AVIUM SUBSP. PARATUBERCULOSIS (MAP)

Johne's disease is a chronic, incurable bowel inflammation caused in cattle, sheep and other ruminants caused by MAP (Taylor 1981). The dairy industry is highly affected due to 10 to 25% decrease in milk production and loss of herds (Losinger 2005). It has been estimated to have a 1.5 billion cost per year in the US (Stephan 2007). MAP has also been directly associated with causing Johne's disease in cattle (Ayele 2004). The bacilli multiply in the mucosa of the intestine and it is shed by ingestion of contaminated hay from faeces or from mother's milk to calves (Gao 2005). The infection can be transmitted from asymptomatic cattle, which are in the earlier stages of the disease, also known as subclinical animals, or by clinically diagnosed animals (Ayele 2001).

In Australia, Johne's disease is monitored through a national program of detection and surveillance of animals, in order to prevent spreading of the disease and ensure animals health by eradicating the problem (Australian Government 2012).

MAP has been suggested to cause disease in humans in addition to its cause of disease in animals (Sutherland 2003). The organism was first mentioned in 1895 but it was not until 1913 when Dalziel noted the similarities between Crohn's disease in humans and Johne's disease in animals (Dalziel 1913). The actual association was not made until MAP was isolated from 3 Crohn's disease patients in 1984 (Chiodini 1989, Feller 2007). More recently, MAP DNA has been tested in Crohn's disease patient using PCR (Polymerase Chain Reaction) method instead of cultural method since it is a faster and highly specific method (Hussein 2009).

There have been several controversial arguments between MAP and it's relation to Crohn's disease, mainly due to the fact that not all Crohn's disease patients are infected with MAP, and MAP has been detected in healthy individuals (Sutherland 2003).

MAP DNA has been detected in between 40 to 50% of Crohn's disease patients in blood and tissue samples (Naser 2004, Autschback 2005, Mendoza 2010). In addition, the studies also reported MAP DNA present in healthy individuals, demonstrating an exposure to the bacteria through the environment; however present in a dormant state, since it was unable to be cultured (Naser 2004, Mendoza 2010).

In contrast, another study showed no detection of MAC in any of the Crohn's disease patients, but a positive result in some of the randomly selected control group, which were healthy individuals (Bernstein 2003).

Finally, there have also been studies that have failed to detect MAP in patients and in control groups (Collins 2000, Clarkston 1998).

Several studies have shown similar results through PCR method (see Table 3).

TABLE 3

STUDIES INVESTIGATING MAP AND CROHN'S DISEASE ASSOCIATION THROUGH PCR TESTING

Study	Year	Method	Patient	Number of Patients	Positives
Sechi	2005	PCR	CD, Control	30, 29	25, 3
Romero	2005	PCR	CD, Control	12, 6	10, 1
Ryan	2002	PCR	CD, Control	15, 34	6, 9
Bull	2003	PCR	CD, Control	37, 29	34, 3
Fujita	2002	PCR	CD, Control	16, 12	0, 0
Cellier	1998	PCR	CD, Control	47, 30	0, 3
Tiveljung	1999	PCR	CD, Control	11, 11	8, 4

(Collins 2000, Clarkston 1998, Slana 2008)

In 2007 a comparison between studies was done, in order to have a clearer view of the evidence available between MAP and Crohn's disease (Feller 2007). They concluded that the evidence showed a substantially greater presence of MAP in Crohn's disease patients than in healthy individuals and that the results were not altered depending on the detection method used in each individual study (Feller 2007).

Another association between MAP and Crohn's disease has been an autoimmune response triggered by the bacteria, although it has yet to be conclusive; even though enzyme-linked immunosorbent assay (ELISA) tests for antibodies of MAP in infected animals, has been successfully adapted for Crohn's disease patients (Suntherland 2003, Singh 2010).

A Japanese study reported Crohn's disease patients had an elevated immune response triggered by MAP (Nakase 2006), however it could be argued that it is caused by other factors and not related to the disease (Singh 2010).

Antibiotic treatments has also been controversial linking MAP to Crohn's disease (Sing 2010). Earlier studies showed promising results against MAP in Crohn's disease patients, since the bacteria were no longer detected after the treatment (Shafran 2002, Borody 2002). Other studies that have reported short-term benefits against MAP, but failed to provide long-term benefits (Selby 2007). In addition to antibiotics, anti-inflammatory treatment used in Crohn's disease patients, has showed to have an effect against MAP as well, but still needs further research (Greenstein 2007).

In 2000, a small MAP detection test was done to two Crohn's disease mother's milk and five healthy mother's milk, resulting in a positive detection in both infected mother's milk and negative for the five healthy ones (Naser and Schwartz 2000).

1.6 MYCOBACTERIA AVIUM COMPLEX IN MILK

One of the first research studies done with MAC and pasteurised milk goes back to 1968 when Harrington and Karlson reported that MAC survived pasteurization method (Chapman 1977), since then many reports of MAC detected in milk have been published (Gill 2011)

MAP specifically, has been isolated from symptomatic and asymptomatic animals using both, culture and PCR methods (Gao 2009), and it has been detected in both raw and pasteurized milk (Grant 2002)

1.6.1 MAP DETECTION IN RAW MILK

Different studies around the world have tested raw milk from individual cows and from bulk tanks (Gill 2011).

In the USA, 61 samples of bulk tank milk from 10 different states were tested for MAP resulting in 68% positive for the bacteria by PCR method (Stable 2002). Another study conducted in the USA reported 50% positive raw bulk tank samples for MAP through PCR (Pillai and Jayarao 2002).

Other studies have shown similar results. Chile tested bulk tank raw milk with at least 80% incidence of MAP (Kruze 2013). Ireland tested 645 raw milk samples, resulting in 12.9% positive for MAP (O'Reilly 2004). Switzerland reported 19.7% positive results from 1384 samples of raw bulk tank milk (Corti and Stephan 2002). Cyprus conducted raw bulk tank milk testing from herds that were not diagnosed with Johne's disease (Botsaris 2010). They reported a total of 28% of positive detections of MAP from 225 samples through qPCR method and 0.9% through culture method (Botsaris 2010). Czech Republic, also reported positive results through PCR, 32% out of 345 samples of raw milk and 44% out of 16 bulk tank samples (Slana 2008). Denmark and India tested raw milk through culture and PCR and got positive results on both, however there was a lower percentage through PCR method, 22% and 6% respectively, compared to culture method which were 55% and 33% respectively, unlike the rest of the countries (Giese and Ahrens 2000, Shankar 2010). A possible explanation could be that there was some problem during the PCR testing (Gill 2010).

A small study was done in Mexico where 100% of the bulk tank milk samples, obtained from 14 cows and 3 goats, were positive for MAP (Favilia- Humara 2010). In addition, milk from 10 cows and 8 goats were individually tested providing the same result (Favilia- Humara 2010). In Mexico there is no prevalence of MAP in

herds and there are no control programs established either (Favilia- Humara 2010).

Recently, a study was conducted in the southern part of Italy, where milk from 780 herds was tested for MAP with PCR method, resulting in 155 (19.9%) with a positive result (Marchetti 2013).

Furthermore, Brazil, Tanzania and Turkey have tested raw milk for other MAC species like *M. fortuitum, M. godonae, M. kansasii* and *M. flavescens* (Kazwala 1998, Mdegela 2005, Leite 2003). In Brazil, 300 samples of raw milk were tested for *Mycobacterium* species resulting 24 to be positive (Franco 2013). Tanzania reported 31 out of 805 samples to be positive for *Mycobacterium* species (Kazwala 1998). Later, in 2005, another study in Tanzania reported 12% positive samples for NTM species (Mdegela 2005). Turkey reported that 6 out of 35 samples tested were positive for NTM and 9 out of 35 were positive for *M. tuberculosis*, all done through culture testing (Konuk 2007).

In Australia there have not been any previous research done in raw milk and its potential contamination with MAC.

1.6.2 MAP DETECTION IN PASTEURISED MILK

Studies have also been done in pasteurised milk with controversial results since some have found positive results and others have not using the same detection

method (Donaghy 2010). Studies have shown that MAP can survive pasteurisation methods (Millar 1996, Van Brant 2011) and in some cases viable MAP has been found in commercially available milk (Ayele 2005, Carvalho 2012, Ellingson 2005).

Other studies have investigated the inactivation of the bacteria though pasteurisation, reporting a reduction of viable cell or a complete elimination of the bacteria (Grant 2005, Lynch 2007, Rademaker 2007, Stabel 2001, Mc.Donald 2005). The concentration of survivors depends on the initial concentration of organisms or due to their clumping mechanism during heating processes (Van Brant 2011, Serraino 2014).

In Iran, a study was conducted in 300 samples of commercially available pasteurised milk. Thirty two (10.7%) out of the 300 samples tested positive for MAP DNA through PCR, however there were no viable bacteria (Anzabi and Hanifian 2012). In Brazil, 1 out of 35 samples tested from commercially available pasteurized milk was positive for viable MAP (Carvalho 2012). In India, commercially available pasteurised milk was also tested, finding 67% of the samples positive for MAP through culture method and 33% through PCR method (Shankar 2010).

In the USA, a study was conducted for viable MAP detection, in commercially available pasteurised milk samples from three of the top milk producing states, California, Minesota and Wisconsin (Ellingson 2005). The study was done over a 12 month period, and a total of 702 pints (332.17 lt) (233 from California, 234 from Minesota and 235 from Wisconsin) of milk were tested (Ellingson 2005). The presence of viable MAP was reported in 2.8% of the pints tested and out of the 22

different brands tested, 12 had at least one sample test positive for viable MAP (Ellingson 2005). In addition, a seasonal effect was noticed since more samples tested positive in the months from July to September (Ellingson 2005).

Ireland reported that 35 of 343 samples of pasteurised milk tested positive for MAP through PCR method (O'Reilly 2004). Argentina also tested commercially available pasteurised milk, however only 1 sample had a positive result (Paolicchi 2003). In the southwest part of Canada, a MAP detection study was conducted with 710 retail and dairy plant milk samples, resulting in 15% (110 samples) positive detections of DNA with no viable bacteria (Gao 2002).

In addition, the UK and Austria conducted long studies for MAP detection in raw and pasteurised milk (Khol 2013, Grant 2002).

In Austria, the study lasted 23 months; milk samples were taken from animals at the beginning and at the end of the study, monthly sampling from 63 animals (33 MAP infected and 30 not infected) and monthly samples from raw and pasteurised bulk tanks (Khol 2013). The results reported 2 to 5% of the individual cows milk samples testing positive for MAP, 13 infected and non infected cows in total, tested positive for shedding MAP in their milk, and there was no positive samples from either of the bulk tanks (Khol 2013). Because individual cows test positive for MAP and bulk tank milk tested negative, it could be suggesting that the MAP dilution in bulk tanks was just too low to detect, and in fact contained the bacteria (Khol 2013)

In the UK, a study lasted for 17 months, with a total 814 cows milk samples (244 bulk raw and 567 commercially available pasteurised milk), from 241 different

dairy processing establishments (Grant 2002). The detection method used was PCR, reporting positive results for MAP DNA in 19 (7.8%) raw milk samples and 67 (11.8%) in pasteurised milk samples (Grant 2002). MAP was also isolated from cultured samples from 4 raw milk samples and 10 pasteurised (Grant 2002, Ruzante 2006). The study finally concluded that MAP might be present in low levels in pasteurised milk (Grant 2002). The variations could have been caused by cross contamination at the moment of the detection proses.

In regards to Australia, there are no previous studies done to determine if pasteurised milk contains traces of MAC; however do to the different conclusions that studies around the world have published, addressing the effectiveness of pasteurisation methods would be essential for a better understanding of MACs survival.

1.6.3 MAP DETECTION IN OTHER DAIRY PRODUCTS

Few studies have been done in cheese; Sung and Collins, made Hispanic cheese with MAP spiked milk showed that if pasteurized cheese is then cured for 60 day, a higher number of MAP inactivated cells were detected. There was not enough data available to conclude MAP detection in cheese made from raw milk (Sung and Collins 2000) and Spahr and Schafroth, made Swiss cheese with MAP spiked milk

found a reduction in the number of organisms as the cheese matured over 3 to 4 months (Spahr and Schafroth 2001).

In Switzerland, 143 raw milk cheeses were tested for MAP through culture and PCR methods and only 6 and 1 respectively had a positive result, confirming the presence of MAP in raw milk and raw milk cheese (Stephan 2007).

More recently, Brazil and Cyprus have also tested for MAP in cheese (Faria 2014, Botsaris 2010). Brazil found 10% of the tested samples to be positive (Faria 2014) and Cyprus had 20% tested positive (Botsaris 2010).

Studies have also been performed in powdered milk in Iraq and Czech Republic. In Iraq, 35% of the tested samples provided positive results for MAP (Hassan 2012). In the Czech Republic, 18 out of 51 samples tested were positive for MAP DNA (Hruska 2011).

1.7 DETECTION METHODS

Mycobacteria are slow growing microorganisms and easy to grow in environments containing carbon, mineral salts and mycobactin, an iron carrier that can not be

synthesized (Lambrecht 1992). For MAC growth, broth and solid agar medias are commonly used, Herrold's Egg Yolk, Lowenstein-Jensen and Middlebrook (Sutherland 2003), however they are normally supplemented with materials to enhance growth or counter act growth inhibitors (Gill 2011). Unfortunately, culture method can take between 8 to 16 weeks to confirm negative results for MAP (Gao 2009). However improvements in the medias Middlebrook7H11 with mycobactin can reduce the time of culture to 3 weeks (Whittington 2011).

Due to Mycobacteria slow growing nature, many samples are lost due to the growth of other organisms and in addition, decontamination processes to control the problem, also inhibit mycobacterium growth (Dzieciol 2010).

Alternatively, DNA based PCR (Polymerase Chain Reaction) and Real- time PCR methods are more resourceful and preferred methods since they are faster and more sensitive (Kralik, 2010; Fang 2002, Dzieciol 2010). The biggest disadvantage of the PCR method, it is impossible to determine the live cells from the dead ones, dye detection has to be used to determine the viability of the bacteria (Kralik 2010, Whiley 2012).

PCR is a DNA sequence amplifier developed in 1983 by Kary Mullis (Calba Tech 2003). It consists in cycles of heating and cooling to react with a specific fragment of DNA targeted with an insertion sequence (Slana 2008)

Quantitative Real-time (QPCR) detection method is the latest modification to simple PCR testing which helps researchers determine the initial level of nucleic acid that will result in the initial microbial load (Khare 2004, O'Mahony 2004).

QPCR has fluorescently labelled probes that monitor the fluorescence emitted during the reaction to indicate the amplicon production in each cycle (Khare 2004). By monitoring these emissions, it is possible to determine the first significant increase in relation to the initial amount of target template thus providing a more accurate quantification (Khare 2004). In addition it is able to detect low levels of bacteria due to the multiple copies of the gene (O'Mahony and Hill 2004).

PCR methods have been used to detect MAP in milk and have been successful in both raw and pasteurized milk (Di Pinto, Ciccarese, Forte Vito 2005; Dzieciol, Volgger, Khol 2010).

1.8 AIMS AND OBJECTIVES

Studies around the world have reported presence of MAC in commercially available milk. However this is the first study conducted investigating Australian milk.

The aim of this study was to test commercially available pasteurised Australian milk, cheese and yoghurt for MAC.

The second aim of the study was to test pasteurisation processes to determine its effectiveness in MAC spiked milk.

This research focus was providing information regarding Australian milk and its relation to MAV and on the effect of MAC concentrations in contaminated milk through two different pasteurisation methods.

The objectives were:

- Use qPCR method to test commercially available pasteurised milk for presence of MAC.
- Use qPCR method to test commercially available pasteurised cheese for presence of MAC.
- Use qPCR method to test pasteurised yoghurt for presence of MAC.
- Use qPCR method to test the effectiveness of pasteurisation on the presence of MAC through the 72°C for 15 seconds method.
- Use qPCR to test the effectiveness of pasteurisation on the presence of MAC through the 72°C for 30 seconds method.

2. MATERIALS AND METHODS

2.1 FACILITIES

Laboratory testing, which included sample preparation and quantitative polymerase chain reaction (PCR) testing, took place at Flinders University in the

School of Environmental Health between September 2015 and February 2016. Samples were collected from supermarkets in Adelaide, South Australia.

2.2 SAMPLE COLLECTION

Samples were collected from supermarkets located in Adelaide, South Australia.

All milk samples were produced in local South Australian farms and went through pasteurisation process. Cheese samples were produced in South Australia, Victoria and New South Wales farms (Table 1 and Table 2). All samples were tested within their expiry date and were stored at refrigeration temperatures between 0°C and 4 °C before and after testing. Opened samples were discarded 5 days after.

Table 1

MILK SAMPLE COLLECTION

SAMPLE	PRODUCTION STATE	EXPIRATION DATE	SERIAL NUMBER
Brand A original milk	South Australia	05/11/15	V52

Brand A Non Fat Milk	South Australia	06/11/15	V61
Brand B Whole Milk	South Australia	04/11/15	V52
Brand B Non fat Milk	South Australia	02/11/15	V51
Brand C Full cream	South Australia	05/11/15	2642295
Brand D Full cream	South Australia	05/11/15	150713040
Brand E Full cream	South Australia	18/11/15	-
Brand E Reduced fat	South Australia	18/11/15	-
Brand F Full cream	South Australia	19/11/15	0956
Brand F Low Fat	South Australia	19/11/15	0613
Brand G	South Australia	14/11/15	2642303

Full cream			
Brand G Reduced Fat	South Australia	19/11/15	2642308
Brand H Full cream	South Australia	16/11/15	V52
Brand H Reduced Fat	South Australia	18/11/15	V50

Table 2

CHEESE AND YOGURT SAMPLE COLLECTION

SAMPLE	PRODUCTION STATE	EXPIRATION DATE
Cheddar Cheese Brand A	South Australia	15/03/16
Cottage Cheese Brand A	South Australia	16/01/16
Yogurt Natural Brand A	South Australia	22/01/16
Tasty Shredded Cheese Brand B	New South Wales	17/05/16

Tasty Cheese Brand C	Victoria	21/05/16
Ricotta Cheese Brand B	New South Wales	16/02/16
Cottage Cheese Brand D	Victoria	11/02/16

2.3 DNA EXTRACTION METHOD

The DNA extraction method was adapted from Thornton (Thorton, MacLellan, Brink 1998) work and was done by Cornejo in 1998 (Cornejo, Sahagun-Ruiz, Suarez-Guemes, 1998), which was later confirmed by Antognoli (Antognoli 2001) and briefly described below (2.3.1, 2.3.2 and 2.3.3).

2.3.1 STOCK SOLUTION 10 X CB-18 BUFFER PREPERATION

The 10 X CB-18 buffer and the TE buffer were prepared in advance as previous reported. The CB-18 buffer contain 78.8g of Tris-HCL (Tris hydroxymethyl amonio methane), 0.06 of NaCl (sodium chloride), 1.35g of CB-18 (adenine) and 8.16 g of N-acetyl –L-cysteine, in 1L of sterilised miliq water.

2.3.2 STOCK SOLUTION TE BUFFER PREPARATION

Briefly the TE buffer contains 10 ml of 1M Tris and 2ml of 500m EDTA. 1M Tris is made with 60.57 g of Tris in 500 ml of water at a 7.5 pH using HCl. The 500 ml EDTA is made with 18.6g in 100 ml of water at an 8 pH using NaOH.

2.3.3 DNA EXTRACTION

In 50 ml test tubes, 27 ml of each milk sample was mixed with 3 ml of 10 x CB-18 buffer (see 2.3.1). Samples were then vortex at a maximum speed for 1 minute and then shaken in an orbital shaker at speed 7 and at 37 ° C for 90 min. Samples were then centrifuged at 3,500 rpm for 20 min. Samples supernatant was discarded, and pellets were resuspended in 100 μL of TE buffer (see 2.3.2). Finally, samples were boiled for 30 min. and stored at 4° C.

Cheese samples were made into a liquid paste by using 15 ml of cheese and 12 ml of sterilised miliq water and homogenising the mixture manually. Samples were then submitted to the same preparation procedure as milk samples.

2.4 QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR) TESTING

MAC qPCR testing was done in triplicate primers reagent equipment cycling data acquired as previously described (Whiley et al., 2014) using MACF primer (5'-CCCTGAGACAACACTCGGTC-3') and MACR primer (5'-ATTACACATTTCGATGAACGC-3') (Park et al., 2000), shown in table 3. The cycles to

which the samples were submitted include a 95 ° C hold for 5 min followed by 45 cycles of 94° C for 15 sec, 50° C for 30 sec and 72° C for 20 sec. (Whiley et al., 2014).

The equipment used was a RotorGene 3000 (Corbett Research, Sydney, Australia) with data gathered at 72 °C on the 6-carboxyfluorescein channel (excitation at 470 nm, detection at 510 nm) at a gain of 5. Melt curve data was also gathered on this channel at gains of 2 and 5 using a ramping rate of 1°C over 60 sec. from 75° C to 95 °C.

DNA testing for MAC was done through qPCR testing in which each qPCR run included a positive and a negative control for MAC. Each sample was mixed in a primer concentration of 30pmol primers (Table 3) and the melt curve was analysed and a positive MAC was confirmed with a melting temperature (Tm) of 88 ± 1°C, 82.5 ± 1 ° C, 85 ± 1° C respectively (Whiley et al., 2012)

Table 3

REAGENTS FOR MAC qPCR REACTIONS

REAGENT	FINAL CONCENTRATION	µL OF WORKING CONCENTRATION ADDED TO 25µL

H20	-	11.55
MgCl2	2.5mM	1.25
PCR Buffer	1X	2.5
dNTP's	0.2mM	0.5
Syto9	2.5µM	2.5
Platinum Taq	1U	0.2
DNA Sample	-	5
MAC F Primer	0.3µM	0.75
MAC R Primer	0.3µM	0.75

2.5 SAMPLE INCUBATION

MAC is a slow growing organism, by incubating samples, the microorganisms would increase in numbers and therefore be easier to detect (Gao 2009).

Briefly, incubated samples were aseptically removed and tested from fresh milk previously tested. It included 27 ml of each milk samples that were placed in 50 ml test tubes and then placed in an incubator at 37 ° C for 3 weeks.

When the incubation period was over, samples followed the same preparation procedure previously done with fresh milk (see 2.3.2).

2.6 SAMPLE PASTEURISATION

To determine efficiency of two pasteurisation methods for MAC disinfection spiked milk samples were used. The test was run twice in order to gather more accurate information.

Milk samples were gathered from commercially available pasteurised milk in South Australia and each sample was done in triplicate.

First test samples were divided into 4 groups made up of three test tubes each. The first group had 27 ml of commercially available pasteurised milk in each test tube. The second group, third group and forth group had 24.3 ml of pasteurised milk and 2.7 ml of MAC dilution in each test tube.

Second test samples were divided into 4 groups made up of three test tubes each. The first group had 27 ml of commercially available pasteurised milk in each test tube. The second, third and forth groups had 26 ml of milk and 1 ml of MAC dilution in each test tube. The second test was made with a higher concentrated dilution of MAC.

No additional process, prior to DNA extraction, was done to the first group of samples or second group of samples.

The third and forth group of samples were submitted to two different pasteurisation methods prior to DNA extraction.

Third group was submitted into 72°C water for 15 seconds. The forth group followed the same procedure as the third group except for an extended time of 30 seconds.

After pasteurisation, DNA extraction method was used (see 2.3.3) and finally qPCR testing was executed (see 2.4)

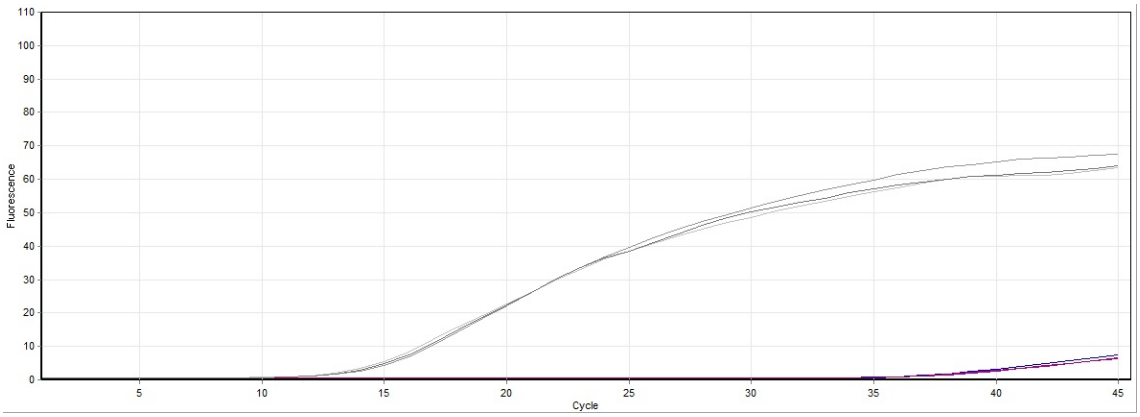
3. RESULTS

3.1 FRESH PASTEURISED MILK MAC DNA TEST RESULTS

Fresh commercially available pasteurised milk in South Australia was tested for presence of MAC. Eight different brands and a total of 14 different samples of milk done in triplicate, were tested. MAC was not detected in any of the samples. Graph 1 and graph 2 show the cycle and melting curve of a positive and negative result.

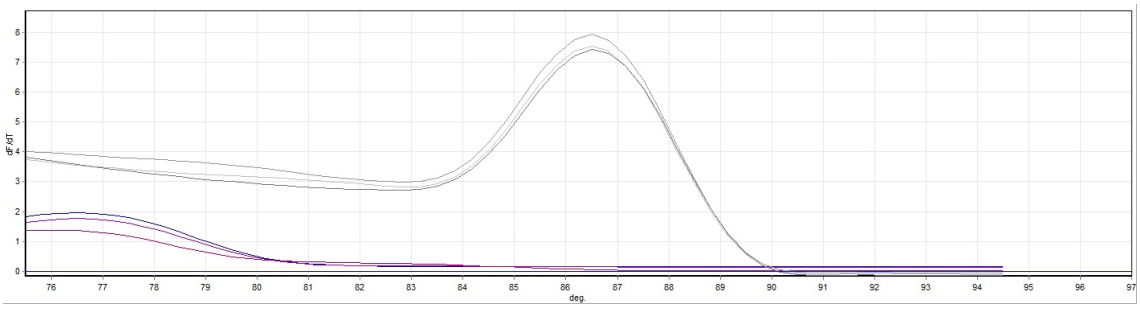
GRAPH 1

qPCR CYCLE CURVE SHOWING POSITIVE AND NEGATIVE RESULTS



GRAPH 2

qPCR MELTING CURVE SHOWING POSITIVE AND NEGATIVE RESULTS



3.2 CHEESE AND YOGHURT MAC DNA TEST RESULTS

Presence of MAC was tested in one commercially available yoghurt brand and three different commercially available cheese brands, giving a total of seven different yoghurt and cheese presentations. Through qPCR testing, no strains of MAC with in detection levels were found.

3.3 INCUBATED MILK MAC DNA TEST RESULTS

Each of the 14 different samples of milk done in triplicates of fresh commercially available milk was incubated in order to enhance bacterial proliferation. After 3 weeks and following the DNA extraction method and qPCR testing, MAC was not detected in any of the samples.

3.4 MAC DNA TEST RESULTS FOR MAC-SPIKED MILK UNDER DIFFERENT PASTERISATION METHODS

The samples of artificially MAC -spiked milk did not show any positive detection of the bacteria in the sample. The samples were divided into commercially available pasteurised milk, artificially spiked milk and two additional different spiked milk groups that where submitted into one pasteurisation process each, 72°C for 15 seconds and for 30 seconds respectively. The qPCR detection showed inconstancies during the testing by showing positive detection of MAC in one cycle and a negative detection of MAC in another cycle, determining that there was an

error during the cycle run. In addition, the artificially spiked milk sample that was not submitted to pasteurisation had a negative result for MAC when it was expected to provide a positive detection of MAC.

A second DNA extraction sampling was done with a higher concentration of MAC to exclude the possibility of a low detection level, however the results were the same as the first samples.

The qPCR test was run 5 times resulting in inconsistencies since some cycles showed positive results and other cycles showed negative results and negative results from all the samples. It was concluded that there was a high probability of an error during the qPCR testing.

A repetition of the full process of sampling, DNA extraction and qPCR testing, is suggested.

4. DISCUSSIONS

MAC is primarily found in water sources and one of the main sources of infection to humans. MAC is also found in soil due to faecal matter contamination (Kubalek 1996). They are a slow growing bacteria with high thermal resistance and an acidic pH for optimal growth. In addition, they are capable of surviving in low oxygen environments (Primm 2004). It is a pathogenic species related to pulmonary

infections, Crohn's disease and cervical lymphadenitis in children (Falkinham 1996).

One strain of MAC, *Mycobacterium avium paratuberculosis* (MAP), has been a main focus of study around the world since it is thought to be a causative agent of Crohn's disease (Hermon-Taylor 2000), which shows similarities with John's disease in cattle (Greenstein 2003). The role of MAP in the disease has been controversial and has not been demonstrated to be the sole factor for the disease (Chamberlin 2001). Crohn's disease has a high financial burden around the world. It is estimated that the United States spends between 10.9 to 15.5 billion dollars, Europe between 2.1 to 16.7 billion euros and Australia 2.7 billion dollars (Peng Yu 2008, Australian Government 2015).

MAC has been tested in milk in several countries like USA, UK, Swizerland, Brazil, Cyprus, Czech Republic, Italy, Tanzania, Austria, Chile, Iran, Iraq, India, Ireland and Turkey.

This is the first study done in Australia in order to detect MAC in milk providing additional information about the safety of commercially available milk.

To perform this study, milk samples were chosen from different South Australian brand in order to have a larger cross section of the market. The detection method chosen was quantitative real-time polymerase chain reaction (qPCR) over culture

method. MAC has a slow growth rate, making the culture detection method harder for an accurate MAC colony count (Gao 2009). QPCR provides a faster and more accurate result (Hussein 2009).

All the milk samples tested, including the fresh commercially available pasteurised milk and the incubated milk showed no detection of MAC. The cheese and yogurt samples showed the same result. This could be due to Australian milk not being contaminated with MAC as a result of the strict and constant surveillance and detection programs established for Johne's disease in cattle and sheep in Australia (Australian Government 2012). Alternatively, the negative results could be explained by a dilution factor that would affect the limit of detection. It could be that MAC is present in cattle but due to the high volume of bulk tank milk, the detection level is not reached. Finally, the reason could be that MAC is in fact present in cattle and milk, but the pasteurisation method in Australia has completely eliminated MAC in milk or lowered the amount of bacteria so that it is under detection levels.

In Australia there are different state surveillance programs for Johne's disease run by state governments, animal heath Australia and national government.

In South Australia, Primary Industry and Regions SA are in charge of prevention and control program for Johne's disease (South Australia Government 2016). The program runs constant testing for MAP on cattle, sheep, goats and alpaca. The program manages the herd and prevents risk of contamination. In case a herd is infected, the program manages and eradicates the infection. The program is based

on the National Johne's disease program (NJDP) in its 8th edition of 2012. The program not only considers the animals that could be at risk of contamination, but it includes the environment by which they can come contaminated from. The guidelines provide a land decontamination process, animal risk factor, quarantine regulations, MAP detection tests done through qPCR, immunisation program, screening definitive testing and plans for managing and eradication (Australian Government 2012).

The NJDP journal published information about known infected cattle with MAP in 2014 (Table 4) (Animal Health Australia 2015).

TABLE 4

NUMBER OF KNOWN INFECTED CATTLE HERDS IN 2014

STATE	MARCH 2014	JUNE 2014	SEPTEMBER 2014	DECEMBER 2014
NSW	124	124	124	124
SA	54	53	52	52
VIC	917	917	919	918
TAS	39	39	39	39
QLD	6	5	5	4

TOTAL	**1140**	**1138**	**1139**	**1137**

However, the programs do not provide MAP tests in milk, only blood test animals. Providing additional information, like adding milk testing for MAP into the program, could lower the probability of furthering contamination to humans by providing an additional safety barrier. In addition, asymptomatic cattle could produce contaminated milk since they may have not been selected for blood testing (Gao 2009).

Several countries have done MAP testing in bulk tank milk, including Austria, Chile, Cyprus, Ireland, Switzerland, USA and UK. All studies showed positive results for contamination of MAP with exception of Austria (Kohl 2013; Kruze 2013; Botsaris 2010; O'Reilly 2004; Corti and Stephan 2002; Grant 2002; Pillai and Jayarao 2002). Austria had no positive results for MAP through qPCR testing even though raw and pasteurized milk was previously collected from individual cattle and up to 5% tested positive for MAP. This study could suggest that the dilution of contaminated milk with non-contaminated milk made MAP detection levels too low to be shown in a qPCR testing. Cyprus study revealed positive results for MAP in raw bulk tank milk through qPCR, however none of the herds tested provided positive results for Johne's disease (Botsaris 2010). The milk could have been contaminated from the environment or the cattle could have been infected with MAP but still had not enough bacteria in the system to show symptoms of Johne's disease (Gao 2005).

MAP detection is based on a relation between volume and bacteria concentration. Detection methods, culture and qPCR, have to concentrate MAP cells in order to carry out the test making it more sensitive for MAP detection. In order to process the milk samples, a centrifuge process is necessary for bacterial concentration, resulting in a 69.4% of cells found in the pellet (Grant 1998). Milk volumes tested have varied between publications from 1 ml to 50 L samples. In the same way, centrifuge times and speed have varied from 15 min to 1 hour and up to 41,000 gr respectively (Slana 2008). PCR detection limits have also varied between publications, ranging from 10^1 to 10^3 CFU/ml (Table 5).

TABLE 5

PCR DETECTION OF MAP IN MILK BY CENTRIFUGATION

MILK	NO. SAMPLES	POSITIVE SAMPLES	%	SENSITIVITY (CFU/ ML)	VOL. (ML)	SPEED (X 1000GR)	TIME (MIN)	REF.
RAW	16	2	13	10^1	10	5	30	Tasara and Stephan 2005
BULK	20	10	50	10^2	50	2	30	Pillai and Jayarao

TANK								2002
	52	35	68	10^2	0.5	150 rpm	5	Stabel 2002
	2	2	100	10^1	10	5	30	Tasara and Stephan 2005
	100	3	3	10^2	10	5	30	Bosshard 2006
	7	5	71.4	10^2	1	-	-	Stratman n 2002
	423	23	5.5	5 x 10^2	1	-	-	Stratman n 2006
PASTE URISED	312	22	7	2 x 10^2	20	41	60	Millar 1996
	710	110	15.5	10^2	1	18	90	Gao 2002

In the present study, all samples contained 27 ml of milk and were submitted to a 3,500 rpm centrifuged process for 20 min in order to achieve a bacterial cell concentration (see 2.3.3); since several studies have shown it to be an essential step for MAC detection in qPCR (see Table 5).

The samples used in this study could have contained very low count of MAC cells resulting in the inability to be detected through qPCR with the possibility they were non existent all together, or they were reduced through a pasteurisation method (Grant 2005, Lynch 2007, Rademaker 2007, Stabel 2001, Mc.Donald 2005).

Pasteurisation processes have been used as a primary method to ensure quality and safety in dairy products since the early 1900's. 'Pasteurisation' means, that every particle of milk or milk product is heated and healed in proper equipment at one of the determined temperatures for at least the specific time (FDA 1999). The International Dairy Federation established that the right temperature- time relation to achieve proper pasteurisation is 63°C for 30 minutes or 72°C for 15 seconds however in Australia, the Food Standards of Australia and New Zealand requires the second option (Australian Government 2007).

Pasteurisation methods kill bacteria present in milk, however MAC has been shown to survive the method (Miller 1996) primarily due to its hydrophobic composition and its clumping formation (Grange 1996). Several studies have shown a reduction of MAP through the process of pasteurisation but not total elimination using two of the most common methods (Table 6). Other industrial scale studies have reported between 4 to 7 log (Grant 2005; Lynch 2007).

TABLE 6

COMPARISON OF INOCULATION OF MAP THROUGH 2 PROCESES OF PASTEURISATION

STUDY	HEAT TREATMENT			
	63° C FOR 30 MIN		72° C FOR 15 SEC	
	Inoculum, ml ^ -1	Number of decimal reductions	Inoculum, ml ^ -1	Number of decimal reductions
Chiodini and Hermon-Taylor 1993	10^4	< 2	10 ^ 4	< 2
Grant 1996	10^6-7 10^3-4	5 – 6 2- 3.7	10 ^ 6-7 10 ^ 3- 4	4.3-6 2-3.7
Grant 1999			10 ^5 -6	5.6 -6
Hope 1996			< 10 ^ 5	≈ 5
Stable 1997			10 ^ 6-7 10^ 5.5 - 6	0.5- 3 > 4.5 -5
Sung and Collins 1998	10^5-6	> 6	10^ 5-6	1-2

Keswani and Frank 1998	10^6-7	> 10	10 ^5	≈ 4

A recent report tested a direct steam injection (DSI) as a treatment in milk for MAP inactivation (Peterz 2016). The study reported that DSI at 105°C for 3 second, a minimum reduction of 5.6 log10 and as high as 7.56 log10 of MAP was obtained, making it another option for MAP treatment in milk (Peterz 2016).

In this study, the negative results could be explained through the effective method of pasteurisation. This test was done with milk samples that have been previously pasteurised before being commercially available (Australian Government 2007). During this process, if there was MAC present in milk, the procedure may have lowered the amount of bacteria resulting under the limit detection. Another possibility is that MAC was not present before the milk went through the pasteurisation process.

5. CONCLUSIONS

MAC in milk is still being researched around the world and there is still information needed to provide conclusions about its effects on human health.

Australia cannot provide conclusive information about its milk and its relation with MAC since very little research has been done to this date.

This research study has been one of the first pieces of information provided about Australian milk and its relation to MAC. The results have provided information that there is no MAC present in Australian milk or that it is under detection limits. All the samples were done in commercially available milk, which under Australian Food Standards, it does not include raw milk.

Further studies are suggested in larger amounts of samples from commercially available pasteurised milk and of raw milk, from bulk tanks and individual cows.

By testing bulk tanks milk and individual cows milk, the dilution factor would be addressed resulting in more accurate conclusion about the presence of MAC in milk. Additional MAC detection tests should also be done in pasteurised and raw milk, to establish the actual effect on the bacteria, of the Australian pasteurisation method used in milk. The tests could also provide additional information about the health status of cattle and the transmission of MAC into their milk.

Finally, MAC detection sampling should be done in animals and milk production plants from different parts of Australia, to get a broader perspective on the milks safety status of the whole country.

MAC continues to be an opportunistic pathogen for human health and a focus of study around the world. By continuing research in this area, there will be a better

understanding of the bacteria and its environment, providing more information to prevent infection resulting in environmental and public health issues.

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