Bacteria associated with *Haemonchus contortus*

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Gajenthiran Sinnathamby

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Abstract

Internal parasitism, a major cause of production losses in sheep, is routinely controlled by anthelmintic drenches, however, alternative control strategies are needed to combat the increasing resistance to these chemicals. A possible novel method of controlling abomasal nematodes, such as *Haemonchus contortus*, is manipulation of their essential resident bacteria, as is currently used to control filarial nematodes. For the first time, bacteria have been identified in the reproductive tract, as well as in the gut, of *H. contortus*, using genetic fingerprinting, light and electron microscopy and fluorescence *in situ* hybridisation (FISH).

PCR-DGGE analysis showed that adult worms had less complex bacterial profiles than did abomasal contents. L3, eggs and adult worms had similar bacterial profiles; 16S rRNA sequences obtained from seven major common DGGE bands were dominated by lactic acid bacterial and Proteobacterial sequences. PCR-DGGE short sequences and clone libraries of nearly full length sequences from all three life-cycle stages contained sequences belonging to *Weissella, Lactococcus, Leuconostoc* and *Streptococcus*. Clone library sequences were used to design group-, class- and species-specific FISH probes to locate bacteria in the parasites.

The gut lumen of adult worms contained a mixed population of Gram-positive and Gram-negative bacteria, which appeared to be multiple morphotypes in TEM images. The FISH probe (EUB338), which targets most bacteria, hybridised with the gut bacteria, but only some of these were targeted by Strc493, which targets most *Streptococcus* sp. and some *Lactococcus* sp. Neither the lactic acid bacterial group- nor the *Weissella* species-specific probes targeted any bacteria in the gut.

A single morphotype of Gram-positive bacteria was seen in large numbers in the distal uterus of female *H. contortus* in the TEM. They were close relatives of either *Lactococcus* sp. or *Streptococcus* sp., as they were targeted by the FISH probe Strc493. These bacteria seemed to be non-pathogenic to the nematodes, as

adult female worms appeared to be healthy (normal in size and active) and carry normal eggs within them. Their roles in worm biology are unknown.

A smaller number of bacteria were seen in the TEM in eggs within female worms. They were closely related to *Weissella confusa,* as all were targeted by lactic acid bacterial group- and *Weissella* species-specific probes, as well as by EUB338. These bacteria were dispersed throughout the eggs, as they could be seen at different focal panels in confocal microscopy. DNA fingerprinting and visualisation of these bacteria in eggs strongly suggest they are maternally transmitted endosymbionts.

As this study was carried out on a parasite strain which has been maintained in the laboratory, practical applications of this research would depend on these bacteria being present in field strains of *H. contortus*.

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List of Abbreviations

A	adenosine
A. aegypti	*Aedes aegypti*
A. albopictus	*Aedes albopictus*
A. cantonensis	*Angiostrongylus cantonensis*
A. stephensi	*Anophleles stephensi*
A. suum	*Ascaris suum*
ATCC	American type culture collection
BLAST	basic local alignment search tool
B. malayi	*Brugia malayi*
B. mucronatus	*Bursaphelenchus mucronatus*
bp	base pair
B. xylophilus	*Bursaphelenchus xylophilus*
C	cytosine
C. elegans	*Caenorhabditis elegans*
CTAB	cetyltrimethylammonium bromide
C. onchophora	*Cooperia onchophora*
Cy3 and Cy5	cyanine
DGGE	denaturing gradient gel electrophoresis
D. immitis	*Dirofilaria immitis*
D. melanogaster	*Drosophila melanogaster*
DNA	deoxyribonucleic acid

dNTP	deoxyribonucleotide triphosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	ethylenediaminetetraacetic
EPN	entomopathogenic nematodes
FISH	fluorescence *in situ* hybridisation
FITC	fluorescein isothiocyanate
g	gram
g	gravitational force
G	guanidine
G. rostochiensis	*Globodera rostochiensis*
h	hour
H. contortus	*Haemonchus contortus*
H & E	hematoxylin and eosin
H. glycines	*Heterodera glycines*
H. goettingiana	*Heterodera goettingiana*
H. polygyrus	*Heligmosomoides polygyrus*
H. pylori	*Helicobacter pylori*
IJ	infective juvenile
Inc	Incorporated
J2	second-stage juvenile
Kg	kilogram
kV	kilovolt
LAB	lactic acid bacteria
LB	Luria Bertani

L1	first stage larva
L2	second stage larva
L3	third stage larva
L4	fourth stage larva
LM	light microscopy
Ltd	limited
M	molar
mg	milligram
MEGA	molecular evolutionary genetics analysis
min	minute
ml	millilitre
ML	maximum likelihood
mm	millimeter
mM	millimolar
MMIC	Manawatu Microscopy and Imaging Centre
M. punctatissima	*Megacopta punctatissima*
MQ	milli Q
N. brasiliensis	*Nippostrongylus brasiliensis*
NCBI	National Center for Biotechnology Information
N. dubius	*Nematospiroides dubius*
ng	nanogram
NJ	neighbour joining
nm	nanometre
O. ostertagi	*Ostertagia ostertagi*
OTUs	operational taxonomic units

O. volvulus	*Onchocerca volvulus*
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
RDP	ribosomal database project
RNA	ribonucleic acid
RO	reverse osmosis
r.p.m	revolutions per minute
R. similis	*Radopholus similis*
SDS	sodium dodecyl sulphate
sec	second
sp	species
T	thymidine
TAE	tris-acetate EDTA
Taq	*Thermus aquaticus*
T. circumcincta	*Teladorsagia circumcincta*
T. colubriformis	*Trichostrongylus colubriformis*
TE	tris EDTA
TEM	transmission electron microscopy
TEMED	tetramethylenediamine
TGGE	temperature gradient gel electrophoresis
T. muris	*Trichuris muris*
T. spiralis	*Trichinella spiralis*

U	unit
UV	ultraviolet
[v/v]	volume per volume
[v/v/v]	volume per volume per volume
[w/v]	weight per volume
X. americanum	*Xiphinema americanum*
X. brevicollum	*Xiphinema brevicollum*
X. rivesi	*Xiphinema rivesi*
µg	microgram
µl	microlitre
µM	micromolar
16S rRNA	small subunit ribosomal RNA

Introduction

Gastrointestinal nematodes of sheep cause health and welfare issues and serious economic losses in pasture-based grazing systems in New Zealand (Leathwick et al., 2001; Vlassoff et al., 2001) and throughout the world. Brunsdon (1988) estimated that about one-third of the New Zealand sheep production was dependent on parasite control, worth $948 million per annum at that time. In Australia, the annual cost associated with parasitism has been assessed at more than $1 billion (Sacket and Holmes, 2006).

The most important nematode parasites that infect sheep and goats are members of the family Trichostrongylidae, particularly *Teladorsagia circumcincta*, *Trichostrongylus colubriformis* and *Haemonchus contortus*. *T. colubriformis* is a small intestinal parasite, whereas *H. contortus* and *T. circumcincta* infect the abomasum. *H. contortus* is a blood sucker and may cause a life-threatening disease in sheep with a severe infection. These species have similar life cycles (Silverman and Campbell, 1959; Urquhart et al., 1987), although each species has its own timing of moults, length of stay in the tissues and prepatent period before egg-laying begins.

Eggs laid by adult female worms pass out in the faeces and, under favourable warm and moist conditions, the eggs hatch into first stage larvae (L1) and develop into L2, both stages feeding on the bacteria present in the faeces. L2 develop into L3, the infective and non-feeding stage, which retains the L2 cuticle as the protective sheath. When the L3 are consumed by an appropriate host, they exsheath, or shed the retained L2 cuticle. This is stimulated in abomasal species by conditions in the rumen and in intestinal species by the conditions in the abomasum. The exsheathed L3 of abomasal parasites then briefly enter the lumen of fundic and antral glands, where they develop to L4, then immature adult worms. *H. contortus* emerge after 2-4 days in the glands as either L4 or immature worms, whereas other species remain longer in the glands and

development may be more advanced. Maturation to adult worms takes place in the gut lumen. The female *H. contortus* is very fecund, laying 5,000-10,000 eggs per day. Eggs begin to appear in the faeces after 12-15 days, although this is variable (Stoll, 1943; Urquhart et al., 1987).

H. contortus female and male adult worms have very distinctive morphological features. The adult female worm is 18-30mm long while the male is only 10-20mm long. Female worms are easily recognised by the "barber pole" colouration, caused by the coiling of the white ovaries around the red gut containing blood. The male worm has a red colour and can also be distinguished by a well-developed copulatory bursa (Urquhart et al., 1987).

In countries where sheep are intensively grazed, chemical control of gastro-intestinal parasites with anthelmintics is still the method of choice (Wolstenholme et al., 2004). The rapid spread of anthelmintic resistance (Prichard et al., 1980) is making it important that research is carried out to find novel alternatives to anthelmintic treatment (Jackson and Miller, 2006), such as grazing management, biological control, nutritional supplementation, vaccination and selective breeding of sheep (Larsen, 2006). A possible novel approach could be manipulation of nematode-associated bacteria, as is currently used for filarial nematodes of humans and animals. A similar approach may be useful for controlling parasites of ruminants, which may also have symbiotic bacteria which can be exploited in a similar way to the filarial *Wolbachia* symbionts.

Nematodes, like all other living organisms, contain bacteria. These bacteria may reside in the gut of the nematode and contribute to the nutrient requirements of the host, help to digest the food taken up by the host or increase the efficiency of nutrient absorption by host gut cells. Bacterial associations may also play crucial roles in worm biology, such as the nematodes needing these bacteria to complete their lifecycle successfully. Most studies of bacterial-nematode associations have been carried out on free-living nematode species, plant parasites,

entomopathogenic nematodes and filaria and relatively little is known about the bacteria in other animal parasitic nematodes.

The aim of the present project was to investigate the bacteria associated with sheep abomasal parasitic nematodes, particularly *H. contortus*. The project will determine whether bacteria are present in tissues other than the adult nematode and L3 gut and identify the bacterial species in the different locations in the parasites.

Chapter 1

Literature Review

1.1. Bacterial associations with eukaryotes

Bacteria live in a wide diversity of environments and have developed close relationships with protozoa, invertebrates, vertebrates and plants (Dale and Moran, 2006; Goodrich-Blair and Clarke, 2007) and even with other bacteria (von Dohlen et al., 2001; Thao et al., 2002). Associations between host organisms and prokaryotic microbes range from parasitism, pathogenesis, mutualism and commensalism to symbiosis, which is classically considered to be a mutually beneficial relationship (Goodrich-Blair and Clarke, 2007).

A precise definition of each of these associations is difficult, as the boundaries between relationships are not clear; this is particularly so for pathogenicity and mutualism in chronic bacterial infections (Dale and Moran, 2006). Parasitism is the most common relationship and it has been suggested that very long-term parasitic relationships evolved to mutualism and commensalism between the partners (Paracer and Ahmadjian, 2000; Dale and Moran, 2006). The classical definitions of bacterial-eukaryote associations have been: in parasitism, the parasite benefits nutritionally and the consequences to the host vary from no effect to death; in symbiosis, both partners benefit; the symbiont benefits in a mutualistic relationship and in a commensal relationship only the symbiont benefits. More recently, the definition of symbiosis has been widened by many investigators to include mutualism and commensalism, so that symbionts are considered to be any organisms living in close association with the host (Moran et al., 2008).

1.1.1. Symbiosis

Symbionts are considered in this review in the wider sense as organisms in intimate association with a host. They can be classified according to their location in the host as either ectosymbionts living on the surface or endosymbionts residing within host tissues. Endosymbionts can further be classified as primary obligate P-endosymbionts, which are vital for host survival, or secondary, facultative S-symbionts, which are not strictly necessary for host survival and can also exist as free-living organisms. Some obligate symbionts live in specialised host structures or organs called bacteriomes, but others are more widely disseminated in host tissues (Dale and Maudlin, 1999; Darby et al., 2005; Dale et al., 2006; Dale and Moran, 2006; Moya et al., 2008).

1.1.2. Evolution of symbiosis

Endosymbiotic relationships are believed to have developed from the entry of ancestral free living bacteria into hosts, after which both host and bacterium underwent functional and genomic changes. Those bacteria which became obligate symbionts lost the ability to exist outside the host and became reliant for spread through the host population on vertical transmission from generation to generation. Their population size became smaller than that of their free-living relatives, the genetic diversity became less and the pool of bacteria for vertical transmission became relatively small (Moran, 1996; Mira and Moran, 2002; Moran, 2003).

During adaptation to endosymbiosis, symbiont bacterial genes not essential in the new environment were lost, while vital genes were retained in a reduced genome (Moya et al., 2008). Symbionts undergoing vertical transmission are selected from a small population, so that deleterious mutations may persist, DNA repair genes can be lost and the reduced genome does not favour a high guanidine and cytosine (G+C) content (Moran et al., 2008; Moya et al., 2008; Bright and Bulgheresi, 2010). There is a good correlation between the length of association

between host and symbiont and loss of bacterial genes. The smallest genomes of endosymbionts are approaching the size of mitochondrial genomes (Andersson, 2006). Endosymbionts have smaller genomes with higher adenosine and thymidine (A+T) contents than free-living relatives, which have moderate percentages of G+C (Moran, 1996; Wernegreen, 2002; Wernegreen and Funk, 2004). Secondary symbionts and more recent primary symbionts have genome sizes and A+T contents intermediate between those of obligate symbionts and free-living organisms (Burke and Moran, 2011). These changes in gene repertoire, genome size and A+T richness occur even in gut residents, such as *Ishikawaella capsulata*, a symbiont of the stinkbug (Nikoh et al., 2011).

During co-evolution of host and symbiont, some bacterial genes have been incorporated into the host genome by horizontal gene transfer (Kondo et al., 2002; Blaxter, 2007). On the other hand, in eukaryotes whole bacteria may become organelles, such as mitochondria and chloroplasts, which are believed to result from the acquisition of a symbiotic bacterial partner that provided new metabolic capabilities to eukaryotic cells, in the form of the mitochondrial respiratory chain and oxygenic photosynthesis in the chloroplast respectively (Margulis, 1993).

1.1.3. Transmission of symbionts

Symbionts are transmitted vertically (from parents, usually the mother), horizontally (from the environment) or in some cases a mixture of horizontally and vertically. The many variations of these forms of transmission are described by Bright and Bulgheresi (2010). Horizontally acquired symbionts are facultative, as there is a free-living population which is the source of infection of the host. Usually there are stages in the host lifecycle where there are no symbionts (aposymbiotic phase), followed by uptake of symbionts from the environment and migration within the host to the housing organ. Recognition of the host by the symbiont usually involves secreted host mucus.

Vertical transmission usually involves no aposymbiotic phase and transmission through the female germ line, although there may be acquisition during mating. There are also unusual ways of maternal transmission, as in the stinkbug (*Megacopta punctatissima*), which harbours extracellular symbiotic Gammaproteobacteria in the midgut cavity (Hosokawa et al., 2005). These bacteria are deposited with the eggs on plants in "symbiont capsules", are eaten by the newly developing nymphs and colonise the midgut of the insect (Nikoh et al., 2011).

1.2. Symbiotic bacteria

A wide diversity of microbes colonise invertebrate and vertebrate mucosal surfaces, including the well-studied mammalian digestive tract, respiratory surfaces and reproductive tract (Lee and Mazmanian, 2010). Symbiotic bacteria have also been studied in some invertebrate groups, particularly insects, entomopathogenic nematodes (EPN) (Section 1.3.4) and filarial nematodes (Section 1.3.5.1), because of their agricultural and health importance. There are very specific invertebrate-microbe associations, such as the generation of bioluminescence by the *Vibrio* genus of bacteria in squid, as well as more widespread provision of nutrients, modulation of the immune and defence systems and manipulation of behaviour and reproduction (reviewed by Ruby, 2008).

The role of commensal gut microbes in fermenting indigestible foods and mucus into usable nutrients for the host is best known from the anaerobic bacteria of the vertebrate colon and rumen, as well as from invertebrate symbionts which synthesise specific nutrients (reviewed by Piel, 2004; Dubilier et al., 2008). In arthropods, some are primary maternally transmitted endosymbiotic bacteria located in specific structures and provide essential nutrients. Others are secondary (facultative) symbionts which can be acquired from the environment, but are often maternally transmitted in insects, and may contribute to host

fitness, reproduction or protection (reviewed by Kikuchi, 2009); these nonessential symbionts infect only some of the host population (Bourtzis and Miller, 2003; Baumann, 2005).

1.2.1. Gut symbionts

Little is known about the symbiotic gut bacteria of nematodes and their role in host nutrition, however, there are ectosymbiotic microbes of marine nematodes known to supply nutrients to their hosts (Dubilier et al., 2008). The best studied gut microbes are the anaerobic bacteria of the colon in hind-gut fermenters and the rumen of foregut fermenters. Much of the detailed knowledge of these organisms has been obtained from the application of molecular techniques to identify uncultivable species. These methods have largely replaced traditional microbiological culture in the study of these organisms.

1.2.1.1. Rumen microbes

As abomasal nematodes are exposed to the microbiota of the rumen and abomasum, the nematode gut symbionts may reflect the external microorganisms which continually pass through their gut. The rumen microbes consist of numerous species of bacteria, protozoa, fungi and archaea and are dominated by bacteria. These microbes are essential for the fermentation, digestion and conversion of otherwise unavailable feed to short-chain fatty acids and microbial proteins (Hungate, 1966; Hobson and Stewart, 1997). The bacterial profile of the rumen differs between species and varies with genotype, age, feed, feed additives, season and geographic location (Ørskov, 1994; Tajima et al., 2001; Zhou et al., 2009).

The rumen microbiota begins establishing in the rumen of lambs soon after birth, before the rumen is functional. Cellulolytic bacteria can survive in the rumen of lambs fed on bovine milk (Fonty et al., 1987). In field-raised lambs, individual bacteria sequentially colonise the rumen: strict anaerobes dominate at day 2; from day 2 to 10, the strict aerobes and

facultative anaerobes are 10 to 100-fold lower than the anaerobes and continue to decrease thereafter; methanogens and cellulolytic bacteria appear by day 3 and reach adult levels by the end of the first week (Fonty et al., 1984; 1987; Yáñez-Ruiz et al., 2010). The dominant bacterial species in the immature rumen belong to *Bacteroides*, *Propionibacterium*, *Clostridium*, *Peptostreptococcus* and *Bifidobacterium* (Fonty et al., 1984; 1987), whereas in adult sheep and goats, the predominant species belong to the genus *Bacteroides* and the phylum Firmicutes (particularly *Clostridium* and *Prevotella*) (Bekele et al., 2010; Cunha et al., 2011).

Culture methods showed the majority of rumen bacteria to be Bacteroidetes, but it is likely that this method over-represents this phylum (Tajima et al., 2001; Firkins and Yu, 2006). Gene sequencing has shown that culturing identifies only a small proportion of species in the rumen, as sequences from known cultivable species represented only 6.5% of total rumen bacterial sequences in databases (Kim et al., 2011). The diversities of rumen bacteria have been more clearly demonstrated by molecular biological approaches, such as PCR amplification of 16S rRNA genes (Whitford et al., 1998; Ramšak et al., 2000), hybridisation studies (Lin et al., 1994; Forster et al., 1997) and denaturing gradient gel electrophoresis (DGGE) (Kocherginskaya et al., 2001). These studies may still underestimate the diversity of specific groups of bacteria (Wintzingerode et al., 1997; Jackson et al., 2000). The increasing numbers of published clone libraries and phylogenetic analyses have resulted in reclassification of species and recognition of distinct species and novel taxonomic groups of rumen bacteria (Krause and Russell, 1996; Avhuatin et al., 1997; Tajima et al., 1999).

Two attempts have been made to collate and analyse the diversity of rumen bacteria from bacterial sequences deposited in public databases (Edwards et al., 2004; Kim et al., 2011), but these analyses are only as reliable as the sequences themselves. Edwards et al. (2004) found the rumen bacterial population to be predominantly composed of two phyla: 54% were Firmicutes and 40% were Bacteroidetes, with the remainder of

sequences belonging to a range of other bacterial phyla. A major limitation of that study was the inability to classify sequences from uncultured bacteria, due to poor similarity with sequences deposited in the GenBank database. Kim et al. (2011) analysed 13,478 bacterial and 3516 archaeal sequences in the ribosomal database project (RDP) database as at November 2010. The diversity was estimated from unique characteristics of bacterial species, by calculating the number of operational taxonomic units (OTUs) at species level. The rumen bacterial sequences were assigned to 19 phyla, with the phyla Firmicutes, Bacteroidetes and Proteobacteria dominant, and the other 16 phyla were less numerous.

1.2.1.2. Human gut microbes

The total population of human gut microbes (10^{14}) outnumbers the somatic and germ cells of the body (10^{13}) (Savage, 1977). The density of microbes increases from the mouth to the large intestine, with about 70% of gut microbes colonising the colon (Whitman et al., 1998). The gut is sterile at birth, colonisation begins immediately after the contact with the mother and the number of bacteria and the diversity of the gut flora increases during postnatal development (Palmer et al., 2007). The bacterial population is influenced by the mode of delivery (vaginal versus caesarean section), diet, age, host genetics (lean versus obese) and country of birth (developed world (Sweden) versus developing world (Pakistan)) (Long and Swenson, 1977; Simhon, 1982; Yoshioka et al., 1983; Adlerberth et al., 1991).

Enumeration of bacteria and molecular analysis of human gut samples have shown that the gut microbiota is comprised of about 35,000 bacterial species belonging to at least 50 bacterial phyla (Schloss and Handelsman, 2004; Frank et al., 2007; Sekirov et al., 2010). The gut bacteria are dominated by members of the phyla Firmicutes and Bacteroidetes and there are relatively small proportions of Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria and Cyanobacteria (Eckburg et al., 2005). The diversity of bacteria in the gut lumen is different from the population

of bacteria attached to the mucus layer and closely associated with the epithelium (Cheesman and Guillemin, 2007; Marchesi, 2011).

Apart from fermenting indigestible foods and mucus into usable nutrients for the host, gut microbes are important to the host in many ways: the commensal bacteria contribute to the development and homeostasis of the immune system; influence epithelial cell proliferation and differentiation and participate in protection against pathogens (Roberfroid et al., 1995; Falk et al., 1998; Guarner and Malagelada, 2003).

1.2.1.3. Gut microbes of the "Ecdysozoa"

In the absence of detailed studies of the gut microbiota of nematodes, the best model may be the symbionts of arthropods, which have been functionally linked with nematodes into a clade called the "Ecdysozoa" (Telford et al., 2008). Although morphologically dissimilar, nematodes and arthropods have many similar features in their endocrinology and physiology, including moulting during development. The gut microbes of insects, which include bacteria, archaea, and eukaryea, are mostly present in the hind gut region (Bourtzis and Miller, 2003). Groups of related insects tend to share related species of symbionts e.g. most pea aphids have primary endosymbionts of the genus *Buchnera* (Douglas, 1998) and tsetse flies harbour *Wigglesworthia* (Rio et al., 2006). In both cases, these are located in specialised cells known as bacteriocytes or mycetocytes (Moran et al., 1993; Douglas, 1998; Shigenobu et al., 2000; Akman et al., 2002). There are also resident populations of microbes in insects which do not have specialised structures; the diversity is usually lower when the gut is a simple tube or there is a high throughput of digesta (Dillon and Dillon, 2004).

Gut microbes form multispecies communities (Paster et al., 1996) and are probably compatible with the host only as complex populations; this was the case in the pea aphid, in which some monocultures of normal gut

organisms were lethal (Harada et al., 1997). The density of microbes can be very high: in the termite hind gut there were 10^7 protozoa and 10^9 to 10^{10} bacteria per ml of gut contents, mostly in the lumen, but some adhering to the gut wall (Smith and Douglas, 1987; Bourtzis and Miller, 2003).

Gut bacteria provide important nutrients for the host (reviewed by Douglas, 1998; Dillon and Dillon, 2004; Zietz et al., 2004). Gut symbiotic bacteria which synthesise essential nutrients for the host allow insects to feed on a wide range of food sources, including sub-optimal diets deficient in nitrogen, specific amino acids, sterols or vitamin B (Cruden and Markovetz, 1987; Douglas and Prosser, 1992). Alternatively, gut microorganisms may provide specialised enzymes, such as cellulase in the termite, which allow digestion of plant material which cannot otherwise be degraded by the insect (Dillon and Dillon, 2004).

A change in diet can cause a rapid change in the microbial population in the hind gut (Santo Domingo et al., 1998); when the cockroach diet was changed to a low-protein, high-fibre diet, there were significant reductions in Streptococci and Lactobacilli in the host (Kane and Breznak, 1991), whereas cellulose-rich diets promoted the proliferation of protozoal populations (Gijzen et al., 1994). Similarly, the bacterial diversities of wild ground beetles were greater than those of laboratory-reared beetles (Zouache et al., 2009) and in field collected *Anophleles stephensi* mosquito larvae than in laboratory strains (Rani et al., 2009; Zouache et al., 2011).

Earlier studies of insect gut microbes had a limited ability to classify cultivable symbionts or those which could be seen microscopically. Culture of pea aphid excreta on nutritional agar identified several species of gram-negative, oxidative-negative, facultative anaerobic, fermentative, motile, rod-shaped Enterobacteriaceae, whose biochemical characteristics showed that they were related to *Erwinia herbicola* and *Pantoea agglomerans*, which are ectoparasites of many plants (Harada et al.,

1997). The hindgut microflora of cockroaches (Bracke et al., 1979) and termites (Honigberg, 1970) consist predominantly of obligate and facultative anaerobes.

More recent studies using denaturing gradient gel electrophoresis (DGGE) fingerprinting techniques and visualisation with fluorescence *in situ* hybridisation (FISH) have been better able to demonstrate in insects the diversity of microbes, most of which are non-cultivable (Baumann and Moran, 1997). The majority of bacteria associated with insects belong to the phyla Firmicutes, Bacteroidetes and Proteobacteria, although there is wide variation in bacterial species within related hosts, location and food source. Some insect species have been extensively studied, including ground beetles and mosquitoes. The gut microbial population of ground beetles included Bacilli, Fusobacteria, Gammaproteobacteria, Alphaproteobacteria, Clostridia and Bacteroidetes, of which the dominant bacteria were bacilli (*Enterococcus casseliflavus*, *Weissella cibaria* and *Enterococcus faecalis*) in wild beetles, but Gammaproteobacteria in laboratory-raised beetles (Lehman et al., 2009). The gut microbes identified in the mosquitoes *A. aegypti* and *A. albopictus* included *Acinetobacter*, *Pseudomonas*, *Asaia*, uncultured Gammaproteobacteria and at the genus level *Bradyrhizobium* sp., *Delftia* sp., *Herbaspirillum* sp., *Rhizobium* sp., *Stenotrophomonas* sp., *Enterobacteriaceae*, *Streptococcaceae* and *Staphylococcus* (Zouache et al., 2011).

1.2.2. Bacterial manipulation of reproduction

The best known of the endosymbionts that alter host reproduction in arthropods are *Wolbachia pipientis*, which use the host insect to spread their progeny, do not provide any benefit to the host, but can alter insect reproduction (Werren, 1997; Werren and O'Neill, 1997). In contrast, some of the supergroups of *Wolbachia* present in filarial nematodes are beneficial to their hosts (Foster et al., 2005; Taylor et al., 2005). Other bacteria, such as the gut commensal bacteria of the fruit fly *Drosophila melanogaster*, influence host mating preferences through the synthesis of

pheromones (Sharon et al., 2010). Another form of host manipulation is shown by symbiotic *Spiroplasma*, which protects female, but not male, *Drosophila hydei* from death from parasitic wasp attack (Xie et al., 2011).

Maternally transmitted "reproductive endosymbionts" are common amongst insects. They manipulate host reproduction to facilitate their own transmission in many different ways, depending on the host and bacterium. Cytoplasmic incompatibility, which causes sterility of hybrids between infected males and females that are either uninfected or infected with a different strain of symbiont, is the most common form (Werren et al., 1995; Stouthamer et al., 1999). Other manipulations reduce the male population: by male killing, which causes males to die during embryogenesis (Hurst et al., 1999); parthenogenesis, the initiation of asexual daughter development, and feminisation, which changes males into females (Breeuwer et al., 1992; O'Neill et al., 1992; Rousset et al., 1992; Stouthamer et al., 1993).

There are recognised inherited bacteria which cause reproductive manipulations of the arthropod host. They belong to seven different bacterial groups, the most common being *Wolbachia* (reviewed in Duron et al., 2008). These authors surveyed 136 arthropod species and found that overall 32.4% of arthropods contained these bacteria, some more than one species. *Wolbachia* were the most common symbionts (22.8%), *Cardinium*, *Arsenophonus* and *Spiroplasma* infected 4-7% and *Flavobacterium* and *Rickettsia* infected a small number of species. These symbionts come from four phyla: *Wolbachia* and *Rickettsia sp* from Alphaproteobacteria; *Arsenophonus* from Gammaproteobacteria; *Cardinium* and *Flavobacterium* from Bacteroidetes and *Spiroplasma* from Mollicutes.

Wolbachia were first recognised microscopically in the 1920s as Rickettsia in insects and arachnid tissues, including eggs, leading to the conclusion that they were maternally transmitted (Cowdry, 1923; Hertig and Wolbach, 1924). Since then, strains of *Wolbachia pipientis* have been

recognised as essential symbionts of filarial nematodes, whereas the strains associated with insects have a variety of effects on insect reproduction (Werren et al., 1995; Jeyaprakash and Hoy, 2000; Bandi et al., 2001; Karr and Ballard, 2001; Fenn and Blaxter, 2004; Hilgenboecker et al., 2008). These bacteria cannot be cultured in defined media and identification of the species is mainly reliant on PCR based analysis of the 16S rRNA gene, as well as the protein-encoding genes *gltA, groEL* and *ftsZ* (Lo et al., 2002; Casiraghi et al., 2005). *Wolbachia* are currently classified into 8 "supergroups" (A to H) (Lo et al., 2007; Ros et al., 2009).

1.2.3. Bacterial manipulation of host fitness

In some instances, host immunity is triggered by symbiotic bacteria, so that the host can control the symbiont population (Rio et al., 2006). This can be directed even against obligate intracellular symbiotic bacteria which cannot live independently (McGraw and O'Neill, 2004) and must avoid the host immune system to survive. Endosymbionts activate the development of the immune system of the tsetse fly (Weiss et al., 2012) and may collaborate with host immunity to protect against parasites and pathogens (Kitano and Oda, 2006; Haine, 2008). Endosymbionts (*Regiella insectocola*) of aphids provide protection to their host against the fungal pathogen *Pandora neoaphidis* (Ferrari et al., 2004; Scarborough et al., 2005). The facultative symbionts *Serratia symbiotica* and *Hamiltonella defensa* protect the host pea aphid from parasitic infection by *Aphidius ervi* and *Aphidius eadyi* (Oliver et al., 2003, 2005; Ferrari et al., 2004). Studies on *Wolbachia* suggest that the extent of their collaboration with the host immune system is determined by the genotypes of host and symbiont (Bourtzis et al., 2000; Fytrou et al., 2005; Pankewitz et al., 2007). *Wolbachia* reduce the ability of *Drosophila simulans* to encapsulate eggs of the parasitoid wasp *Leptopilina heterotom*, but do not affect host defences against an entamopathogenic fungus (Fytrou et al., 2005).

Outcompeting invaders for resources inside the host is one way symbionts protect the host (Haine, 2008). Another strategy of symbionts to assist host defences is through synthesis of toxic chemicals. Wolf spiders predate on *Paederus* beetles and the symbionts associated with these beetles are capable of producing toxins which can protect their host larvae from the wolf spider predators (Kellner and Dettner, 1996; Kellner, 1999). A similar situation has developed in crustaceans. Symbiotic microbes produce antifungal metabolitites to protect *Palaemon macrodactylus* and *Homarus americanus* from the fungus *Lagenidium callinectes* (Gil-Turnes et al., 1989; Gil-Turnes and Fenical, 1992).

Some secondary endosymbionts contribute to host fitness by increasing heat tolerance in some environments (Russell and Moran, 2006). Two secondary symbionts of pea aphids, one a Rickettsia and the other a Gammaproteobacterium, have been shown to allow the insects to reproduce under heat stress and therefore to adapt to a greater range of environments (Montllor et al., 2002).

1.3. Bacteria associated with nematodes

1.3.1. Free-living soil nematodes

Studies of the interactions of terrestrial nematodes with bacteria have principally focused on bacteria as a food source, but more recently also on pathogenic bacteria as a model of host-pathogen interactions and as a possible nematode biocontrol strategy.

Bactiverous nematodes are selective in their choice of microbes as a food source; different nematode species feed only on some types and sizes of bacteria, which they can identify from the chemical cues emitted by the bacteria (Katznelson and Henderson, 1962; Venette et al., 1998; Moens et al., 1999; Rodger et al., 2004; Salinas et al., 2007). Many nematodes also

use negative chemical cues to avoid taking in pathogenic bacteria (Zhang et al., 2005; Hasshof et al., 2007; Rae et al., 2008).

Identification of symbiotic bacteria in bactiverous nematodes is difficult using 16S rRNA sequences, as this method does not distinguish between those consumed as food, surface contaminants, pathogens and symbionts (Standing et al., 2006; Treonis et al., 2010). Symbionts have, however, been identified in free-living nematodes. Grassland soil nematodes with five different feeding habits all had bacterial profiles which were clearly different from those of their environments (Ladygina et al., 2009). There was also less bacterial diversity in the nematodes, as the soil samples had bacteria from 10 phyla, compared with 3 phyla in the nematodes. The most abundant soil bacteria were Alphaproteobacteria, whereas Gammaproteobacteria (mostly *Pseudomonas* sp.) dominated in the nematode bacterial profiles. Actinobacteria were either absent or represented a very low percentage of the nematode bacterial profile. Acidobacteria and Gemmatimonadetes were not present in any of the nematode bacterial profiles, but were present in the soil bacterial communities.

The host immune response to bacteria and the establishment of pathogens have been a recent focus of nematode-bacterial interactions, using *Caenorhabditis elegans* as a host model organism (Kurz and Ewbank, 2000). Microorganisms used in these studies include *Pseudomonas aeruginosa* (Tan et al., 1999), *Salmonella typhimurium* (Labrousse et al., 2000), *Escherichia coli* (Anyanful et al., 2005), *Serratia marcescens* (Kurz and Ewbank, 2000; Mallo et al., 2002), *Microbacterium nematophilium* (Hodgkin et al., 2000), *Burkholderia pseudomallei* (Garsin et al., 2001), *Acinobacter baumanii* (Peleg et al., 2008), gram positive bacteria (Couillault and Ewbank, 2002) and fungi (Mylonakis et al., 2002). One of these laboratory studies of *C. elegans* showed that the ability of different bacteria to out-compete resident founder species was mainly dependent on close interaction of the bacterium with host intestinal cells (Portal-Celhay and Blaser, 2012).

More recently, another free-living nematode *Priostionchus pacificus* has been used to determine host-bacterial interactions, in this case susceptibility to *Bacillus* sp. (Rae et al., 2010). *Priostionchus* is a genus of necromenic nematodes found in close association with specific insects (Herrmann et al., 2006) on which they feed after the death of the insect (Rae et al., 2008).

1.3.2. Free-living marine nematodes

The association of sulphur-oxidising, chemoautotrophic bacteria with sediment inhabiting microscopic marine nematodes is one of the many examples of symbiosis between marine invertebrates and chemosynthetic bacteria (Cavanaugh et al., 1988; Cavanaugh, 1994; reviewed by Dubilier et al., 2008). By migrating between reduced and oxidised sand layers, the nematode provides oxygen and sulphide to the symbiont, which in turn provides food for the host (Ott et al., 1991; Polz et al., 1992).

The cuticle of Stilbonematinae carry sulphur-oxidising ectosymbionts (Ott et al., 1991; Polz et al., 1992); these bacteria have been shown by $\delta^{13}C$ analysis to have been acquired as free-living environmental bacteria in the sediments. The nematodes feed on these bacteria, which can be seen attached to the gut of these nematodes (Ott et al., 1991). Highly species-specific symbiont-nematode attachments are mediated by interactions of sugars on the bacteria with specific pattern recognition Mermaid molecules on the surface of the nematodes (Nussbaumer et al., 2004; Bulgheresi et al., 2011).

Whereas ectosymbiotic microbes may be dominated by one species, as on *Laxus oneistus* and *Robbea* sp. (Bayer et al., 2009), other nematodes, such as *Eubostrichus dianae*, have a mixed population of bacteria (Polz et al., 1999). Based on electron microscopy, 16S rRNA libraries and FISH analysis, each of three *Robbea* sp. was covered by only one distinct

bacterial morphotype (Bayer et al., 2009). Known ectosymbionts are morphologically diverse: there were both a long and filamentous dominant species and smaller rod-shaped bacteria on *E. dianae* (Polz et al., 1999); coccoid bacteria formed a multilayered coat on all but the head and the tip of the tail on the *Stilbonema* (Polz et al., 1992) and rod-shaped bacteria formed a monolayer on *Laxus* sp. (Polz et al., 1994). Analysis of 16S rRNA sequences showed that the ectosymbionts of *Laxus* sp. (Polz et al., 1994), three *Robbea* sp. (Bayer et al., 2009) and *E. dianae* (Polz et al., 1999) belonged to Gammaproteobacteria, however, the sequences from the dominant filamentous bacteria on *E. dianae* were not represented in the clone libraries.

As the gut is absent or non-functional in nematodes of the genus *Astomonnema*, their sulphur-oxidising symbionts are internal (Musat et al., 2007). Microscopically, these bacteria filled almost the entire worm and 16S rRNA sequence analysis showed them to be closely related to the ectosymbionts of *Stilbonema*.

1.3.3. Plant parasitic nematodes

1.3.3.1. *Xiphinema* and Verrucomicrobia

The plant parasitic nematodes *Xiphinema americanum*, *Xiphinema rivesi* and *Xiphinema brevicollum* each harbours its own species of maternally transmitted intracellular bacterium belonging to the Verrucomicrobial clade, designated respectively *Candidatus* Xiphinematobacter americani sp. nov., *Candidatus* Xiphinematobacter rivesi sp. nov. and *Candidatus* Xiphinematobacter brevicolli sp. nov (Coomans et al., 2000; Vandekerckhove et al., 2000). The mature symbionts seen by Vandekerckhove et al. (2000) were 0.7-1.0μm wide, 2.1-3.2μm long, rod-shaped bacteria with rounded ends; they were clustered around the developing oocytes, embedded mainly inside the epithelial wall of the ovaries in adult female nematodes. Bacteria populated the gut cells of juvenile worms; during the moult to adults,

bacteria concentrated around the developing oocytes, which took up only a few bacteria. These proliferated, so that mature eggs contained several hundred bacteria. In males, the bacteria remained in the gut and appeared to be latent and not dividing and did not enter the testes (Vandekerckhove et al., 2002).

1.3.3.2. Cyst forming nematodes

Endosymbiotic rod-shaped (0.3-0.5μm diameter, 1.8-3μm long) bacteria were first seen in the 1970s in electron microscopic images of adult and second-stage juvenile (J2) nematodes *Globodera rostochiensis* (potato cyst nematode), *Heterodera goettingiana* (pea cyst nematode) (Shepherd et al., 1973) and *Heterodera glycines* (soybean cyst nematode) (Endo, 1979). Noel and Atibalentja (2006) confirmed the presence of bacteria in the *H. glycines* intestine, pseudocoelome, oocytes, ovaries, spermatozoa, hypodermal cords of males and in hypodermal cords, intestinal tissue and pseudocoelom of J2. Analysis of bacterial 16S rRNA and *gyr*B gene sequences placed these bacteria in the phylum Bacteroidetes. They proposed the name *Candidatus* Paenicardinium endonii for this endosymbiont from structural similarity and gene homology to *Candidatus* Cardinium hertigii, an endosymbiont of wasps of the genus *Encarsia* (Noel and Atibalentja, 2006).

1.3.3.3. *Radopholus similis*

Bacteria characterised as *Wolbachia* have been seen by transmission electron microscopy and fluorescent immunolocalisation in the reproductive tract in the burrowing nematode *R. similis*, a parasites of bananas (Haegeman et al., 2009). Sequencing of the 16S rRNA, *fts*Z and *gro*EL genes showed the endosymbiont of *R. similis* to be distantly related to the known *Wolbachia* supergroups and was placed in a new supergroup I. The role of these bacteria in plant parasitic nematodes is unknown, but could be similar to that in filarial nematodes (Haegeman et al., 2009).

1.3.3.4. Pine wilt nematodes

Pine wilt disease is a disease of conifers caused by a combination of the nematodes *Bursaphelenchus xylophilus* or *Bursaphelenchus mucronatus* and the bacteria they carry. The pathogenicity of the disease appears to result from toxins secreted by the bacteria, which vary with the geographical location (Oku et al., 1979; Mamiya, 1980; Cao et al., 2001; Han et al., 2003). Using bacterial culture and morphological criteria, three of these bacteria were identified by Han et al. (2003) as *Pseudomonas fluorescens* biotypes I and II and *Pantoea* sp. The bacterial diversities of a Chinese and a Japanese isolate of *B. mucronatus* were compared morphologically, by their metabolic characteristics and by 16S rRNA gene sequences: clones clustered into Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Bacteriodetes (Tian et al., 2011). Alphaproteobacteria and *Rhizobium* sp. dominated in the Japanese clone library, whereas the four groups were numerically similar in the Chinese library, with *Pedobacter* sp. the slightly dominant species.

1.3.4. Entomopathogenic nematodes

Entomopathogenic nematodes (EPN) are lethal insect parasitoids, which have been used effectively for the biological control of lepidopteran, dipteran, and coleopteran pests (Burnell and Stock, 2000). Steinernematidae and Heterorhabditidae are the most extensively studied and widely exploited families of these nematodes (Kaya and Gaugler, 1993). Their symbiotic bacteria, *Photorhabdus* and *Xenorhabdus* respectively, are motile, Gram-negative Gammaproteobacteria belonging to the family *Enterobacteriaceae* (Akhurst, 1980; Boemare and Akhurst, 1988), which colonise the intestines of the infective soil-dwelling stage of the EPNs (Goodrich-Blair and Clarke, 2007). These bacteria play different roles in their insect and nematode hosts: pathogens in the insect host, but mutualists in the nematode (Fig. 1.1) (reviewed by Boemare et al., 1996; Forst et al., 1997; ffrench-Constant et al., 2003; Herbert and Goodrich-Blair, 2007).

The nematode life cycle starts with the laying of eggs in the soil and the eggs developing into L1, L2 and L3, which is non-feeding and known as the infective juvenile (IJ) (Sicard et al., 2004b). The symbiotic bacteria *Photorhabdus* or *Xenorhabdus* colonise the anterior intestine of IJ in a structure called the vesicle (Morgan et al., 1997; Sicard et al., 2004a). The IJ penetrate through the cuticle of insects into the haemolymph, from where the IJ regurgitate the symbiotic bacteria (Ciche and Ensign, 2003), which multiply and release toxins and exoenzymes that are lethal to the host insects. The insects usually die of septicaemia within 24-48 hours. The symbiotic bacteria also provide nutrients for the nematodes, which resume their life cycle, the IJ moulting to L4 and becoming adult worms within 2-3 days. The nematodes multiply and reproduce for 2-3 generations until the nutrients are exhausted and further nematode development is inhibited, causing IJ to accumulate. The bacteria re-colonise the intestine of the IJ before they emerge from the insect cadavers into the soil, where they can survive for several months awaiting a suitable host (Wang and Bedding, 1996; Goodrich-Blair and Clarke, 2007).

The toxins produced by *Photorhabdus* and *Xenorhabdus* bacteria are not only toxic to a range of insects, but also to other plant parasitic nematodes, suggesting they could have a practical use in controlling these parasites (Lewis et al., 2001; Jagdale et al., 2002; ffrench-Constant et al., 2007).

1.3.5. Nematodes of mammals

Studies on the associations of bacteria with nematode parasites of animals and humans are few, apart from those on *Wolbachia* in filarial nematodes and biocontrol of agriculturally important species by nematode pathogens, such as *Bacillus thuringiensis* (Kotze et al., 2005). It has long been known that the establishment of several species of gastrointestinal nematodes is lower in germ-free mice (Wescott and Todd, 1964; Stefanski and Przyjalkowski, 1965; Wescott, 1968), but only recently has the

interaction of the host gut, commensal bacteria and metazoan parasites been investigated (Hayes et al 2010; Walk et al., 2010).

1.3.5.1. *Wolbachia* in filaria

Wolbachia were first seen in nematodes in the 1970s, when Rickettsia were reported within the hypodermal cords and eggs of filaria, suggesting they were maternally transmitted symbionts (Kozek, 1970; Lee, 1975). These organisms were later identified as *Wolbachia* using 16S rRNA gene sequencing (Sironi et al., 1995) and partial sequencing of the *ftsZ* gene (Bandi et al 1998).

Symbiotic *Wolbachia* have been detected in the majority of filarial nematode species, including the human parasites *Brugia malayi*, *Onchocerca volvulus, Wuchereria bancrofti* and *Mansonella ozzardi* (Bandi et al., 1998), the dog heart worm *Dirofilaria immitis* (Kramer et al., 2003) and the bovine parasite *Onchocerca ochengi* (Hansen et al., 2011). Symbiotic *Wolbachia* are absent from a smaller number of filarial species, including *Onchocerca flexuosa* (Bandi et al., 1998), *Setaria equine* (Chirgwin et al., 2002) and *Loa loa* (McLaren et al., 1975; Cross et al., 2001). It was suggested by Taylor et al. (1999) that all female *B. malayi*, but only 25% of males, were infected with *Wolbachia*, however, a later unpublished study by the same group using nested PCR with eubacterial primers and *Wolbachia* specific primers identified that most of the male worms contained bacteria (M.J. Taylor and H. Cross, unpublished, cited in Taylor and Hoerauf, 1999).

In those filaria in which *Wolbachia* are present, they are obligate mutualistic endosymbionts which are essential for worm embryogenesis, development and adult survival (Taylor and Hoerauf, 1999; Hoerauf and Pfarr, 2007). The bacteria appear to supply essential nutrients to the nematodes: sequencing the complete genome of the *Wolbachia* strain from *B. malayi* revealed that the bacterium carried the genes required to synthesise riboflavin, haem, glutathione, glycolytic enzymes and

compounds necessary for the biosynthesis of purines and pyrimidines, whereas these genes were absent from the host (Foster et al., 2005).

Antibiotic therapy has proved useful in treating human and animal filarial infections and doxycycline treatment is now widely recommended in endemic areas (Hoerauf et al., 2003; Hoerauf and Pfarr, 2007). As symbiotic *Wolbachia* are mainly responsible for chronic inflammation and pathology in filariasis (Taylor et al., 2000; Keiser et al., 2002), antifilarial drug treatment and release of bacteria causes adverse reactions; these reactions can be reduced by prior or concurrent elimination of the bacteria (Brattig et al., 2000; Cross et al., 2001; Punkosdy et al., 2003). Prolonged antibiotic treatment of filarial-infected animals with anti-rickettsial and/or antibacterial drugs (doxycycline, tetracycline and rifampicin) caused delayed moulting, reduced growth rates and failure of embryogenesis in the parasites and eventually death of the microfilariae. In contrast, similar treatment of animals that were parasitised by filarial worms free of endosymbiotic *Wolbachia* was without effect on the parasites (Hoerauf et al., 1999; 2000; Casiraghi et al., 2002; Fenn and Blaxter, 2006). Treatment of infected humans with doxycyline, tetracycline, rifampicin and chloramphenicol effectively reduces the L3 to L4 moult, causes stunted larvae (Smith and Rajan, 2000) and impairs worm embryogenesis (Hoerauf et al., 1999).

1.3.5.2. *Wolbachia* in Strongyloidae

It has generally been accepted that *Wolbachia* are not present in nematodes other than the filaria (Bordenstein et al., 2003; Duron and Gavotte, 2007). Tsai et al. (2007) reported that *Wolbachia ftsZ*, WSP and 16S rRNA sequences could be obtained by amplification of genomic DNA of the rat lungworm *Angiostrongylus cantonensis*, a member of the Metastrongylidae, however, this could not be replicated by PCR and immunohistochemistry by Foster et al. (2008), who suggested the earlier finding could have resulted from contamination with DNA from arthropods and filarial nematodes.

1.3.5.3. *Ascaris suum*

The bacteria in Ascarids have been studied only by culture-based methods, which detect only the few cultivable species in a bacterial population (Amann et al., 1995). Facultative anaerobes from the intestine of *A. suum* were cultured aerobically on brain heart infusion agar as part of an investigation on possible serotonin synthesis by the nematode intestinal microflora (Hsu et al., 1986). The bacterial species cultured were *Escherichia, Enterobacter, Klebsiella, Acinetobacter, Citrobacter, Pseudomonas, Aeromonas* and *Shigella.* Seventeen cultures secreted serotonin into the culture medium (Hsu et al., 1986) and incubation of worms with antibiotics showed that bacteria could contribute to the serotonin levels in the worm intestinal tissue (Shahkolahi and Donahue, 1993).

1.3.5.4. *Heligmosomoides polygyrus*

The bacterial diversities of L3 and adult *H. polygyrus* were determined as part of a study investigating the effects of this small intestinal parasite on ileal and caecal bacterial populations of mice (Walk et al., 2010). Despite the bacteria in two groups of mice varying considerably before infection with *H. polygyrus*, introduction of the parasite increased the population of Lactobacillaceae in both groups of mice. The construction of 16S rRNA sequence clone libraries of bacteria in the infected ileal tissues, adult worms and L3 revealed that the bacterial profiles of adult worms were very similar to those in the infected ileum, with the majority of bacterial species belonging to the phylum Firmicutes (Lactobacillaceae). Both profiles were different from the L3 bacterial profile, which was mainly comprised of species of the phylum Proteobacteria (mostly family Pseudomonadaceae).

1.3.5.5. *Trichuris muris*

Host gut bacteria appear to be necessary for the development of some gastro-intestinal parasitic nematodes. The mouse large intestinal whip

worm *T. muris* has recently been used by Hayes et al. (2010) to investigate the well-known phenomenon of lower establishment of nematode parasites in germ free mice. Species known to be affected by the germ-free status of the host include *Nippostrongylus brasiliensis* (Wescott and Todd, 1964), *H. polygyrus* (formerly *Nematospiroides dubius*) (Wescott, 1968) and *Trichinella spiralis* (Stefanski and Przyjalkowski, 1965). Hayes et al. (2010) observed that fewer embryonated eggs hatched in mice in which the gut microbes had been reduced by antibiotic treatment.

Bacteria were shown to facilitate *T. muris* egg hatching in both caecal explants containing mixed bacteria and in cultures of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium* or *Pseudomonas aeruginosa* (Fig. 1.2). *Escherichia coli* interacted with the polar operculum of the egg through type 1 fimbriae, which are adhesion organelles used by many gram-negative bacteria to facilitate their adherence to mucosal surfaces and inflammatory cells

1.3.5.6. *Haemonchus contortus*

There appear to be few reports of studies designed to identify the bacteria naturally associated with any sheep gastrointestinal nematode parasite. An attempt has been made to culture potentially pathogenic bacteria of livestock from L3 obtained by *in vitro* culture of faeces collected in the U.K. from animals infected with the parasitic nematodes *O. ostertagi, Cooperia onchophora* and *H. contortus* (Lacharme-Lora et al., 2009). The bacterial species identified in *H. contortus* were *Sphingobacterium multivorum* and *Streptococcus macacae*. *Sphingobacterium multivorum* is an opportunistic pathogen, while *Streptococcus macacae* is a commensal bacterium of humans and sheep.

The main focus of studies on bacterial interactions with *H. contortus* has not been on symbiotic bacteria, but rather on potential pathogens, such as the soil bacterium *Bacillus thuringiensis*, which could be exploited to

control the nematodes. Promising candidates are the toxic *Bacillus thuringiensis* crystal proteins (Kotze et al., 2005; O'Grady et al., 2007; Linares et al., 2008), which are commonly used in the biological control of insects (Siegel, 2001). The efficacy of *Bacillus thuringiensis* and its toxins have been compared with thiabenzadole and levamisole as anthelmintics against larval and adult stages of the three main sheep nematode parasites *H. contortus*, *T. colubriformis* and *T. circumcincta* (Kotze et al., 2005) with encouraging results.

1.4. Exploring the bacterial profile of *Haemonchus contortus*

The aim of the present project was to investigate the resident bacterial populations of adult worms and infective larvae (L3) *H. contortus*. Culture-independent methods were used, as more than 99% of bacterial species cannot be cultured by conventional culture-based methods (Amann et al., 1995). This is particularly so for endosymbionts, which frequently cannot be grown outside the host organism. Molecular fingerprinting and phylogenetic analyses were the methods of choice, as they had been successfully used to explore the composition of microbial communities and identify species previously unknown from culture methods (Ward et al., 1990; Hugenholtz and Pace, 1996).

Identification of bacteria most often involves analysis of 16S rRNA, or the gene encoding it, as the phylogeny of this gene is representative of the phylogeny of the organism (Fox et al., 1980). Although three bacterial ribosomal RNAs, the 5S, 16S and 23S subunits, can be used for this purpose, 16S rRNA is the most commonly used, as it is a relatively large molecule (1500 nucleotides) and contains sufficient information for meaningful phylogenetic analysis, yet is small enough for practical handling (Woese et al., 1975; Stahl et al., 1985; Olsen and Woese, 1993). 5S rRNA (250 nucleotides) does not contain sufficient information to

distinguish between closely related organisms, while the larger 23S rRNA has a high rate of change of the information it carries. The gene which encodes for the16S rRNA contains both highly conserved and highly variable regions. The highly conserved regions are common to all bacteria and are used to analyse distantly related bacteria, whilst even closely related organisms can be distinguished from each other by analysing the highly variable regions (Woese et al., 1975; Stackebrandt and Woese, 1981).

Microbial communities can be studied using the genetic fingerprinting techniques DGGE and temperature gradient gel electrophoresis (TGGE) to analyse PCR products amplified by universal bacterial primers from mixed bacterial 16S rDNA fragments (Muyzer et al., 1993; Muyzer and Smalla, 1998). DGGE separates sequences on the basis of their melting behaviour at different levels in a linear denaturing gradient, after which DNA in bands of interest can be extracted, cloned and sequenced. Sequences from these bands can be compared with those in online sequence databases (Muyzer et al., 1993; Amann et al., 1995; Wu et al., 2001). The success of these methods depends on efficient DNA extraction from the microorganisms which are associated with the host cells. The advantages and limitations of these methods have been reviewed by Muyzer and Smalla (1998), Muyzer (1998), and Burr et al. (2006). They concluded that the advantage of PCR-DGGE is that it identifies the most abundant microbial sequences, but the major limitation is the shortness of the sequences involved, which restricts meaningful phylogenetic analysis.

To investigate the bacteria associated with *H. contortus*, initially parasites were examined by transmission electron microscopy (TEM) and light microscopy (LM) to establish their presence in the nematodes (Chapter 2). After developing appropriate protocols (Chapter 3), PCR-DGGE was used to compare the bacterial communities associated with *H. contortus* life-cycle stages and determine the phylogenetic affiliations of ~190bp bacterial sequences in excised DGGE bands, by matching them to entries in online sequences databases (Chapter 4). Nearly full length 16S

rRNA sequences (~1400bp) were used to make clone libraries and construct phylogenetic trees for more meaningful analysis of the phylogenetic relationships of bacterial sequences obtained from *H. contortus* (Chapter 5). Finally, the locations of the bacteria in the nematode gut and reproductive tissues were visualised by FISH, using universal, group-, and class- and species-specific probes (Chapter 6).

Chapter 2

Visualisation of Bacteria using Transmission Electron and Light Microscopy

2.1. INTRODUCTION

Many of the bacterial symbionts visualised in nematodes are Gram-negative bacteria belonging to the phylum Proteobacteria. *Wolbachia* seen in developing eggs, lateral cords and germ cells of filaria (Chapter 1, Section 1.3.5.1) and in the reproductive tract of the plant parasitic nematode *R. similis* (Section 1.3.3.3) have a similar ultrastructure. They are usually rod-shaped and up to 1.5μm long, although some are smaller and spherical, and have the typical Alphaproteobacterial Gram-negative cell wall structure of two layers of non-continuous envelope separated by a clear interspace of 30-200nm (Lee, 1975; Kozek, 1970; Taylor et al., 1999). The entomopathogenic nematodes carry symbiotic Gammaproteobacteria (Section 1.3.4). These *Photorhabdus* or *Xenorhabdus* are present in a specialised vescicle in the anterior intestine of IJ entomopathogenic nematodes (Morgan et al., 1997; Sicard et al., 2004a).

Other nematodes contain symbionts not affiliated to the Proteoeobacteria. The plant-parasitic *Xiphinema* nematodes (Section 1.3.3.1) carry rod-shaped Verrucomicrobia 0.7-1.0μm wide and 2.1-3.2μm long (Coomans et al., 2000; Vandekerckhove et al., 2000, 2002) forming clusters around developing oocytes in female worms. The oocytes contain few bacteria, but bacterial proliferation results in mature eggs containing several hundred bacteria. The cyst nematodes *G.rostochiensis* (potato), *H. goettingiana* (pea) and *H. glycines* (soybean) (Section 1.3.3.2) contain endosymbiotic bacteria of the phylum Bacteroidetes, which are also rod-shaped, 0.3-0.5μm diameter and 1.8-3μm long (Shepherd et al., 1973; Endo, 1979). Ectosymbionts of free-living Stilbonematinae marine nematodes (Section 1.3.2) are morphologically diverse: there are both a long and filamentous dominant species and smaller rod shaped bacteria on *E. dianae* (Polz

et al., 1999); coccoid bacteria formed a multilayered coat on all but the head and the tip of the tail on the *Stilbonema* (Polz et al., 1992) and rod-shaped bacteria formed a monolayer on *Laxus* sp. (Polz et al., 1994).

Little is known about bacteria associated with *H. contortus* and other nematode parasites of sheep. A large number of species of bacteria would be expected to inhabit the gut of both adult and L3 *H. contortus*. The L3 feed on bacteria in faeces and bacteria can be seen in the gut (S. Reinhardt, personal communication). There is also the possibility of finding symbionts in other tissues, such as the maternally transmitted symbionts which manipulate reproduction in filaria (Bandi et al., 2001). As a first step in determining the locations of any bacteria in adult *H. contortus*, they were examined by light microscopy (LM) and transmission electron microscopy (TEM).

2.2. MATERIALS AND METHODS

2.2.1. Sheep and parasites

Romney or Romney crossbred sheep were housed indoors and fed *ad libitum* lucerne chaff harvested from paddocks not grazed by sheep (Denver Stock Feeds, Palmerston North, N.Z.); water was freely available. All sheep were drenched on arrival (2ml/5kg) and the following day (1ml/5kg) with a broad spectrum anthelmintic (Matrix, Merial Ancare, N.Z.), containing abermectin, oxfendazole and levamisole. Two weeks later, the absence of nematode infection was confirmed by a faecal float (Appendix 2.1). Animal experiments were approved by the Massey University Animal Ethics Committee (MUAEC 06/117).

A total of 12 sheep provided adult worms for histology (Appendix 1). Worms of both sexes were used for LM sections (Sheep #2-4, 20-21). Female worms were selected for TEM (Sheep #3-8, 12, 16 and 19). Adult worms were collected after recovery from agar blocks (Appendix 2.4).

2.2.2. Light microscopy

Adult worms were fixed overnight in 10% [v/v] neutral buffered formalin (Appendix 3.1). Specimens were routinely processed in a Leica TP1050 Tissue

processor (Global Science and Technology, Auckland, New Zealand) and paraffin embedded (Leica Histo Embedder, Germany). Sections 5μm thick were cut on a Leica RM 2235 manual rotary ultramicrotome (Wetzlar, Germany), using a S35 Feather disposable microtome blade (Osaka, Japan). Sections were de-waxed and stained with hematoxylin and eosin (H & E) using a Leica Autostainer XL (Global Science and Technology, Auckland, New Zealand). De-waxed sections were stained by the Gram Twort method (Appendix 3.2). Slides were washed, dried and a drop of Entellan immersion oil (Merck New Zealand Ltd, Auckland, and NZ) was added as a mounting solution. Sections were covered with cover slips and viewed under the LM (OLYMPUS BX61, Tokyo, Japan) at 20x, 40x, 60x and 100x magnification.

2.2.3. Transmission electron microscopy

Adult female worms were sliced with a clean surgical blade into small pieces 6-8mm long. Tissues were fixed for 2-3 days in 3% glutaraldehyde and 2% formaldehyde in 0.1M phosphate buffer (Na_2 $HPO_4.12H_2O$ and KH_2PO_4), pH 7.2 and post-fixed in 1% OsO_4 in phosphate buffer (provided by the Manawatu Microscopy and Imaging Centre, Massey University, Palmerston North, New Zealand (MMIC)). Specimens were then embedded in resin and 1μm thick sections were cut with a diamond knife on an Ultra-microtome (Leica Microsytems, Wetzlar, Germany). Sections were stained with 0.05% toluidine blue and an area of interest was chosen by light microscopy. Sections 100nm were cut and double stained with uranyl acetate and lead citrate and observed by electron microscopy (Philips CM10 Transmission Electron Microscope with SIS Morada high-resolution digital imaging) at 60kV at the MMIC. Full details of tissue processing for TEM are given in Appendix 3.3.

2.3. RESULTS

2.3.1. Light microscopy

Numerous bacterial cells were seen in the gut lumen (Fig. 2.1A) and within the uterus (Fig. 2.1B), whereas no bacterial cells were clearly evident in the eggs.

There was a mixed population of Gram-positive and Gram-negative bacteria in the gut lumen (Fig. 2.2A) of worms of both sexes. The uterus contained only Gram-positive bacteria (Fig. 2.2B) in the distal uterus but not near the ovaries. Bacteria in the uterus were seen around the eggs (Fig. 2.1B) and along the wall of the uterus (Fig. 2.2B).

2.3.2. Transmission electron microscopy

A small number of spherical bacteria 800-1000nm in size (Fig. 2.3) were seen dispersed within the egg. They had a clearly defined cell wall and some of them contained nuclear-like material. Two of 28 egg sections contained clearly identifiable bacteria. These bacteria did not have noticeably thick cell walls, as was the case for all bacteria in other locations. Bacteria were seen in the eggs, not in the oogonia or columnar epithelial cells of the ovary.

A densely grouped population of a smaller (300-500nm) morphotype was seen adjacent to the inner uterine wall (Fig. 2.4) and outside the eggs in almost all worms examined. These bacteria could be clearly distinguished from sperm adjacent to eggs in EM sections (Fig. 2.5A), as sperm were bigger and contained a defined nucleus, membranous organelles and mitochondria (Fig. 2.5B).

The gut lumen of single worms contained diverse bacterial morphotypes (Fig. 2.6), but these differed in individual worms. Bacteria were not attached to the microvilli.

2.4. DISCUSSION

Bacteria were seen in *H. contortus* adult worms, not only in the gut, but surprisingly also within the uterus and eggs in female worms. Bacteria in the gut and uterus were seen in both LM and TEM sections, but bacteria were recognised in eggs only in TEM images (illustrated in Fig 2.7). Bacteria inside the eggs and within the uterus were different morphotypes, whereas there were more diverse morphotypes in the gut in a single worm and differed amongst worms.

The bacteria in the gut were both Gram-positive and Gram-negative (Fig. 2.2A) and had various morphotypes (Fig. 2.6), as would be expected of a population of gut bacteria (Ladygina et al., 2009). None had a thick cell wall in TEM images, which is a characteristic for Gram-positive bacteria, although some cultured Gram-positive bacteria have thin cell walls (Hespell et al., 1993; Tocheva et al., 2011). There were no apparent specialised structures containing bacteria, which were seen in the gut lumen and not attached to microvilli.

The bacteria in the uterus were Gram-positive and in TEM images were a smaller morphotype than those in the eggs and gut. They were numerous in the uterus and present in most worms examined. These bacteria may be horizontally acquired from their environment in the abomasum, because they were seen in the distal uterus and not proximally near the ovaries.

Bacteria in the eggs were only one morphotype and appeared to be different from those in the uterus. It could not be determined whether they were Gram-positive or Gram-negative, as they did not stain with either gram stain or H&E. The bacteria in the eggs may have evolved into intracellular endosymbionts which may not stain properly with gram stain, as is the case for intracellular endosymbiotic *Wolbachia* (Fenollar et al., 2003). Lack of clarity of the bacterial cell wall could also be the result of poor penetration of fixative and stain, as these worms have tough cuticles. As they were present in egg in female worms, there is the possibility of maternal transmission. However unlike other maternally transmitted endosymbionts, these bacteria were not clustered in the egg tissue and very few were recognised in each egg.

In work described in the following Chapters, the bacteria seen in *H. contortus* have been genetically characterised by molecular biological techniques frequently used to study the bacterial profile of complex microbial communities. First, protocols were developed to remove externally associated bacteria from the parasites and for DNA fingerprinting methods (PCR-DGGE) (Chapter 3). Optimised DNA fingerprinting methods were then applied to *H. contortus* life-cycle stages to analyse their bacterial communities (Chapter 4).

Chapter 3

Development of Protocols for DNA Fingerprinting

3.1. INTRODUCTION

The bacterial profile of microbial communities, such as those in *H. contortus*, can be investigated using PCR amplified 16S rRNA sequences, which are then analysed by DGGE. Currently this is the most reliable approach to explore microbial diversity and to determine the species composition. The DGGE band patterns reflect the bacterial diversity of a sample. Each band can be excised aseptically and the DNA re-amplified to identify the bacterial sequences in the band (Muyzer et al., 1993; Muyzer and Smalla, 1998; Yoshie et al., 2001; Liu et al., 2011). This method is more likely to be successful than conventional bacterial culture methods, as many bacterial species need fastidious growth conditions, especially those endosymbionts associated with nematodes and insects (Amann et al., 1995).

In spite of the popularity and wide application of the PCR-DGGE method in microbial ecology, it is not free from biases and errors. Biases can be introduced by storage conditions, DNA extraction methods, PCR inhibitors in the samples and preferential amplification of rRNA genes by PCR (Reysenbach et al., 1992; Suzuki and Giovannoni, 1996). In addition, chimeric and heteroduplex molecules can be formed during PCR amplification, which leads to incorrect interpretation of DGGE results (Liesack et al., 1991; Kopczynski et al., 1994). PCR is a powerful method for detecting the presence or absence of bacterial sequences, but will provide information about the relative abundance of the bacterial species only if there is equal efficiency of PCR amplification of small subunit (SSU) rDNA sequences by the universal bacterial primers, which may not always be the case (Farrelly et al., 1995; Fogel et al., 1999; Klappenbach et al., 2000; Ishii and Fukui, 2001).

Separation of DNA fragments depends on differences in their melting behaviour in the denaturing gradient. It is not always possible to separate DNA fragments which have particular differences in their sequences (Vallaeys et al. 1997), as they may have similar melting behaviour and co-migrate in the gel (Muyzer and Smalla, 1998; Jackson et al., 2000). Meaningful phylogenetic analysis of bacterial sequences obtained from DGGE bands is limited by the shortness (<500bp) of the sequences (Myers et al., 1985).

Before the bacteria in *H. contortus* samples could be examined, protocols needed to be developed to extract bacterial DNA from *H. contortus*, primers for PCR-DGGE and phylogenetic analysis must be selected and the optimal DGGE conditions (denaturing gradients and voltage) need to be determined. One of the important PCR conditions to be determined was the annealing temperature at which there is good, specific primer annealing, resulting in PCR products of the expected size without non-specific bands (Ishii and Fukui, 2001). In addition to developing the molecular biological protocols, the removal of loosely adherent bacteria during the recovery of L3 and adult worms was also assessed.

3.2. MATERIALS AND METHODS

3.2.1. Sheep

A total of 11 sheep (#1-11, Appendix 1) provided parasites or abomasal fluid samples for the development of PCR-DGGE protocols:

(a) abomasal contents were collected from 2 sheep;
(b) mixed sex adult worms were recovered from the abomasa of 11 sheep;
(c) L3 were cultured from faeces of 5 sheep.

3.2.2. *Haemonchus contortus*

The laboratory strain of *H. contortus* was originally obtained from the field and maintained by infection of nematode-free sheep and routine laboratory culture of L3 (Appendix 2.3.1-2.3.2). L3 were stored at 10°C in reverse osmosis (RO) water and L3 viability was assessed microscopically prior to infection of

sheep. Approximately 10,000 L3 were given orally to infect the sheep using a syringe. Infected sheep were housed separately from uninfected sheep. Infection was confirmed by faecal floatation on Day 21 post infection (Appendix 2.1).

L3 for experiments were recovered from faeces, using the sieve method (A) (Appendix 2.3.1).

Adult worms were routinely recovered from the abomasal contents on Day 21 post infection, using the technique of Simpson et al. (1999) (Appendix 2.4). Infected sheep were killed by stunning with a captive bolt, followed by exsanguination. The abomasum was removed, its contents and washings were mixed 2:1 [v/v] with 3% agar and the agar blocks were placed in saline in trays. Worms were collected from the saline after they migrated out of the agar blocks. Some samples of adult worms were manually collected from abomasal contents. Male and female populations were separated using a dissecting microscope, based on their morphology (the male has a copulatory bursa and the female has a barber's-pole appearance).

3.2.3. Collection of sheep gut samples

Faeces were collected from faecal bags and stored at -20°C for up to 12 days. Abomasal contents were collected from euthanased sheep into sterile pottles.

3.2.4. DNA free water and equipment

Ultrapure water was used for all protocols. This was prepared by filter sterilisation, autoclaving and ultraviolet (UV) treatment for 48h of milliQ (MQ) water. All laboratory plastic-ware used for molecular biology was autoclaved and UV treated overnight. Pipette tips contained filters (Labcon, Petaluma, CA, USA.).

3.2.5. Development of molecular biological protocols

3.2.5.1. Extraction of DNA

Five methods of DNA extraction were used: cetyltrimethylammonium bromide (CTAB) extraction; phenol-chloroform extraction; physical disruption followed by extraction with a QIAamp DNA stool-kit (Qiagen, Hilden,

Germany); long enzymatic digestion and freezing-thawing cycles, followed by extraction with a QIAamp DNA stool-kit (Qiagen); physical disruption and short enzymatic digestion, followed by DNA extraction with a QIAamp DNA stool-kit. 0.5g of abomasal contents and adult worms (either fresh or frozen) were used to develop appropriate DNA extraction methods.

The concentration of the extracted DNA was measured and the purity was determined using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) according to the manufacturer's instructions. 5µl of extracted DNA was also visualised on 1% agarose gels after electrophoresis at 100V for 30min to assess the quality of extracted DNA.

3.2.5.1.1. Cetyltrimethylammonium bromide

Approximately 0.5g of clumped adult worms was washed three times in absolute ethanol and finely ground in liquid nitrogen using a sterile pestle and mortar. 0.1ml of ground sample was transferred to a 1.5ml sterile, DNA-free microcentrifuge tube (Eppendorf AG, Hamburg, Germany) and 0.8ml of 0.2µm filter-sterilised CTAB buffer (100mM tris-HCl pH 8.0, 1.4M NaCl, 20mM NaEDTA, 2% hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO, USA)) was added and vortexed for 2min. Samples were incubated at 70°C for 30min, with vortexing for 5sec after the first 15min of the incubation. At the end of the incubation, 0.5ml of chloroform was added and the tubes vortexed until a white emulsion formed. The sample was centrifuged at 13,000*g* for 10min so that an aqueous layer formed above the chloroform. The aqueous layer was carefully aspirated into a sterile microcentrifuge tube. 0.3ml of isopropanol was added, the tube was inverted gently and then incubated at room temperature for 10min. DNA was pelleted by centrifuging at 13,000*g* for 10min. The supernatant was removed and the DNA pellet was washed using 70% [v/v] ethanol; flicking or briefly centrifuging the tube ensured efficient washing. The pellet was incubated at 70°C for 10min or until it became white and more clearly visible. The pellet was dried at room temperature and dissolved in 50 or 100µl filter-sterilised 10mM tris-HCl pH 8.0 (Sigma). Tubes were warmed to 37°C to increase the solubility, if required. For downstream PCR applications, a dilution of 1:25 in DNA-free water was used.

3.2.5.1.2. Phenol-chloroform method

Approximately 0.5g of clumped adult worms was washed three times in absolute ethanol, then washed three times with 10ml of NTE buffer (100mM NaCl, 50mM tris, 20mM ethylenediaminetetraacetic (EDTA)) (Sigma). Most of the fluid was removed and the pellet was frozen at -20°C for 1-2h. The frozen pellet was dissolved in 500µl of lysis buffer (1% SDS, 20mM tris, 20mM EDTA, pH 8 (Sigma) containing 100µg/ml proteinase K (Life Technologies, New York, NY, USA) and incubated on a shaker at 37°C overnight. After 10-12h, 15µl of 3M sodium acetate and 515µl of 25:24:1 [v/v/v] phenol/chloroform/isoamyl-alcohol were added and vortexed for 1min and then centrifuged at 2000*g* for 10min. The aqueous layer was transferred to a sterile, DNA-free microcentrifuge tube (Eppendorf), an equal volume of phenol/chloroform/isoamyl-alcohol (25:24:1) was added and the tube was vortexed for 5sec. The tube was centrifuged at 2000*g* for 10min and the aqueous layer transferred to a sterile, DNA-free microcentrifuge tube (Eppendorf). The aqueous solution was again mixed with an equal volume of phenol/chloroform/isoamyl-alcohol and centrifuged at 2000*g* for 10min. The aqueous layer was transferred into a sterile, DNA-free microcentrifuge tube and mixed with 2 volumes of ice-cold 100% ethanol and incubated on ice for 30min. The mixture was centrifuged at 3000*g* for 10min at 4°C. The supernatant was removed and half the volume of ice-cold 70% ethanol was added and incubated on ice for 30min. After vortexing for 5sec, the tube was centrifuged at 17,100*g* for 10min at 4°C, the supernatant was carefully removed and the DNA pellet was dried overnight at room temperature. After 10-12h, the DNA pellet was re-suspended in 60µl of TE buffer (10mM tris-HCl, 1M EDTA, pH 8) (Sigma). The DNA sample was stored at -20°C or kept at 4°C for immediate use.

3.2.5.1.3. QIAamp DNA stool-kit method

Approximately 0.5g abomasal contents, adult worms or larvae (L3) was frozen in liquid nitrogen and then ground with a frozen pestle and mortar. A QIAamp DNA stool-kit (Qiagen) was used according to the manufacturer's instructions to extract DNA.

3.2.5.1.4. QIAamp DNA stool-kit method with enzymatic digestion and/or physical disruption

To attempt to increase the DNA yield, the stool kit method included: (1) a long enzymatic incubation (overnight) was followed by freezing-thawing cycles or (2) a shorter (2-3h) enzymatic incubation of gut samples or unfrozen parasites which had been homogenised with a micro-homogeniser.

(1) **Long enzymatic incubation plus freezing-thawing cycles**

Samples of 0.5g of 4% sodium hypochlorite (Clark Product Ltd, Rotorua, New Zealand) washed adult worms, sheathed and exsheathed L3 were incubated on a shaker ($\frac{\text{MaxQ}}{2000}$ Barnstead International, IA, USA) at 200 r.p.m. at 37°C overnight in a lysis solution, which contained 10mM EDTA, 20mM trizma and 1% sodium dodecyl sulphate (SDS) (Sigma), plus 20µl of 20mg/ml proteinase K solution (Life Technologies). After 10-12h, 120µl of 10mg/ml lysozyme (Sigma) was added to each sample and samples were further incubated for 2h at 37°C. Samples were then frozen in liquid nitrogen for 10min and then heated to 65°C for 10min. This freezing-thawing cycle was performed three times. After the freezing-thawing cycles, 20µl 20mg/ml proteinase K (Life Technologies) was added to each sample and the samples were incubated at 60°C for 30min. The extracted DNA was stored at -20°C and used as a template for PCR to amplify the 16S rRNA sequences from the bacterial population.

(2) **Physical disruption with short enzymatic incubation**

Samples of 0.5g of 4% sodium hypochlorite (Clark Product Ltd) washed adult worms and exsheathed larvae were homogenised using a sterile micro-centrifuge pestle, incubated in lysis solution, plus 20µl of 20mg/ml proteinase K (Life Technologies) solution for 2.5h at 37°C. DNA was extracted from 200µl samples, as described in the manufacturer's manual of the QIAamp DNA stool-kit (Qiagen). The extracted DNA was stored at -20°C and used as a template for PCR to amplify bacterial 16S rRNA sequences.

3.2.5.2. Primer selection

DNA, extracted from adult worms using the stool kit with a long enzymatic incubation time plus freezing-thawing cycles, was amplified with 6 sets of

universal bacterial primers (Table 3.1) to select suitable primer pairs for PCR-DGGE and phylogenetic analysis without primer biases. The primer 338f had a 50bp GC clamp to retain amplified products in the DGGE gel. Bacterial 16S rRNA genes were amplified in a 50µl reaction mixture containing: 5.1µl 10X PCR buffer with Mg^{2+}, 5µl of 2mM dNTP, 0.5µl of 10µM forward primer and 0.5µl of 10µM reverse primer, 2.5U (0.5µl) of native Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), 5µl of template and 33.4µl of ultrapure water. The following touchdown PCR was used: stage I: 3min initial denaturation at 95°C, followed by stage II: 10 cycles of denaturation (30sec at 95°C), initial annealing (30sec at 54-62°C), -0.5°C/cycle, and elongation (30sec at 72°C), followed by stage III: 26 cycles of denaturation (30sec at 95°C), annealing (30sec 57°C) and elongation (30sec at 72°C) with final elongation at 72°C for 10min.

Amplified DNA was analysed by agarose gel electrophoresis. The agarose gels (1.5% [w/v]) were made up in 1X TAE buffer, which contained 40mM tris (hydroxymethyl) aminomethane, 1mM EDTA and 65mM acetic acid and adjusted to pH 8.0 with NaOH. 5µl aliquots of each PCR amplified product was loaded into a well with 1µl of 20% [v/v] Orange G loading buffer (0.1mM EDTA, 40% [v/v] glycerol, 0.15% [w/v] Orange G (Sigma)); a 1 Kb plus marker (Invitrogen, Carlsbad, CA, USA.) was also run on each gel. The agarose gels were electrophoresed in 1X TAE buffer at 100V for 30min. Gels were stained with SYBR safe DNA stain (Invitrogen) (10µl of stain per 100ml of agarose solution) and visualised using UV trans-illumination and photographed using a BIO-RAD Molecular Imager® Gel Doc™ XR (BIO-RAD Laboratories, Milan, Italy).

3.2.5.3. PCR-denaturing gradient gel electrophoresis (PCR-DGGE)

For DGGE, DNA was amplified by PCR with the universal bacterial 16S rRNA primer set F, forward 338f and reverse 518r. This primer set targets gene sequences corresponding to the V3 region of the *Escherichia coli* 16S rRNA ribosomal locus. The forward primer (338f) had a 40-nucleotide GC-clamp

added to the 5'-end. DGGE was carried out using a CBS Scientific DGGE gel system (CBS Scientific Company, Del Mar, CA, USA).

3.2.5.3.1. PCR using primer set 338f and 518r

Bacterial sequences were amplified in 50µl reaction mixtures containing 5.1µl of 10X PCR buffer with 1.5M Mg^{2+}, 5µl of 2mM dNTP, 0.5µl of 10µM 338f (with 40bp GC-clamp), 0.5µl of 10µM 518r, 2.5U (0.5µl) of native Taq DNA polymerase (Roche), 5µl of template and 33.4µl of ultrapure water. The touchdown polymerase chain reaction was used at the initial annealing temperature of 62°C. Amplified DNA was verified by electrophoresis on an agarose gel (Section 3.2.5.2).

All PCR products were purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The DNA concentrations of the PCR amplicons were measured and the purity confirmed using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies), according to the manufacturer's instructions.

3.2.5.3.2. Selection of denaturing gradients, electrophoresis time and voltages

PCR-amplified bacterial sequences from adult worms (sodium hypochlorite washed) and L3 (sodium hypochlorite exsheathed, and sodium hypochlorite and ultrapure water washed) were initially used to optimise the gradient range. The 6% [w/v] polyacrylamide gels were made with either a 30 to 55% [v/v] or a 30 to 45% [v/v] urea and formamide denaturing gradient. DGGE was carried out using a CBS Scientific DGGE gel system (CBS Scientific Company). Details of denaturing gradient solutions, buffers and gel casting methods are described in Appendix 4.

PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). 300ng of purified PCR products were mixed with equal volumes of DGGE loading dye (0.05% [w/v] bromophenol blue, 0.05% [w/v] xylene cyanol, 70% [w/v] glycerol in water, pH 8.0) and loaded into the DGGE gel wells. A 1 Kb plus marker (Invitrogen) was also run on each gel. The gels were electrophoresed with 1X TAE buffer which contained

40mM tris (hydroxymethyl) aminomethane, 65mM acetic acid and 10mM EDTA, adjusted to pH 8 with 5M NaOH. The electrophoresis was performed at 100V and 60°C for 12h or 200V and 60°C for 5h. After the electrophoresis, gels were stained with 3µl of SYBR Gold (Invitrogen) in 600ml of MQ water for 20min on a shaker and then de-stained overnight. The gel was visualised using UV trans-illumination and photographed.

3.2.5.4. Evaluation of bacterial contamination of reagents and solutions

PCR water, MQ water, RO water, QIAamp DNA stool-kit buffers, lysozyme, lytic solution, saline and agar solutions were subjected to DNA extraction and PCR amplification with the universal bacterial primers (338f and 518r) in order to determine whether there were bacterial contamination. In addition, all reagents listed above were used directly without DNA extraction as the template for PCR amplification. Lytic solutions were also incubated with yeast extract to provide nutrients for any bacteria present.

3.2.6. Bacterial profiles of L3 and adult worms during recovery from gut contents

An assessment was made of the effectiveness of routine laboratory recovery and cleaning protocols in reducing environmental and loosely associated bacteria of L3 and adult worms. The bacterial communities in samples collected at major steps were determined by PCR-DGGE. DNA was extracted using a long enzymatic digestion, freezing-thawing and the QIAamp DNA stool-kit (Section 3.2.5.1.4). A 30-55% denaturing gradient was used for DGGE gels.

3.2.6.1. Collection of samples during larval culture

Samples were collected for DNA extraction at several points in the recovery of L3 from faeces (Fig. 3.1); this protocol was based on the sieve method A (Appendix 2.3.1).

1. **Sample A** was a faecal sample (30g) collected on Day 21 post-infection from a faecal collection bag and stored at -20°C.

2. Faeces collected at the same time were incubated for 10-12 days in trays and faecal **Sample B** (30g) was collected after 12 days.

3. Faeces were then placed in a sieve covered with tissue paper. The sieve was placed in a bowl containing RO water and left for one day at room temperature. The larvae migrated through the tissue paper and settled on the bottom of the bowl (**Sample C**) (1.5ml).

4. The water in the bowl was passed through a sieve (pore size 20μm) and larvae retained on the sieve surface were collected for **Sample D** (1.5ml).

5. Larvae were washed off the sieve with RO water and placed in a funnel. **Sample E** (1.5ml) was collected after 12h from larvae which settled in the clamped tube connected to the funnel.

6. The water containing larvae in the funnel was poured through a 20μm sieve and the retained larvae were collected into a beaker of RO water. One aliquot of the larvae (1.5ml) were again collected on a sterile 20μm sieve and washed three times with absolute ethanol (**Sample F**).

7. Another aliquot of larvae (1.5ml) in the beaker was exsheathed by incubating at 37°C in 0.05% sodium hypochlorite solution (Clark Product Ltd) 15-20min. After confirming microscopically that 95-100% had exsheathed, they were Baermannised (Step E, Fig. 3.1) in ultrapure water for 12h. The larvae were collected on a sterile 20μm sieve and washed briefly with 4% sodium hypochlorite, then with ultrapure water and these steps were repeated 5 times. **Sample H** was then collected aseptically into a sterile centrifuge tube.

8. **Sample G** was laboratory RO water (1.5ml). **Samples I and II** were washings (1.5ml) with RO water of the equipment used: the bowl (I) and the funnel, tubing and filter paper (II) respectively (Fig. 3.1).

3.2.6.2. Collection of samples during adult worm recovery

Samples were collected for DNA extraction during adult worm recovery from the abomasum (Fig. 3.2); this method is described in full in Appendix 2.4.

1. The abomasum was opened on a tray; **Sample 1** (30g) was collected from abomasal contents and may have contained a few worms.

2. **Sample 2** (5g) was manually collected worms from the abomasal mucus.

3. The abomasal surface was washed with saline 2-3 times and washings were collected in a graduated beaker. Worms in the graduated beaker were collected using a pipette (**Sample 3**) (~1.5g).

4. Abomasal contents and washings were set in agar blocks, from which the worms migrated into a saline bath. Worms were collected from the saline (**Sample 4**) (~1.5g).

5. Worms recovered from the saline were thoroughly washed alternately 5 times in 4% sodium hypochlorite and ultrapure water. **Sample 5** (5g) was aseptically collected into a sterile microcentrifuge tube.

6. **Sample 6** (1.5ml) was collected from a stock solution of 0.9% [w/v] sodium chloride solution used for adult worm recovery.

7. **Sample 7** (1.5ml) was a sample of agar powder (0.5g) used to make the 3% agar solution in RO water.

8. **Samples III, IV and V** (1.5ml) were washings with saline of the equipment used: the tray (III); graduated beaker (IV) and the tray (V) respectively (Fig. 3.2).

Three further populations of adult worms were used to compare the bacterial profiles of adult worms which had been manually collected from abomasal mucus (Sample 2) with worms which had undergone the whole recovery process and washing with sodium hypochlorite (Sample 5). No samples were collected at intermediate steps.

3.3. RESULTS

3.3.1. Development of the experimental protocol

3.3.1.1. DNA extraction

The criteria for selection of a suitable method of DNA extraction were adequate DNA yield, an absorbance ratio 260/280 of >1.8, sharp bands of genomic DNA on agarose gels and clear separation of PCR products in DGGE gels. The CTAB yielded <1ng/µl DNA, an absorbance ratio of 1.27 and there

was either no product or smearing on agarose gels. Although the phenol-chloroform method yielded 25.43ng/µl DNA with an absorbance ratio of 1.94, there was smearing on agarose gels. The DNA extracted using both method in downstream PCR amplification gave unsatisfactory results.

DNA extracted with QIAamp DNA stool-kit with an additional lysis step (either long or short incubation) fulfilled all criteria. DNA extracted with the QIAamp DNA stool-kit alone yielded sufficient DNA (>96ng/µl) from sheep gut contents, but not from *H. contortus* samples (<1ng/µl). Addition of a lysis step and freezing-thawing cycles gave the best yield of DNA: 5-17ng/µl and absorbance ratio >1.8 for adult worms and L3. The length of the enzymatic digestion (10-12h or for 2h) did not influence the yield and quality of DNA and DGGE band patterns of *H. contortus* samples. For parasite samples, the best method was determined to be the QIAamp DNA stool-kit plus lysis and freezing-thawing and was used for subsequent parasite DNA extraction.

3.3.1.2. Primer selection

Primer sets D and F were selected for downstream PCR amplifications at an initial annealing temperature of 62°C. PCR products generated by primer sets A to F at 5 annealing temperatures are shown in Fig. 3.3 and summarised in Table 3.2. The criteria for primer selection were: greater specificity of primer at the annealing temperature of 62°C; expected size (Table 3.2) of the PCR products without non-specific bands (Fig. 3.3 and Tables 3.1-3.2). Primer set D, which amplified nearly complete length 16S rRNA (~1400bp) sequences, was selected for making clone libraries and primer set F (~190bp) for DGGE experiments.

3.3.1.3. Denaturing gradients, electrophoresis time and voltages

In both 30-45% and 30-55% gradients, adult worms and L3 gave similar DGGE band patterns to each other (Fig. 3.4). The 30-45% gradient range gave better band separations than 30-55% after electrophoresis at 200V for 5h.

3.3.1.4. Evaluation of bacterial contamination in reagents

There was no evidence of bacterial 16S rRNA sequences in the PCR water, MQ water, QIAamp DNA stool-kit buffers, lysozyme, lytic solution, and saline

solutions, which did not produce any PCR products (Fig. 3.5). No bacterial sequences were amplified in the lytic solution with addition of yeast extract. RO water and tap water produced faint bands. Washings of equipment (Samples I-V) produced no PCR products.

3.3.1.5. Bacterial profiles of adult worms and L3 during recovery from gut contents

The DGGE band patterns of L3 at the first (Fig. 3.6, lane C) and last steps (Fig. 3.6, lane F) of the Baermannisation process were similar, indicating little removal of bacteria by either passage through tissue paper or subsequent ethanol washing. The intention was to follow the process at each step of the recovery of L3 from faeces and finally after washing with MQ water and ethanol. However, the communities present in L3 at the intermediate stages (lanes D and E) could not be determined, as too few L3 were collected and thus too little DNA was extracted to give clear DGGE profiles. In contrast, exsheathing and sodium hypochlorite washing reduced bacterial diversity: the DGGE profile of sodium hypochlorite and washed exsheathed L3, analysed in a separate gel (Fig. 3.6, lane H), was different from that of ethanol washed sheathed L3 (Fig. 3.6, lane F), particularly having fewer bands.

The DGGE band patterns of the faecal samples (Fig. 3.6, lanes A-B) were indicative of poor separation of the PCR products.

The direct comparison of the bacterial communities of 3 samples each of manually recovered and agar recovered, sodium hypochlorite washed adult worms (Figs 3.7-3.8) showed the latter produced fewer DGGE bands (Fig. 3.7). Collection of adult worms during the recovery steps from the agar blocks did not yield convincing results, although again there appeared to be fewer bands after the final step (Fig. 3.8, lane 5). Generally too few worms and thus too little DNA were available to give clear DGGE profiles (Fig. 3.8). The bacterial profile of abomasal contents showed more diversity than that of sodium hypochlorite washed adult worms (Fig. 3.8, lanes 1 and 5).

3.4. DISCUSSION

3.4.1. DNA fingerprinting protocols

To explore the unknown profile of microbial communities in worm samples, as in environmental or tissues samples, the extracted DNA should ideally be representative of all the bacterial species present, therefore an important step using DNA fingerprinting techniques is efficient recovery of DNA from the microbes (Zhou et al., 1996; Frostegård et al., 1999; Hurt et al., 2001). If all bacterial DNA were able to be extracted, it would quantitatively reflect the genomes and their relative abundance in worm samples. The extraction method should also cause the least damage to the DNA.

Five methods of DNA extraction were compared, based on adequate DNA yield, an absorbance ratio 260/280 of >1.8, sharp bands of genomic DNA on agarose gels and clear separation of PCR products in DGGE gels. Of the methods tested physical cell disruption and enzymatic lysis (either 2h or for 10-12h incubation), followed by DNA extraction with the QIAamp DNA stool-kit, gave the highest yield of bacterial DNA from parasites and the PCR products separated well on DGGE gels. The QIAamp DNA stool-kit is routinely used and is designed to reduce PCR inhibitors in the extracted DNA and for extraction of bacterial DNA from gut samples. It proved suitable for extracting bacterial DNA from within nematodes and their eggs, which have very tough cuticles and eggshells, which must be broken before the cell walls of the bacteria within them can be disrupted mechanically or enzymatically to extract the microbial DNA. The inclusion of a lysis step, however, did not appear to be appropriate for faecal samples, as the PCR products were poorly separated on the DGGE gels.

The possibility of introducing bacterial contamination in lytic solutions, enzymes and buffers used for DNA extraction was discounted, as none contained bacterial DNA detectable by PCR, while only RO water and tap water contained very small amount of bacteria DNA which is unlikely to contribute significantly to the bacterial DNA extracted from the parasites. After provision of yeast extract as a source of nutrients to promote bacterial growth, no bacterial sequences were amplified in the lytic solution after PCR amplification.

Six sets of published, routinely used universal bacterial primers (Tables 3.1 and 3.2) were assessed to determine the optimal annealing temperature/s for parasite samples. The best results were obtained at 62°C initial annealing temperature with primer sets 27f + 1492r (primer set D) and 338f + 518r (primer set F), which gave the expected size PCR products (~1400bp and ~190bp respectively). The primer set 338f + 518r (F) was selected for PCR-DGGE analysis, while primer set 27f + 1492r (D) was selected for clone library construction and phylogenetic analysis, which requires longer sequences. However, even using an optimal annealing temperature, biases in PCR amplification of a mixture of 16S rDNA fragments can be caused by differences in the number of small subunit ribosomal DNA copies, the varying genome size among organisms, the G+C content of sequences, template reannealing and different primer binding energies (Dutton et al., 1993; Farrelly et al., 1995; Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998; Fogel et al., 1999; Klappenbach et al., 2000; Ishii and Fukui, 2001).

3.4.2. Parasite recovery

Each of the life cycle stages of *H. contortus* was separated from its microbial rich environment using routine parasitological techniques, followed by washing with sodium hypochlorite to reduce external bacteria. In the absence of similar studies to remove bacteria from parasitic nematodes, two methods of cleaning were compared. The recovery protocols and cleaning process used here effectively reduced the bacterial diversity of L3 and adult worms (Figs 3.6-3.8), probably by removing bacteria from the external surfaces. Preliminary experiments showed that washing with sodium hypochlorite was more effective than an ethanol wash. Exsheathing L3 further reduced the number of DGGE bands (Fig. 2.6, lanes F and H). Despite attempts to clean parasites, all three life-cycle stages still appeared to carry some environmental bacterial DNA.

These protocols for recovery of parasites and optimised DNA fingerprinting methods were applied to investigating the bacteria associated with *H. contortus* by PCR-DGGE (Chapter 4) and phylogenetic analyses of their 16S rRNA sequences (Chapter 5).

Chapter 4

Identification of Bacteria Associated with *Haemonchus contortus* Using DNA Fingerprinting

4.1. INTRODUCTION

Bacterial associations with host organisms can range from mutualism, in which they provide nutrients to the host, to lethal pathogenic relationships (Goodrich-Blair and Clarke, 2007). These symbioses between prokaryotes and eukaryotes are very intimate and in most cases both partners are required for survival, making the symbionts uncultivable using conventional microbiological techniques (Schleifer, 2004; Slatko et al., 2010). Apart from the filarial-*Wolbachia* symbiosis, there are only a few studies of bacteria associated with animal parasites, including the gut bacteria of *A. suum* (Hsu et al., 1986) and *H. polygyrus* (Walk et al., 2010). Published studies on bacterial communities associated with ruminant parasites appear to be limited to a culture-based study of potential host pathogens carried by L3 (Lacharme-Lora et al., 2009).

The bacterial profile of complex microbial communities, such as those in *H. contortus*, can be initially explored using DNA fingerprinting methods, most commonly PCR-DGGE using 16S rRNA sequences. DGGE band patterns reflect the bacterial diversity of a sample. Each band can be excised aseptically and DNA from this band can be re-amplified to identify the specific bacterial species it contains (Muyzer et al., 1993; Muyzer and Smalla, 1998; Yoshie et al., 2001; Liu et al., 2011). The methodology required for exploring the bacterial communities in abomasal contents and adult worms, L3 and eggs has been developed and reported in Chapter 3.

4.2. MATERIALS AND METHODS

4.2.1. Sheep

A total of 15 experimentally-infected sheep (#12-26, Appendix 1) provided parasites or abomasal fluid samples for the PCR-DGGE experiments:

(a) abomasal contents were collected from 3 sheep;
(b) mixed sex adult worms were recovered from the abomasa of 15 sheep;
(c) female and male worms were separated from a mixed population after recovery from agar blocks (3 individual sheep);
(d) L3 were cultured from the faeces of 9 sheep;
(e) eggs were collected from the faeces of 5 sheep and *in vitro* laid eggs from adult worms recovered from 9 sheep.

4.2.2. Samples used for DNA extraction

Faeces and abomasal contents were collected as described in Section 3.2.3. L3 were cultured from infected faeces and recovered as described in Appendix 2.3. Mixed sex adult worms were recovered from the abomasal mucosa as described in Appendix 2.4. Male and female worms were separated under a dissection microscope. 500-600 eggs, either collected from faeces or laid *in vitro* (Appendices 2.5-2.6), were used for DNA extraction. Before the DNA was extracted, *H. contortus* adult worms, exsheathed L3 and eggs were washed 5 times with sodium hypochlorite. The parasites were briefly (5-10sec) washed with 4% sodium hypochlorite and rinsed with approximately 200ml ultrapure water on a sterile filter; both steps were repeated 4 more times.

4.2.3. Extraction of DNA

DNA was extracted as described in Section 3.2.5.1.4. The quantity and the purity of the DNA were determined using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies) or using the Qubit TM ds DNA HS Assay Kits on a Qubit fluorometer (Invitrogen) according to the manufacturer's instructions. Samples were stored at -20°C for downstream applications

4.2.4. PCR-DGGE

PCR-DGGE amplifications were carried out with the universal bacterial primers, 338f and 518r, as described in Section 3.2.5.2, at a 62°C initial annealing temperature. The forward primer (338f) had a 40-nucleotide GC-clamp added to the 5'-end. DGGE was carried out using a CBS Scientific DGGE gel system using a 30 to 45% denaturing gradient (Section 3.2.5.3.2).

4.2.5. Sequencing DNA fragments in excised DGGE bands

4.2.5.1. DNA extraction and PCR

Bands of interest in adult, L3 and egg samples were excised using new sterile surgical scalpel blades and aseptically transferred into sterile centrifuge tubes. 100µl ultrapure water was added to the tube containing the excised band and vortexed for 1min, then the water was removed. Gel slices were finely broken in 50µl of ultrapure water using sterile pipette tips. Tubes were incubated overnight at 4°C and the following day were vortexed for 5sec and centrifuged at 28,000*g* for 1min. The supernatants were transferred to sterile centrifuge tubes and the DNA fragments in the supernatant were re-amplified with the primers 338f and 518r, using the touchdown PCR protocol with an initial annealing temperature of 62°C (Section 3.2.5.2).

4.2.5.2. Cloning and sequencing

Purified PCR products were cloned by ligation into a plasmid vector (pCR 2.1TOPO-TA cloning vector, Invitrogen) and transformation into chemically competent *Escherichia coli* TOP-10 cells, using a TOPO-TA cloning system (Invitrogen) according to the manufacturer's instructions. Transformed cells were plated onto Luria Bertani (LB) agar plates containing ampicillin (50mg/l) (Sigma) and X-gal (40mg/l) (Promega) and incubated overnight at 37°C. Clones were screened for blue (not containing insert) and white (containing insert) colonies; white colonies were randomly selected as templates for PCR amplification with the primers GEM2987 and TOP168r (Table 4.1). The PCR mixture (50µl) contained 5µl of 10X reaction buffer without $MgCl_2$, 4µl 1.5mM $MgCl_2$ (Invitrogen), 5µl of dNTP, 0.5µl of 1µM of each primer, 0.5µl of 2.5U of

Taq DNA polymerase (Roche) and 34.4µl DNA free ultrapure water. The amplification was done using the touchdown PCR programme described in Section 3.2.5.2 with an initial annealing temperature of 62°C. Amplified PCR products were sequenced using the primer M13f by Macrogen Inc. (Seoul, Republic of Korea).

4.2.5.3. DNA extraction and sequencing of Band 8 in DGGE gels

An intense band (Band 8, Fig. 4.2) was present in one L3 and all adult worm samples. Because of the amount of DNA in this band, it was processed differently, in that, instead of direct cloning and sequencing, it was confirmed in another DGGE gel that cloned PCR products migrated at the correct position.

Band 8 was excised from one L3 and 3 adult worm samples. DNA was extracted as described in Section 4.2.5.1. Extracted DNA from these samples was re-amplified with the primer set 338f (without GC-clamp) and 518r. PCR products were cloned using the TOPO-TA cloning system (Invitrogen). Only white colonies were selected and colonies were re-grown on LB plates (without X-gal). After 1-12h, 12 well grown colonies were individually scooped and suspended in 100µl ultrapure water and heated at 100°C for 10min and centrifuged at 20,800*g*. The supernatant (DNA) was transferred into sterile microcentrifuge tubes and used as template in downstream PCR amplification for DGGE. PCR was carried out with the primers 338f and 518r; the forward primer had a 40bp GC-clamp.

For each of the 4 samples (one L3 and 3 adult worm samples), PCR products of 12 colonies were loaded, along with the original sample, on a 30-45% polyacrylamide denaturing gradient gel. Electrophoresis was performed at 200V and 60°C for 5h. All clones producing a band at the same level as Band 8 were submitted for sequencing by Macrogen Inc.

4.2.6. Sequence editing and analysis

Vector sequences were removed using MEGA version 5 (Molecular Evolutionary Genetics Analysis) (Tamura et al., 2011). The sequences were checked for quality of the chromatogram (evenly-spaced peaks) and mis-called nucleotides. Good quality sequences were analysed with known sequences

available in the GenBank database. The BLASTn search option of the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov) was used to compare sequences of close evolutionary relatives with sequences obtained from the DGGE bands (Altschul et al., 1990, 1997).

4.3. RESULTS

4.3.1. *Haemonchus contortus* and abomasal contents

DGGE profiles of sodium hypochlorite washed adult worms were different from those of abomasal contents, whilst sharing similar profiles with eggs and L3 (Fig. 4.1). Abomasal contents and manually collected worms produced similar DGGE band patterns, suggesting that they have similar bacterial profiles.

4.3.2. Eggs, adult worms and L3

The DGGE band patterns of adult worms, L3 and eggs (Fig 4.2) were similar, all the lifecycle stages sharing major intense bands at the same level, but some bands were unique to each life-cycle stage.

In vitro laid eggs and the female worms which laid them had similar bacterial profiles e.g. the band patterns of HA1 and HE2 were similar, except for a greater intensity of the band in adult worms which is arrowed in Fig. 4.2. This intense band (Band 8) in adult worms was also present in L3 and all egg samples, but fainter than adult worms, except for one L3 sample. The band patterns produced by eggs extracted from faeces (HE1) and *in vitro* laid eggs (HE2) were not identical, as the former had intense bands (circled), which were not detected in the *in vitro* laid eggs (Fig. 4.2).

4.3.3. Male and female worms

The band patterns of male and female *H. contortus* samples from 3 different sheep were very similar, suggesting that worms of the two sexes have similar bacterial communities (Fig. 4.3).

4.3.4. Sequences from DGGE bands in *Haemonchus contortus* adults, L3 and eggs

There were 7 major bands common to all three life-cycle stages (Fig. 4.4). The short sequences (~190bp) obtained from the bands of interest were compared with those in the GenBank database (Benson et al., 2008), using the BLASTn option. The sequences fell into the 3 phyla Firmicutes, Proteobacteria and Bacteroidetes (Table 4.2). In addition, a few 18S rRNA partial sequences belonging to *H. contortus* were also identified in adults, L3 and eggs, more frequently in the egg samples. Band 8 was intense in one L3 and some adult worm samples. This band contained mainly the sequences belonging to *Escherichia* and *Rhizobium* sp.

4.3.4.1. *Haemonchus contortus* adult worms

A total of 44 bacterial sequences were obtained from the 7 common bands from 3 separate DGGE gels (Fig. 4.4). These are listed in Appendix 8. The dominant phylum was Firmicutes (27), followed by Proteobacteria (10) and Bacteroidetes (7).

Of the 27 sequences from the phylum Firmicutes, 11 were from *Lactococcus* sp. and 6 from *Weissella* sp. The other bacterial species belonging to the phylum Firmicutes were *Veillonella* sp. (5), *Enterococcus* sp. (2), *Lactobacillus* sp. (1), *Leuconostoc* sp. (1) and *Clostridium* sp. (1). *Weissella* sp. was mainly identified in Bands 1 and 2 and occasionally in Band 5.

In the phylum Proteobacteria, *Acinetobacter* sp. (6) were the dominant bacteria, while a single sequence of each of *Stenotrophomonas* sp., *Delftia* sp. *Comamonas* sp. and *Pseudomonas* sp. was identified.

In the phylum Bacteroidetes, *Flavobacteriaceae* sp. or *Chryseobacterium* sp. (4) *Prevotella* sp. (2) and an uncultured rumen bacterium (1) were identified in several bands.

A total of 45 bacterial sequences were obtained from Band 8 in DGGE gels from 3 adult worms. The sequences were identified as *Escherichia* (29), *Rhizobiaceae* sp. or uncultured *Rhizobiales* sp. (8), *Citrobacter* sp. (3),

Acinetobacter sp. (1) *Helicobacter* sp. (1), *Corynebacterium* sp. (1), *Lactococcus* sp. (1) and *Bacillus* sp. (1). One of the 3 samples also had 2 18S rRNA *H. contortus* sequences.

4.3.4.2. *Haemonchus contortus* L3

The 7 common bands were excised and cloned from 3 separate DGGE gels, giving a total of 21 bands and a total of 35 bacterial sequences. The phylogenetic affiliations of sequences in each band are presented in Fig. 4.4 and the summary of affiliations in Table 4.2. The dominant phylum was Firmicutes (29), followed by Proteobacteria (6).

The sequences from phylum Firmicutes were principally from *Lactococcus* sp. (14) and *Weissella* sp. (8). The other bacterial species belonging to the phylum Firmicutes were *Streptococcus* sp. (2), *Veillonella* sp. (3), *Butyriovibrio* sp. (1) and *Catonella* sp. (1). *Weissella* sp. was identified only in Bands 1 and 2.

The most numerous sequences of the phylum Proteobacteria belonged to *Acinetobacter* sp. (4), while *Pseudomonas* sp. (1) and *Comamonas* sp. (1) were also identified.

A total of 18 bacterial sequences were obtained from Band 8 of one L3 sample (HL2), which was excised from a DGGE gel (Fig. 4.4). The other 2 L3 samples did not produce an intense Band 8 with DNA for sequencing. The sequences were identified as *Escherichia* (10), *Rhizobium* sp. or uncultured *Rhizobium* sp. (7) and *Acinetobacter* sp. (1).

4.3.4.3. *Haemonchus contortus* eggs

All seven common bands were excised and sequenced. The lower five bands contained only *H. contortus* 18S rRNA sequences or there was no band visible. A total of 23 bacterial sequences were obtained from the upper 2 common bands from 4 gels. The phylogenetic affiliations of sequences in each band are presented in Fig. 4.4 and the summary of affiliations in Table 4.2. The dominant phylum was Firmicutes (13), followed by Proteobacteria (10).

Two egg samples were collected from faeces and two were samples of eggs laid *in vitro* by female worms. Proteobacteria (10) were associated with eggs

from both sources, whilst bacterial species belonging to Bacteroidetes were not recorded from eggs from either source. *Weissella* sp. (5) and *Pseudomonas* sp. (6) were associated with both eggs extracted from faeces and laid *in vitro* eggs, while *Clostridium* sp. (3) was found only in the eggs extracted from faeces.

4.4. DISCUSSION

This study is the first which used DNA fingerprinting to identify bacteria associated with *H. contortus*, a nematode parasite of the abomasa of sheep and goats in tropical and sub-tropical areas of the world. Adult worms collected from the abomasa of infected sheep had less complex bacterial profiles than abomasal contents. Lactic acid bacterial and Proteobacterial sequences dominated those identified from adult worms, L3 and eggs (Table 4.2). Some of these bacteria (*Weissella* sp. and *Lactococcus* sp.) are not usually found in the rumen microbiota (Kim et al., 2011), suggesting that *H. contortus* does not acquire them from their environment. About half of the ~190bp sequences were matched to the phylum Firmicutes and the rest were consistent with sheep gut or ubiquitous environmental bacteria.

Lactic acid bacterial and Proteobacterial sequences dominated those from adult worms, L3 and eggs (Table 4.2). Both of these bacterial groups have been previously identified residing in the gut of many invertebrate animals (Rani et al., 2009) and Proteobacteria have been associated with other tissues, such as reproductive organs (Zouache et al., 2009). Alpha-, Beta- and Gamma-proteobacteria are ubiquitously present in aquatic environments (Glöckner et al., 1999), therefore the possibility of environmental contamination cannot be ruled out at this stage. This would be unlikely from laboratory reagents, which were shown not to be contaminated. Bacterial species belonging to Bacteroidetes were identified in adult worms, but not L3 or eggs (Fig. 4.4 and Table 4.2). These are typical ruminant foregut bacteria (Tajima et al., 1999; Sundset et al., 2007; Kim et al., 2011; Leng et al., 2011), which either could have been consumed by adult worms and merely passing through their gut or adhere to the external surfaces of the parasites. Rumen bacteria can remain alive in abomasal contents (Simcock et

al., 1999, 2006) and either dead or live rumen bacterial cells could contribute to the DNA and the sequences subsequently identified in the adult worm samples.

Eggs would not be expected to have many bacteria associated with them if the eggshells had been effectively cleaned. This was clearly not the case after sodium hypochlorite washing, perhaps because bacteria or DNA were firmly attached to the carbohydrate coat on the surface of the eggs (Hillrichs et al., 2012). Both egg samples (extracted from faeces and laid *in vitro*) appeared to have bacterial contamination, the former acquired from faeces and the latter from gut contents of the mother during egg-laying. During the egg-laying period, the female worms probably excreted some gut contents containing bacteria which may have firmly attached to the egg surface, resulting in similar band patterns for *in vitro* laid eggs and adult worms (Fig. 4.2, lanes HA1 and HE2). Sheep faecal bacteria appeared to contaminate eggs collected from the faeces, as the DGGE band patterns differed slightly from those of the *in vitro* laid eggs and also contained bacterial sequences of *Clostridium* sp., which are typical bacteria associated with faeces (Finegold et al., 1983; Leser et al., 2002; Eckburg et al., 2005; Rajilić-Stojanović et al., 2007).

Bacteria could either be located internally or externally on adult worms, eggs and L3 *H. contortus*. Free-living L1 and L2 feed on bacteria in the faeces, but after moulting, the L3 becomes a non-feeding dormant stage covered with the retained L2 cuticle. Some bacteria in the gut are likely to persist in the L3 and may continue to live permanently within the gut of the adult *H. contortus* and establish a mutualistic relationship. The location of these bacteria can be determined using FISH (Chapter 6).

PCR-DGGE analysis is particularly useful as an initial step to compare and identify the bacterial community composition in PCR generated samples. However, identical band patterns for two samples are indicative, but do not necessarily prove they are identical bacterial communities. Sequences which are different in G+C content and/or belong to different bacterial species can be isolated in one band e.g. both *Escherichia* and *Rhizobium* sp. were identified in the intense Band 8 present in adults and L3 (Fig. 4.4). On the other hand, the same bacterial species or identical sequence can be located in more than one

band from a single sample or in different bands in a DGGE gel from two samples. 16S rRNA gene sequences belonging to specific bacterial species can be found in more than one band in a DGGE gel due to the presence of multiple rrn operons, e.g. sequences belonging to *Weissella* separated at 3 different levels of the gel, probably because of slight sequence heterogeneities between operons (Fig. 4.4).

Despite its limitations, DGGE is still a useful technique to compare and analyse the bacterial profiles of sheep gut contents and *H. contortus*. The primer set 338f and 518r, which was used in the present experiment, is widely used for analysing bacterial sequences by the DGGE finger printing method (Muyzer et al., 1996; Watanabe et al., 2001), but generates only relatively short sequences of ~190bp. A limitation of PCR-DGGE is that ~500bp sequences are the maximum length which can be analysed in DGGE (Myers et al., 1985), which is inadequate for a detailed phylogenetic analysis and identification of species.

To support and verify the DGGE results, clone libraries were constructed using the universal bacterial primer set (27f and 1492r) to amplify nearly complete length (~1400bp) 16S rRNA sequences (Chapter 5). Amplified bacterial 16S rRNA sequences were used to analyse phylogenetic evolutionary relationship between sequences. FISH probes were selected, based on the sequences identified (both ~190bp and ~1400bp) from *H. contortus*, in order to determine the exact location of the bacterial species in *H. contortus* (Chapter 6).

Chapter 5

Phylogenetic Analysis of Bacteria Associated with *Haemonchus contortus*

5.1. INTRODUCTION

Bacterial sequences belonging to the phyla Firmicutes and Proteobacteria were identified amongst short sequences from DGGE bands from adult worms, L3 and eggs. The most frequently identified sequences were from lactic acid bacterial groups. There were fewer sequences from the phylum Proteobacteria than from the phylum Firmicutes, especially in the adult worms and L3. More precise identification of bacterial species can be inferred from phylogenetic trees representing the evolutionary relationship between the sequences analysed. Detailed phylogenetic identification of the bacteria in *H. contortus* can be achieved by comparison of nearly full length 16S rRNA sequences, a powerful method to infer phylogenetic relationships (Fox et al., 1980; Weisburg et al., 1991; Amann et al., 1995).

Phylogenetic analysis is an estimation of the evolutionary history of particular sequences, not the organism itself, and cannot be done absolutely, as the past history is not known. Several methods are used to compare aligned sequences and to deduce the phylogenetic affiliation of bacterial sequences. For bacterial 16S rRNA sequence data sets, alignments at highly conserved regions are straightforward, while the highly variable regions are more difficult. Commonly, phylogenetic relationships are inferred from the highly variable regions of aligned sequences, not the conserved regions, using distance (neighbour joining (NJ)), parsimony and maximum likelihood (ML) analyses (Holder and Lewis, 2003; Osborn and Smith, 2005; Hall, 2008). Each of these methods employs algorithms

based on certain assumptions which are not necessarily valid for all sequences and organisms and therefore may introduce biases into the analysis.

Unlike parsimony methods, both NJ, a popular distance method (Saitou and Nei, 1987), and ML methods (Felsenstein, 1981) compare each nucleotide position and require an evolutionary model. Parsimony methods map the history of gene sequences on to a tree. All possible trees at each site in aligned sequences are evaluated and the most likely tree is the one that requires the fewest number of changes to explain the data in the alignment. Limitations are the assumption that all sites evolved independently and underestimation of change caused by ignoring superimposed changes.

In NJ, pairwise comparisons of aligned sequences are converted into a distance matrix representing an estimate of evolutionary distance between sequences. The evolutionary distance is the number of changes that occurred as the two sequences diverged and is expressed as the percentage of the site which is different: sequences which differ in smaller percentages are more closely related than those that differ by higher percentages. Distance methods use base substitution models and take account of multiple substitutions at a single nucleotide position. The most commonly used model in the NJ method, the Jukes and Cantor model (Jukes and Cantor, 1969), assumes that changes at each site are independent and occur with equal probability; the evolutionary distance between sequences can be underestimated when these assumptions are not correct. The advantage of this method is that it is comparatively fast and is relatively reliable for recently diverged organisms (Saitou and Nei, 1987).

ML methods link the NJ and parsimony methods and test the probability of an evolutionary tree being the best of many alternative trees and the one which maximizes the probability of observing the aligned sequences. It involves comparison of each tree with an initial tree and is therefore highly dependent on the selection of the initial tree for accuracy. This bias can be minimised by using different initial trees. A disadvantage of ML methods is the huge number of trees generated when analysing large sequence data sets (Rogers and Swofford, 1998).

In the present study, DNA extracted from all three life-cycle stages was amplified with the universal bacterial primer set (27f and 1492r) and also with the universal bacterial primer 27f and the phylum Firmicutes-specific primer 1040firmR. Clone libraries were constructed for each primer set. The sequences obtained from these primers were used to construct bacterial 16S rRNA gene phylogenetic trees using NJ, ML and parsimony methods.

5.2. MATERIALS AND METHODS

5.2.1. *Haemonchus contortus* DNA

A clone library of nearly complete length (~1400bp) 16S rRNA sequences was constructed from adult worms (recovered from agar blocks) from 10 sheep (#14-15, 17, 20-26), eggs laid *in vitro* from 5 sheep (#14, 20, 23-25), eggs collected from faeces from 4 sheep (#20, 23-25) and L3 (ex-sheathed) from 6 sheep (#14, 17, 20, 23-25) (Appendix 1). Adult worms from sheep #24-25 and L3, eggs laid *in vitro* and faecal eggs from sheep #24 were used to make a clone library of partial (~1000bp) 16S rRNA gene sequences (Appendix 1). DNA was extracted as described in Section 3.2.5.1.4.

5.2.2. PCR, cloning and colony PCR

Nearly complete length (~1400bp) 16S rRNA gene sequences were amplified using the universal bacterial primer set 27f and 1492r. Partial length (~1000bp) 16S rRNA gene sequences were amplified using the universal primer 27f and the phylum Firmicutes-specific primer 1040firmR (Table 5.1). The PCR (25µl) mixture contained 2.6µl of 10X reaction buffer with 1.5M $MgCl_2$, 2.5µl of 2mM dNTP, 0.5µl of 0.5µM of each primer, 0.5µl of 2.5U of Taq DNA polymerase (Roche), and 15.9µl DNA free ultrapure water. The PCR was carried out using the touchdown PCR programme described in Section 3.2.5.2 with an initial annealing temperature of 62°C. Amplified DNA products were verified by electrophoresis (100V for 30min) of 5µl aliquots of each PCR product on 1.5% agarose gel in 1X TAE buffer. Gels were stained with SYBR® safe DNA gel stain

(Invitrogen), visualised using UV trans-illumination and photographed using a BIO-RAD Molecular Imager® Gel Doc™ XR (BIO-RAD Laboratories).

Cloning and colony PCR were carried out as described in Section 4.2.5.2. Each positive clone was sequenced using both M13f and M13r at Macrogen Inc.

5.2.3. Sequence editing

The forward and reverse sequences from each clone obtained using M13f and M13r were assembled using the Contig Express programme of Vector NTI11 (Invitrogen) according to the supplier's manual. The assembled sequences in the contig file were saved in the FASTA format and vector sequences were removed using MEGA 5.0 (Tamura et al., 2011). Sequences were checked for quality based on the chromatogram (evenly-spaced peaks) and mis-called nucleotides. Poor quality sequences were omitted from further analysis.

5.2.4. Clone library and phylogenetic analysis

Bacterial sequences retrieved from *H. contortus* using the universal bacterial primer set (~1400bp) and the phylum Firmicutes-specific primer (~1000bp) were matched with those in the GenBank database (Benson et al., 2008) using the BLASTn option (Altschul et al., 1990). The initial taxonomic identification was determined for each sequence from the closest match in the BLASTn search, however, the closest match in the GenBank database is not always correct, due to the wrong name or phylogenetic affiliation having been given to sequences deposited.

Additionally, the sequence match option of Ribosomal Database Project (RDP) was used to compare the bacterial sequences from *H. contortus* to obtain information on the closest type strain. For phylogenetic tree building, sequences were obtained from the GenBank database. These included uncultured bacterial sequences with similarity >99%, those from the closest cultured isolate and type strain sequences which had the highest similarity to the sequence. Bacterial sequences obtained from the GenBank database and *H. contortus* were combined for the subsequent analysis. Sequences were globally aligned with ClustalW using the MEGA 5.0 software package (Tamura et al., 2011). The alignment was

carefully examined and unnecessarily inserted gaps were removed manually to ensure proper alignment of all sequences based on previously identified conserved regions.

The online chimeric detection programme Bellerophon (Huber et al., 2004) was used to identify chimeric sequences. The phylogenetic affiliations of sequences were created with the phylogeny option of MEGA 5.0 (Tamura et al., 2011), using the default settings for the NJ, ML and parsimony methods. The number of bootstrap replications was 500. Phylogenetic trees of ~1400bp sequences obtained from NJ, ML and parsimony were compared with each other to verify the robustness of tree topology. Additionally, two NJ trees were created from the first and last ~400bp of aligned ~1400bp sequences and compared with each other for further detection of chimeric sequences. Potential chimeric sequences were excluded from further tree building analysis. Distance matrices of aligned sequences were also made using the Geneious software package (Kearse et al., 2012). The same procedure was carried out for ~1000bp sequences.

The final dendrograms of sequences of phylum Firmicutes (~1400bp and ~1000bp) were inferred using the NJ, ML and parsimony methods. Each analysis included sequences from the closest cultured and type strains and bacterial sequences identified in *H. contortus*.

5.3. RESULTS

5.3.1. Clone libraries

Initial taxonomic identification of bacterial 16S rRNA sequences of ~1400bp (amplified by 27f and 1492r) and ~1000bp (amplified by 27f and 1040firmR) is shown in Table 5.1App (Appendix 5). After the detailed phylogenetic analysis, 2 chimeric sequences were removed from the ~1400bp 16S rRNA initial clone library but none in ~1000bp clone library.

Leuconostoc, Weissella, Lactobacillus and *Streptococcus* sequences were identified in the ~1000bp clone library. Only *Leuconostoc* and *Weissella*

sequences were found in all life-cycle stages, *Streptococcus* sequences were not identified in eggs laid *in vitro* whilst *Lactobacillus* sequences were found only in L3.

Except for the eggs collected from the faeces, most of the phylum Firmicutes ~1400bp sequences belonged to lactic acid bacterial group, particularly from *Lactococcus, Weissella* and *Leuconostoc*. Nearly equal numbers of bacterial sequences belonging to *Weissella* were identified from all life-cycle stages except eggs collected from the faeces in which there was none. *Leuconostoc* sequences were also found all three life-cycle stages, except in the eggs collected from the faeces. The majority of the *Lactoccocus* sequences were identified from DNA from adult worms. Most *Clostridum* sequences were identified from DNA from eggs collected from faeces.

Mesorhizobium, *Stenotrophomonas*, *Pseudomonas*, *Comamonas* and *Rhizobium* were the dominant bacterial genera among the phylum Proteobacteria. No bacterial sequences were common to adult worms, L3 and eggs. *Mesorhizobium* was found only in adult worms and L3, while the majority of the *Stenotrophomonas* was identified in eggs laid *in vitro* and *Pseudomonas* was identified in eggs collected from faeces.

Bacterial sequences belonging to the phylum Bacteroidetes were identified only in adult worms and the majority were identified as uncultured rumen bacteria. Only a single *Spiroplasma* sequence was identified, in eggs collected from faeces.

5.3.2. Phylogenetic analysis of phylum Firmicutes ~1400bp 16S rRNA sequences

Bacterial sequences identified in *H. contortus* were assigned into 13 clusters by distance matrix analysis of the 66 clone sequences from *H. contortus,* their close relatives and cultured and type strains. The names were assigned as abbreviations of the nearest type strain (Table 5.2). Of the 66 sequences, 33 had more than 99% 16S rRNA gene sequence similarities with the validly described, named, cultured type strains *Lactococus plantarum* (cluster CLP), *Lactococcus raffinolactis* (cluster CLR), *Lactococcus lactis* subsp. *lactis* (cluster CLL),

Weissella confusa (cluster CWC), *Leuconostoc citreum* (cluster CLC) and *Streptococcus infantarius* subsp. *coli* (cluster CSI). Five sequences with similarities between 93.5-98% with known cultured and type strain sequences (3 from *in vitro* laid eggs, 1 from faecal eggs and 1 from L3) are likely to originate from 5 different species. The 28 sequences which had <93% similarities with known cultured and type strain sequences are probably from bacteria in genera that have not yet been named and described. Sequences belonging to genera *Clostridium* III (cluster CCT), *Catabacter* (cluster CCH), *Veillonella* (cluster CVP), *Clostridium* XI (cluster CET) and *Eubacterium* or *Robinsoniella* (cluster CEF) had considerable variation in sequence similarities with known cultured and type strain sequences (Table 5.2).

Phylogenetic trees generated by the ML method are shown in Fig. 5.1-5.3 and those created using the NJ and parsimony methods in Appendix 5. All three methods of analysis resulted in the same phylogenetic affiliations for the phylum Firmicutes sequences. Both NJ and ML analyses identified 13 clusters of bacterial sequences obtained from *H. contortus* with their close relative and cultured and type strain sequences; this is summarised in Fig. 5.1. These sequences always clustered into stable groups in ML and NJ analyses; these groups had high bootstrap values (shown at each node in Figs 5.1-5.3 and Appendix 5).

Most of the clone sequences within the CCT cluster grouped separately from the known type strain *Clostridium thermocellum* and the cultured species *Clostridium sufflavum*. These clone sequences had a low bootstrap value (55) with their type strain and cultured strain sequences in the phylogenetic tree (Fig. 5.3).

5.3.3. Phylogenetic analysis of ~1000bp 16S rRNA gene sequences

Bacterial sequences identified in *H. contortus* were assigned into 4 clusters (CFLC, CFWC, CFLF and CFSE) by distance matrix analysis of the 38 clone sequences from *H. contortus,* their close relatives and cultured and type strains. All 38 sequences had >99% sequence similarity with sequences from known cultured strains (*Leuconostoc citreum* (cluster CFLC), *Weissella confusa* (cluster CFWC), *Lactobacillus fermentum* (cluster CFLF) and *Streptococcus equinus*

(cluster CFSC)). Only 28 of these sequences had >99% similarity with sequences from the type strains *Leuconostoc citreum* and *Weissella confusa*, whereas the other 10 sequences had 94.5-97.9% sequence similarity with type strain sequences belonging to the genera *Lactobacillus* and *Streptococcus* (Table 5.3).

Phylogenetic trees generated by the ML method are shown in Fig. 5.4 and those created using the NJ and parsimony methods are given in Appendix 5. The topology and robustness of the trees were similar in both ML and NJ methods. Both NJ (Appendix 5) and ML phylogenetic tree analyses of the 38 bacterial sequences from *H. contortus* also identified 4 groups which clustered with their respective reference sequences (Fig. 5.4).

5.4. DISCUSSION

Detailed phylogenetic analysis was carried out for phylum Firmicutes bacterial sequences obtained from adult worms, L3 and eggs of *H. contortus*, as only these sequences were identified in all three life-cycle stages. These sequences grouped into 13 clusters in the phylogenetic analysis of ~ 1400bp sequences (sequences amplified using 27f and 1492r) and 4 clusters in the analysis of ~1000bp sequences (sequences amplified using 27f and 1040firmR). Sequences belonging to the lactic acid bacteria *Lactococcus, Streptococcus, Leuconostoc* and *Weissella* were identified in adult worms, L3 and eggs, similar to the findings from short sequences from DGGE bands (Chapter 4).

Bacterial sequences of the phylum Proteobacteria were not subjected to detailed analysis because of the high sequence variation identified by the universal bacterial 16S rRNA primer pair and vertical transmission being unlikely, as they were not detected in all life cycle stages. There is a greater likelihood of acquisition from their environment onto the surface of parasites, as Proteobacteria are ubiquitous in the environment (Kersters et al., 2001). On the other hand, *Mesorhizobium, Stenotrophomonas* and *Pseudomonas* may be present in the parasite gut, as these bacteria have been reported to be gut microbes in many insects (Zouache et al., 2011).

Regardless of the analysis employed (NJ and ML), the topology and robustness of phylogenetic trees composed of bacterial sequences of phylum Firmicutes were similar. Nearly full length (~1400bp) and partial (~1000bp) 16S rRNA bacterial sequences were grouped into 13 (Table 5.2) and 4 (Table 5.3) clusters respectively; this was consistent regardless of the algorithm used in the phylogenetic analysis. Sequences belonging to the lactic acid bacterial group had over 99% sequence similarity with those from type strains identified as *Weissella confusa, Leuconostoc citreum, Lactococcus plantarum, Lactococcus raffinolactis, Lactococcus lactis,* and *Streptococcus infantarius*. These findings are in agreement with results of PCR-DGGE (Chapter 3). There are no previous reports of lactic acid bacteria associated with nematodes. These bacteria inhabit a variety of environments, including fermented foods (Rantsiou et al., 2005; Rantsiou and Cocolin, 2006), the microbial flora of the honey stomach of the honey bee (Olofsson and Vásquez, 2008) and human faeces (Hermie et al., 1999; Harmsen et al., 2002). *Weissella confusa* has been associated with fatal endocarditis in humans (Flaherty et al., 2003).

Detailed phylogenetic analysis of bacterial sequences of the order Clostridiales showed that many may belong to yet to be described genera or species, because these sequences had very low sequence similarity with type strain sequences and their groups also had low bootstrap values.

Despite the relatively small number of sequences in the clone libraries, detailed phylogenetic analysis suggested that source of some the bacteria in the different life-cycle stages of *H. contortus* was the sheep gut microflora. Bacterial sequences belonging to the genus *Clostridium* were found only in adult worms and faecal eggs. This is not surprising, as *Clostridium* spp. are mammalian gut residents (Finegold et al., 1983; Leser et al., 2002; Eckburg et al., 2005; Rajilić-Stojanović et al., 2007) that could contaminate faecal eggs and adult worms. No sequences belonging to *Clostridium* spp. were identified in L3, despite their development in faeces, probably due to removal of bacteria by exsheathing of L3. Bacterial sequences belonging to the genus *Lactococcus* were present in both adult worms and eggs, suggesting that sheep gut bacteria may be present on the surface of the parasites or in the worm gut.

Sequences (~1400bp) from the genera *Weissella* and *Leuconostoc* were found in all life-cycle stages, except eggs collected from faeces. This is not unexpected, as most of the bacterial sequences of faecal eggs belonged to *Clostridium* spp. The large number of sequences of *Clostridium* spp. could be caused either by primer bias or presence of many of these bacteria in the egg sample. Nevertheless, the partial 16S rRNA bacterial sequences (~1000bp) using the phylum Firmicutes-specific primer confirmed that *Weissella* and *Leuconostoc* bacterial sequences can be obtained from all life-cycle stages, including eggs collected from faeces. Strikingly, those sequences amplified from *in vitro* laid eggs using the phylum Fimicutes-specific primer were all *Leuconostoc* or *Weissella*.

The information from the above phylogenetic analysis was used to select group-, class- and species-specific FISH probes to identify the morphology and locations of bacteria in *H. contortus*.

Chapter 6

Fluorescence *in situ* Hybridisation

6.1. INTRODUCTION

In *H. contortus*, the most frequently identified bacterial 16S rRNA sequences were from the phyla Firmicutes and Proteobacteria in both DGGE short band (~190bp) and nearly complete length (~1400bp) sequences. Many of the bacterial sequences from the lactic acid bacterial group belonged to *Lactococcus* sp., *Weissella* sp. and *Leuconostoc* sp. Most of the bacterial sequences belonging to the Alpha-, Beta- and Gammaproteobacterial classes were identified as *Mesorhizobium* sp., *Comamonas* sp., *Pseudomonas* sp. and *Stenotrophomonas* sp. The goals of the experiments reported in this Chapter were to investigate the location, morphology and abundance of selected bacteria associated with *H. contortus* adult worms, L3 and eggs.

FISH was the preferred method to demonstrate the location of the microbial community of *H. contortus.* This method has been used to identify the morphology, localisation, abundance and activity of microbes in various environments, which in turn contributes to an understanding of the organisms and their interaction with the environment (DeLong et al., 1989; Amann et al., 1995; Wagner et al., 2003). As 16S rRNA genes have both conserved and variable regions, FISH probes can be designed to identify microorganisms at species, genus, family, order or domain level, depending on the degree of conservation of the target sequences. The higher the degree of conservation, the more difficult it is to differentiate organisms, such as strains of a particular bacterial species (Loy et al., 2003).

The basic steps of FISH involve sample fixation to stabilise cell morphology and facilitate permeabilisation of the cell membrane, hybridisation with probes, washing to remove excess probe and then viewing the signals under epifluorescence or confocal laser microscopy (Amann et al., 2001; Amann and Fuchs, 2008). More information is obtained if multiple probes labelled with different fluorochromes, such as fluorescein isothiocyanate (FITC) and cyanine (Cy3 and Cy5), are used on the same section. This would be possible only if the probes require the same formamide concentration for optimal hybridisation stringency. A limitation of using FISH is that the failure to detect signals does not necessarily mean that the target bacterial species are not present. Low intensity of signals can be due to low ribosomal content, impenetrability of the cell envelope to probes due to over-fixation and inaccessibility of the probe to the target region because of secondary and tertiary structures of the rRNA and/or ribosomal protein (Amann and Ludwig, 2000; Zwirglmaier, 2005).

To identify the bacteria associated with *H. contortus* adult worms, L3 and eggs, eubacterial probes and class- and species-specific probes were selected based on bacterial sequences identified in clone libraries (Chapter 5). The probes were chosen using a literature search and the online probeBase database (Loy et al., 2007). In each experiment, universal and specific bacterial probes with different fluorochrome labels were combined. The specificity and hybridisation stringency of each probe were first optimised against relevant reference bacteria combined with non-target organisms.

Table 6.1. Conditions used for culture of reference bacteria.

Bacterial species	Medium (broth)	Growth environment	Temperature
Weissella confusa **DSM 20196 548-D**	MRS	Microaerophilic	30°C
Weissella paramesenteroides **DSM 20283**	MRS	Microaerophilic	30°C
Leuconostoc citreum **DSM 5577 B2399**	MRS	Microaerophilic	28°C
Lactobacillus acidophilus **NCFM***	MRS	Anaerobic	37°C
Lactococcus lactis **subsp. *lactis* DSM 20481**	Corynebacterium broth	Anaerobic	30°C
Streptococcus mutans **ATCC 19434**	Trypticase soy yeast extract medium	Microaerophilic	37°C
Enterococcus faecalis **ATCC 25175**	Trypticase soy yeast extract medium	Microaerophilic	37°C
Comamonas testosteroni **DSM 50244**	Nutrient broth	Aerobic	30°C
Pseudomonas beteli **DSM 21257**	Nutrient broth	Aerobic	28°C
Aminobacter niigataensis **DSM 7050 DM81**	Nutrient broth	Aerobic	30°C

Abbreviation: MRS: de Man, Rogosa and Sharpe. *provided by Todd Klaenhammer, North Carolina State University

6.2. MATERIALS AND METHODS

6.2.1. Parasite samples

Adult female worms (recovered from agar blocks; Appendix 2.4), eggs (laid *in vitro* or collected from faeces; Appendices 2.5-2.6) and L3 (ex-sheathed; Section 3.2.6) were collected from 2 sheep (#19-20). Male worms were also collected from sheep #20. A further 5 sheep (#14-15, 21-23) provided female worms (Appendix 1). Adult female worms were also collected directly from the abomasal mucosa of two sheep and fixed immediately to prevent emptying of their gut bacteria.

6.2.2. Reference bacterial cultures

Cultures of *Weissella confusa* DSM 20196^T 548-D, *Weissella paramesenteroides* DSM 20288^T R80, *Leuconostoc citreum* DSM 5577^T B2399, *Lactococcus lactis* subsp. *lactis* DSM 20481^T, *Comamonas testosteroni* DSM 50244^T, *Pseudomonas beteli* DSM 21257^T, and *Aminobacter niigataensis* DSM 7050^T DM81 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany). *Lactobacillus acidophilus* NCFM (provided by Todd Klaenhammer, North Carolina State University), *Streptococcus mutans* ATCC 25175, and *Enterococcus faecalis* ATCC 25175 were obtained from the AgResearch Ltd Grasslands (Palmerston North) bacterial culture collection.

6.2.3. Bacterial culture

Details of culture conditions used for reference bacterial species are given in Table 6.1. The growth media used were those recommended by DSMZ for their organisms and culture was carried out according to the supplier's instructions. The bacteria obtained from AgResearch Ltd were cultured as shown Table 6.1. To maximise the cellular rRNA content,

Table 6.2. Oligonucleotide probes used for fluorescence *in situ* hybridisation of sections of *H. contortus* and bacterial reference cultures.

Probe	Target	Sequences (5'-3')	Target site (rRNA positions)	Fluorochrome attached to the probe	References
EUB338	most bacteria	GCTGCCTCCCGTAGGGT	16S, 338 - 355	Cy3 or FITC	Amman et al. (1990)
Non-EUBb338	Negative control	ACTCCTACGGGAGGCAGC	16S, 338 - 355	Cy3 or FITC	Wallner et al. (1993)
Lab158	Lactic acid bacteria	GGTATTAGCAYCTGTTTA	16S, 176 - 195	Cy3 or FITC	Harmsen et al. (2002)
Wgp	*Weissella* sp.	TTATCCCCYRCTAAGAGGT AGG	16S, 150 - 171	Cy3	Collins et al. (1993)
S-G-Wei-0121-a-S-20	*Weissella* sp.	TAAGAGGTAGGTTTCCCG	16S, 141 - 160	Cy3	Jang et al. (2002)
Strc493	Most *Streptococcus* sp. and some *Lactococcus* sp.	GTTAGCCGTCCCTTTCTG	16S, 493 - 511	Cy3 or FITC	Franks et al. (1998)
ALF73a	Some Alphaproteobacteria	TTCCGTCTAACCGCGGG	23S, 2043 - 2059	Cy3	Manz et al. (1992)
Beta1	Betaproteobacteria	CCCATTGTCCAAAATTCC CC	16S, 359 - 378	Cy3	Ashelford et al. (2002)
SteMal_439	*Stenotrophomonas maltophilia*	GCT GGA TTT CTT TCC CAA CA	16S, 439 - 458	Cy3	Piccini et al. (2006)

bacteria were harvested 24h after inoculation into the media, except *Enterococcus faecalis*, which was harvested after 2 days.

Aliquots of bacterial cultures were cryo-preserved: 500µl of the bacterial culture was added to 30% [v/v] 500µl of sterile glycerol in sterile water in a cryo-vial and vortexed. Vials were stored at -20°C.

6.2.4. Fixation of bacterial cells

Bacterial cells were fixed in cold 4% [v/v] paraformaldehyde in PBS (PFA) (Appendix 6.1.1). Three volumes of 4% PFA were added to one volume of microbial culture and kept at 4°C for 2h. 750µl of fixed cells were transferred into sterile microcentrifuge tubes (Eppendorf). The tubes were centrifuged at 5000*g* for 5min and the fixative was removed. The cells were washed with 300µl 1X PBS by brief vortexing and centrifuging at 5000*g* for 5min. The PBS was removed and cells were re-suspended in 300µl 1X PBS and 300µl 100% ethanol and vortexed briefly. Samples were stored at -20°C.

6.2.5. Oligonucleotide probes

Group-, class- and species-specific probes were selected from the literature (Table 6.2) to target the bacterial species identified from DGGE band short sequences (~190bp) and nearly complete length bacterial (~1400bp) sequences. These probes were analysed using the probeBase website (Loy et al., 2007). Bacterial sequences obtained from DGGE bands (~190bp) and nearly complete length (~1400bp) 16S rRNA bacterial sequences from *H. contortus* were compared with sequences deposited in the RDP and NCBI databases. Bacterial sequences were retrieved from the GenBank database of uncultured bacteria with similarity >99%, the nearest cultured isolate and type strain sequences which had the highest similarity to the bacterial sequences from *H. contortus*. Sequences were globally aligned with ClustalW using version 5 of the MEGA software package (Tamura et al., 2011).

Table 6.3. Specificity, target and non-target species chosen to optimise bacterial probes used for fluorescence *in situ* hybridisation.

Probe	Specificity	Target species	Non-target species
1. **Lab158**	Most lactic acid bacteria	*Lactobacillus acidophilus*, *Enterococcus faecalis*, *Leuconostoc citreum*, *Weissella confusa*	*Weissella paramesenteroides, Streptococcus mutans, Lactococcus lactis* subsp. *lactis*
2. **Wgp**	*Weissella* sp.	*Weissella confusa*	*Streptococcus mutans, Enterococcus faecalis,*
3. **S-G-WEI-0121-A-S-20**			
4. **Strc493**	Most *Streptococcus* sp., some *Lactococcus* sp.	*Streptococcus mutans, Lactococcus lactis* subsp. *lactis*	*Lactobacillus acidophilus Enterococcus faecalis, Leuconostoc citreum, , Weissella paramesenteroides Weissella confusa*
5. **Alf73a**	Alphaproteobacteria	*Aminobacter niigataensis*	*Streptococcus mutans* *Weissella confusa*
6. **Beta1**	Betaproteobacteria	*Comamonas testosteroni*	
7. **SteMa1_439**	Gammaproteobacteria	*Pseudomonas beteli*	

The following probes, Lab158, Wgp, S-G-Wei-0121-a-S-20, Strc493, Alf73a, Beta1 and SteMal_439, were selected, compared and analysed with the MEGA 5 aligned sequence database. Included in each experiment were: the universal bacterial EUB338 probe, which targets most bacteria; a negative control Non-EUBb338 probe to detect nonspecific binding and a no probe control to detect autofluorescence. Labelled (Cy3 or FITC) probes were purchased from Eurofins MWG Operon (Ebersberg, Germany) and Sigma Aldrich (Auckland, New Zealand). The details of oligonucleotide probes are shown in Table 6.2.

6.2.5.1. Optimisation of hybridisation stringency for FISH probes

The specificity and optimum hybridisation stringency of the 16S rRNA-targeted oligonucleotide probes were determined against the relevant species. Probe specificity, target and non-target species of each probe are summarised in Table 6.3. In the absence of a suitable 16S rRNA probe for Alphaproteobacteria, a 23S rRNA targeted probe was selected. The optimum formamide concentration for each probe was determined using a series of formamide concentrations (Appendix 6.2). The optimal hybridisation stringency was determined to occur at the formamide concentration immediately below that in which specific signals decreased and there were no non-specific signals from non-target species.

Before determining the optimum hybridisation stringency of Lab158, Strc493, Wgp and S-G-Wei-0121-a-S-20 probes, the effect of lysozyme treatment, which usually increases probe penetrability through the Gram-positive cell wall, was assessed using PFA fixed bacterial cells treated with and without lysozyme. Gram-positive bacterial (lactic acid bacterial group) cells were treated with 1mg/ml lysozyme solution (in ultrapure water) for 10min prior to hybridisation. Gram-negative bacteria (Proteobacteria) were not treated with lysozyme.

6.2.5.2. FISH of PFA fixed reference bacteria

FISH was carried out as described by Hugenholtz et al. (2002). The wells of teflon-coated slides (Thermo Fisher Scientific New Zealand Ltd, Auckland, New Zealand) were spotted with 3-5µl of PFA fixed bacterial cells. The slides were air-dried thoroughly and dehydrated through a series of ethanol solutions (3min each in 50, 80 and 98% ethanol). The slides were thoroughly air-dried and the wells containing bacteria were covered with 1mg/ml lysozyme (Sigma) for 10min, except for the proteobacterial cells (Gram-negative). The wells were covered with tris-based hybridisation buffer containing formamide and the labelled probe. The hybridisation procedures, hybridisation buffers and washing buffers are described in detail in Appendix 6.

The optimum hybridisation stringency and probe specificity were determined at a constant incubation temperature of 46°C, using a series of hybridisation buffers with formamide concentrations of 0-40% (0, 5, 10, 15, 20, 25, 30, 35 and 40%). After 2h incubation, the wells were washed in appropriate washing buffers (Appendix 6.1.3) at 48°C for 15min. In each experiment, a control for autofluorescence (buffer without probe), a negative control (Non-EUB338) and both target and non-target bacterial species for each probe were included.

The hybridisation and washing procedures for the proteobacterial class-specific probes were carried out as described above, but omitting lysozyme treatment. Initial optimisation of hybridisation stringency was carried out using 0-30% (0, 10, 20, and 30%) formamide concentrations in hybridisation buffer. After the initial optimisation, fine optimisation was performed using 0-20% (0, 5, 10, 15 and 20%) formamide in hybridisation buffer.

Table 6.4. Characteristics of fluorochromes attached to the probes used in fluorescence *in situ* hybridisation.

Fluorochromes	**Excitation wavelength (nm)**	**Emission wavelength (nm)**
FITC	470-495	510-550
Cy3	535-555	570-625

6.2.5.3. Epifluorescence microscopy

A thin film of Vectashield® mounting medium for fluorescence (Vector Laboratories, Burlingame, CA, USA) was applied to the wells containing bacterial cells. The slides were covered with a large cover slip and the edges were sealed with nail polish. Hybridised samples were observed under an epifluorescence microscope (OLYMPUS BX61, Tokyo, Japan) at 20x, 40x, 60x and 100x magnification. Characteristics of the fluorochromes attached to the probes used in FISH are given in Table 6.4.

6.2.5.4. Confocal microscopy

Reference bacterial cells were hybridised with EUB338 Cy3 and FITC labelled probes. These bacteria were viewed under a confocal laser scanning microscope (Leica TCS SP5 DM 6000B Leica Microsystems, Germany) to define the excitation and emission wave lengths in order to avoid non-specific signals.

6.2.6. *Haemonchus contortus* tissue sections

6.2.6.1. Adult worms

Adult worms were collected after recovery from agar blocks (Appendix 2.4). Both female and male worms were selected for FISH. The worms were straightened by incubating for approximately 12h at 4°C in PBS and then fixed overnight at 4°C in PFA. Female worms were also collected directly from the abomasa of two sheep and fixed in PFA immediately to reduce emptying of their gut bacteria. After residual PFA had been removed by washing twice with PBS, the worms were transferred to 70% ethanol and stored at -20°C until further processing. Worms were paraffin embedded (Leica Histo Embedder, Wetzlar, Germany) after automated, routine histological processing through graded alcohol solutions and 100% xylene (Leica TP1050 tissue processor, Wetzlar,Germany).

6.2.6.2. Eggs and L3

Eggs collected from faeces (Appendix 2.6), *in vitro* laid eggs (Appendix2.5) and ex-sheathed L3 (Section 3.2.6.1) were fixed overnight at 4°C in 4% PFA in microcentrifuge tubes (Eppendorf). Residual PFA was removed by twice washing with PBS and centrifuging for 3-5sec. The samples were dehydrated by passing sequentially through 50, 75 and 100% ethanol solutions. Each alcohol solution was added to the sample and left for 3min at room temperature, then the tube was centrifuged at 17,100*g* for 1min and the supernatant removed. This was followed by serial immersion of the samples in ethanol-xylene solutions of 3:1, 1:1 and 1:3 [v/v] and finally in 100% xylene for 10min. After removing the xylene, the samples were embedded in paraffin blocks (Leica Histo Embedder).

6.2.6.3. Preparation of sections for FISH

Sections 3µm-thick were cut on a manual rotary ultramicrotome (Leica RM 2235, Wetzlar, Germany) using a FEATHER (Osaka, Japan) disposable Microbe Blade. Two sections, each containing 3 adult worms, 100-200 eggs or L3, were placed on each slide (Menzel-Glaser Superfrost, Lomb Scientific Pty Ltd, Sydney, Australia). Sections were de-parafinised prior to FISH. Slides were placed on a heating block at 100°C until the paraffin had melted (3-5sec). The slides were then immersed in 100% xylene for 15min and then in 100% ethanol for 15min. These two steps were repeated twice and then the slides were washed in MQ water. The slides were thoroughly air-dried and treated with 1mg/ml lysozyme (Sigma) for 10min for the Lab158, Strc493, Wgp and S-G-Wei-0121-a-S-20 probes, but not Proteobacterial class-specific probes. Lysozyme was removed under running tap water and the slides were air-dried thoroughly.

Table 6.5. Probe combinations, fluorochrome labels and formamide concentrations for optimal hybridisation stringency of bacterial species-, group- and class-specific probes used to identify bacteria in *H. contortus*.

Probe combination	Optimised formamide concentration (%)
EUB338 FITC and Lab158 Cy3	25
EUB338 Cy3 and Lab158 FITC	25
EUB338 FITC and S-G-Wei-0121-a-S-20 Cy3	25
EUB338 FITC and Wgp Cy3	25
EUB338 FITC and Strc493 Cy3	35
EUB338 Cy3 and Strc493 FITC	35
EUB338 FITC and ALF73a Cy3	5
EUB338 FITC and Beta1 Cy3	10
EUB338 FITC and SteMal_439 Cy3	10

6.2.6.4. FISH on *Haemonchus contortus* sections

Multiple slides were used, each slide containing two consecutive serial sections of either 3 adult worms, 100-200 eggs or 100-200 L3. Details of probe combinations used to identify the location of bacteria in *H. contortus* are summarised in Table 6.5, along with optimised formamide concentrations determined from reference cultures. Details of the components of the hybridisation and washing buffers for each probe are described in Appendix 6.3. Each experiment included a control for autofluorescence (hybridisation buffer without probes) and a negative control (Non-EUB338 labelled with FITC or Cy3).

The hybridisation oven was pre-warmed to 46°C. Hybridisation buffers were freshly prepared in 2ml microcentrifuge tubes. Using a Liquid Blocker-Super PAP pen (Daido Sangyo, Tokyo, Japan), circles were drawn around the nematode sections to form hydrophobic barriers to keep the buffer on the nematode sections. The slides were placed on a paper towel in a sealable plastic box. Hybridisation buffer was added to cover each section on the slide; the volume of buffer required in each case was recorded. For every 8µl of hybridisation buffer, 0.5µl (50ng/µl) of probe was added and mixed carefully with a filtered pipette tip without touching the cells. The residual hybridisation buffers were poured on to the paper towel to prevent the evaporation of hybridisation buffer on the slides during the incubation period. The lid was placed loosely on the box, which was then placed in the hybridisation oven for 2h. Appropriate washing buffers were prepared in 50ml centrifuge tubes and placed in the oven to warm. Following hybridisation, each slide was rinsed immediately using a pipette containing the appropriate washing buffer (pre-heated to 48°C) and then placed in a tube containing washing buffer at 48°C for 10-15min. After removal from the washing buffer, the slides were immediately rinsed briefly in a beaker of ice-cold distilled water and then thoroughly air-dried.

6.2.6.5. Epifluorescence and confocal laser scanning microscopy

Sections were mounted and viewed under phase contrast and the appropriate wave lengths (Table 6.4) and photographed at 100x, as described in Section 6.2.5.3. Prior to viewing the *H. contortus* sections, EUB338 Cy3 and FITC labelled reference bacteria were viewed under the confocal laser scanning microscope (Leica TCS SP5 DM 6000B Leica Microsystems, Germany) to define the excitation and emission wave lengths in order to avoid non-specific signals. Selected *H. contortus* tissue sections were viewed under a confocal laser scanning microscope at the excitation and emission wave lengths optimised to avoid non-specific signals (Section 6.2.5.4.). Images were analysed using the Leica LAS AF and Leica LAS AF lite (Leica Microsystems CMS GmbH, Wetzlar, Germany) imaging software packages.

6.3. RESULTS

6.3.1. Optimisation of hybridisation stringency

Lysozyme treatment of Gram-positive bacteria prior to hybridisation increased the probe penetrability (Lab158, Strc493, Wgp and S-G-Wei-0121-a-S-20 -0121-a-S-20), as signals were of higher intensity from cells treated with lysozyme than from untreated cells. Detailed results for Lab158 probe are given in Appendix 7.1.

The formamide concentrations for optimal hybridisation stringency for all probes are shown in Table 6.5. Detailed optimisation results are given in Appendix 7. Lab158, Wgp, ALF73a Beta1 and SteMal_439 were very specific to the relevant target species at specific formamide concentrations (Table 6.5). The S-G-Wei-0121-a-S-20 probe bound to target bacterial species throughout the entire range of formamide concentrations used (0-40%), but not to the non-target species. The optimal hybridisation stringency for Strc493 was determined to occur at 35% formamide, but

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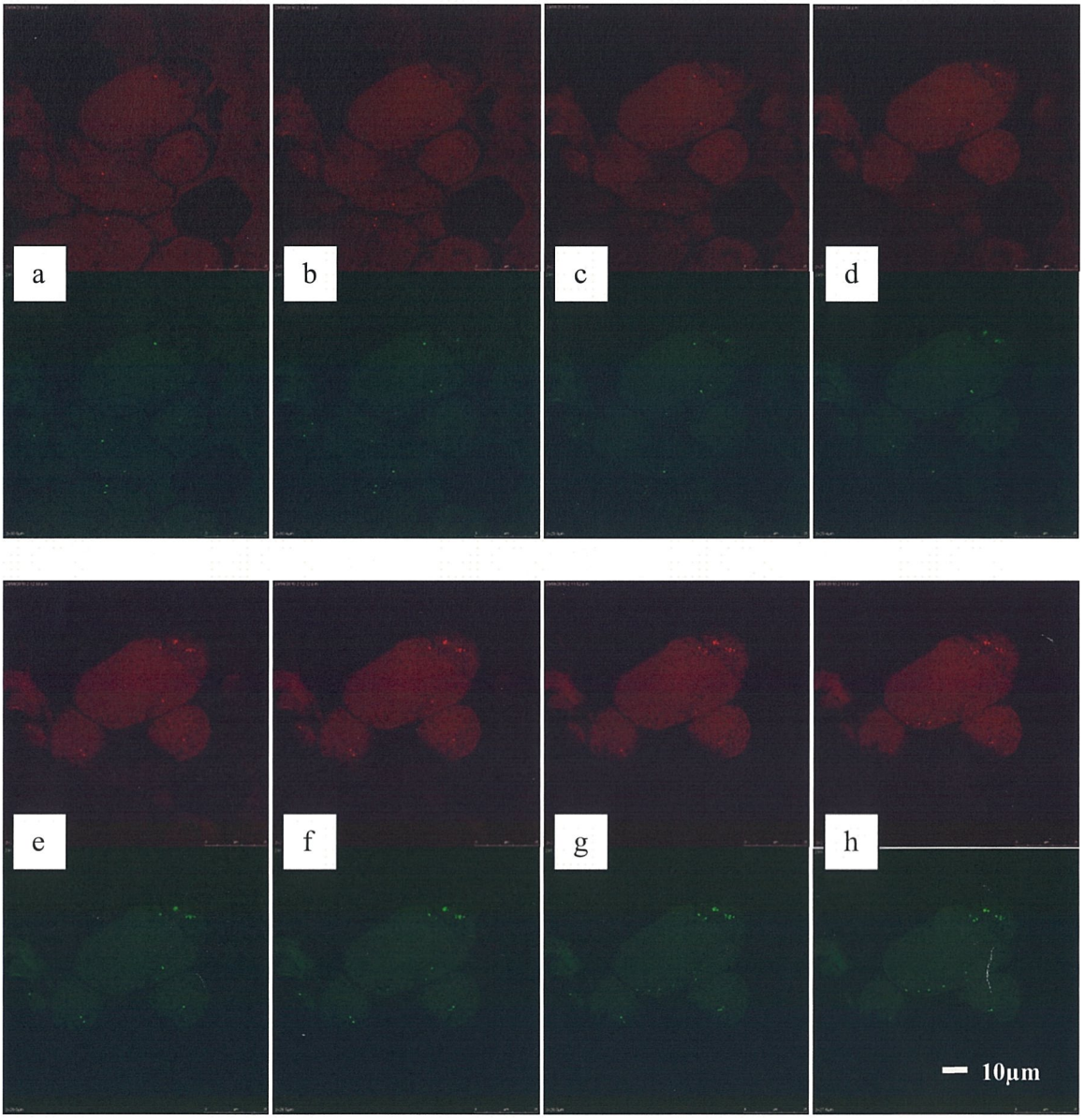

Fig. 6.1. Bacteria within eggs in adult *H. contortus*. The layers of egg images were generated by optical sectioning at the same location in the worm, using a confocal microscopy. The stack was collected in 200nm steps; it is represented as a-h. Bacteria in the eggs were targeted by EUB338 (FITC labelled, green signals) and Lab158 (Cy3 labelled, red signals) probes. The EUB338 probe targets most bacteria and Lab158 targets most lactic acid bacteria.

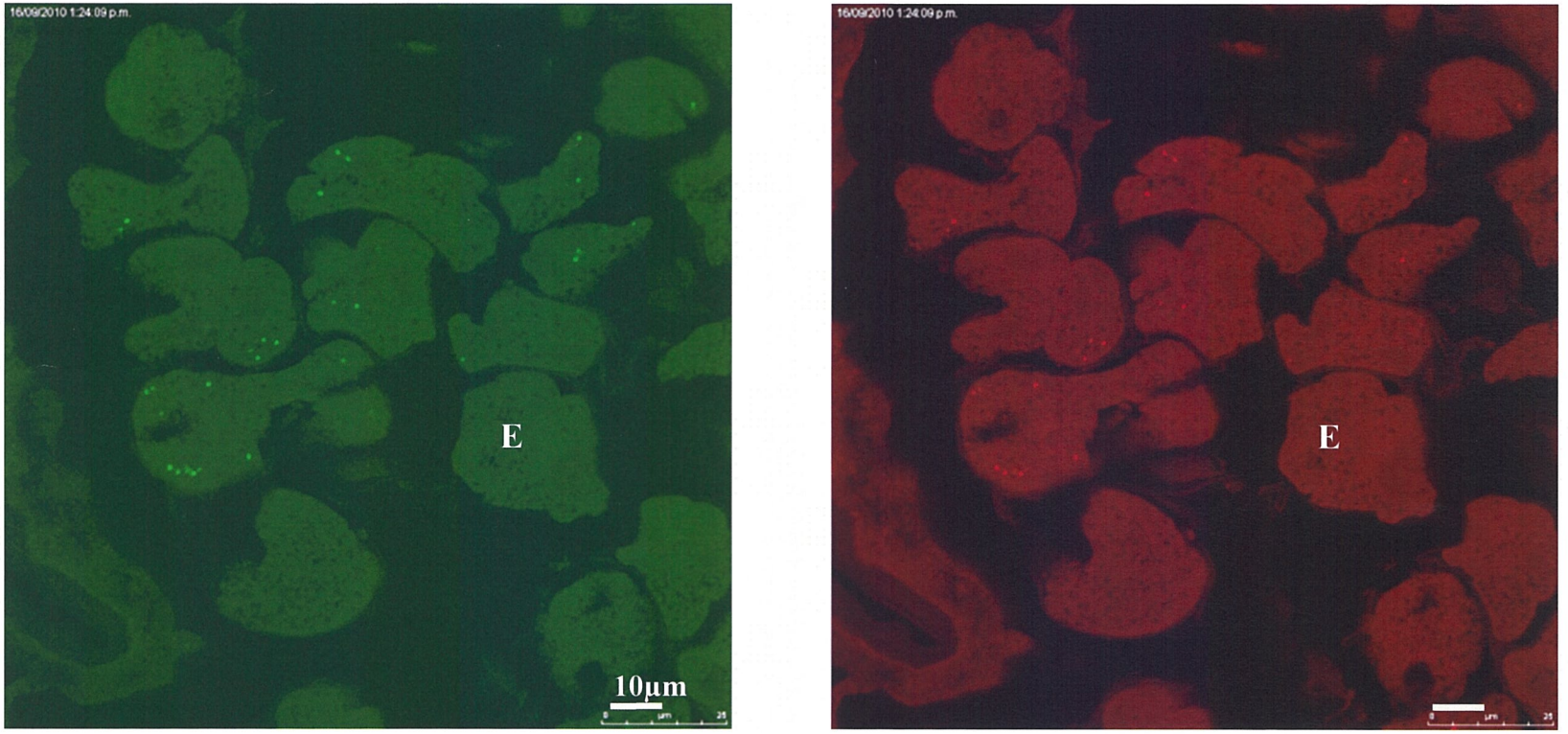

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Fig. 6.2. Bacteria within fertilised eggs (E) (presence of morula) in *H. contortus*. Bacteria in the eggs were targeted by both EUB338 (FITC labelled, left) and Lab158 (Cy3 labelled, right). The EUB338 probe targets all bacteria and Lab158 probe targets most lactic acid bacteria. The contrast of these images has been enhanced for printing.

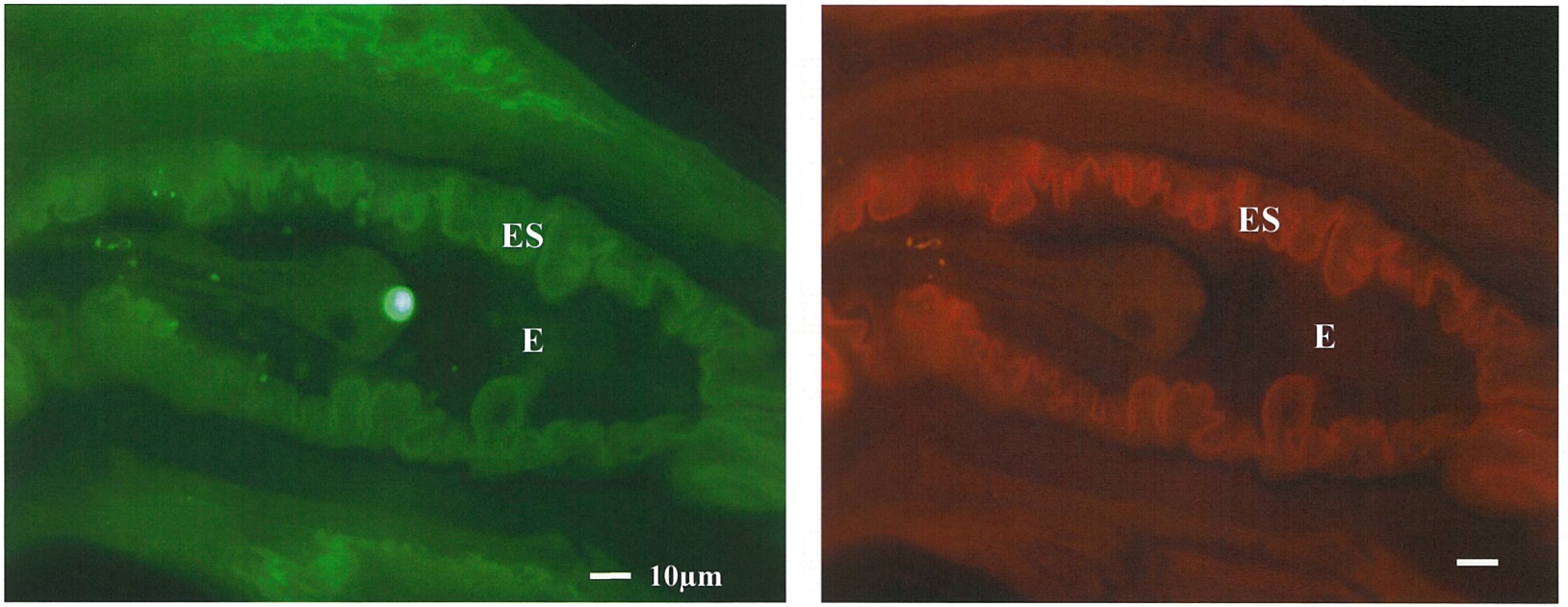

Fig. 6.3. Bacteria inside an egg near the ovipositor. Bacteria were targeted by EUB338 (FITC labelled, left) and Wgp (Cy3 labelled, right) probes. The EUB338 probe targets all bacteria and the Wgp probe targets *Weissella* sp. Not all bacteria were targeted by the Wgp probe. E: egg; ES: egg shell.

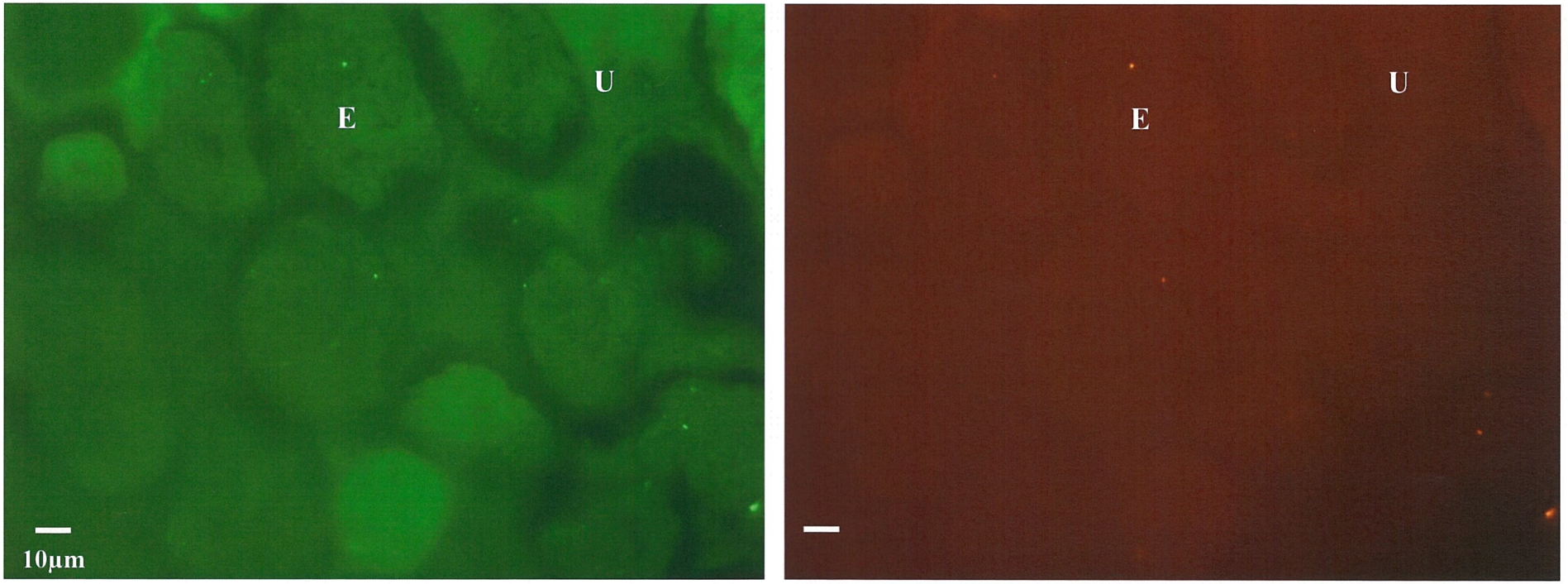

Fig. 6.4. Bacteria inside eggs in a female *H. contortus*. Bacteria were targeted by EUB338 (FITC labelled, left) and S-G-Wei-0121-a-S-20 (Cy3 labelled, right) probes. The EUB338 probe hybridises with all bacteria and the S-G-Wei-0121-a-S-20 probe hybridises with *Weissella* sp.. Not all bacteria were targeted by the S-G-Wei-0121-a-S-20 probe. E: egg; U: uterus.

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Fig. 6.5. Bacteria inside an egg near the ovipositor of a female *H. contortus*. Bacteria were targeted by EUB338 (FITC labelled, left) but not by SteMA1_439 (Cy3 labelled, right). The EUBB338 probe targets all bacteria and the SteMA1_439 probe targets *Stenotrophomonas maltophilia*. E: egg; ES: egg shell.

under these conditions, this probe targeted *L. citreum*, one of the non-target species (Appendix 7.3).

6.3.2. Identification of bacteria in *Haemonchus contortus*

In female worms, bacteria were seen in eggs, within the uterus and in the gut. Bacteria were also seen in laid eggs, but not in L3. Only gut bacteria were seen in male worms. The *Weisella* species-specific (Wgp and S-G-Wei-0121-a-S-20) and lactic acid bacterial group-specific (Lab158) probes hybridised with bacteria inside the eggs. The Strc493 probe, which targets most *Streptococcus* sp. and some *Lactococcus* sp., hybridised with bacteria within the uterus. The EUB338 probe, which targets most bacteria, hybridised with all of these bacteria. The Proteobacterial class-specific probes did not hybridise with any bacteria associated with *H. contortus*.

6.3.2.1. Bacteria in female worms

6.3.2.1.1. In eggs

Only those lactic acid bacteria which could be targeted by Lab158 were present in eggs, as both the numbers and distributions of Lab158 and EUB338 probe signals were identical (Fig. 6.1). This was also the case in eggs containing morulae (Fig. 6.2). Most of the bacteria within the eggs could also be targeted by both *Weissella* species-specific probes, Wgp (Fig. 6.3) and S-G-Wei-0121-a-S-20 (Fig. 6.4). The Wgp and S-G-Wei-0121-a-S-20 probes hybridised with most, but not all, of the bacteria within the eggs, as there were more signals from EUB338 than from Wgp (Fig. 6.3) or S-G-Wei-0121-a-S-20 (Fig. 6.4). No Proteobacterial class-specific probes hybridised with the bacteria within the egg (Fig. 6.5).

Using confocal microscopy, signals from eggs were photographed at successive focal planes at the same site (Fig. 6.1), building a three dimensional image (see enclosed CD). Bacteria were scattered throughout, densely clustered in some parts of the egg, and also found individually.

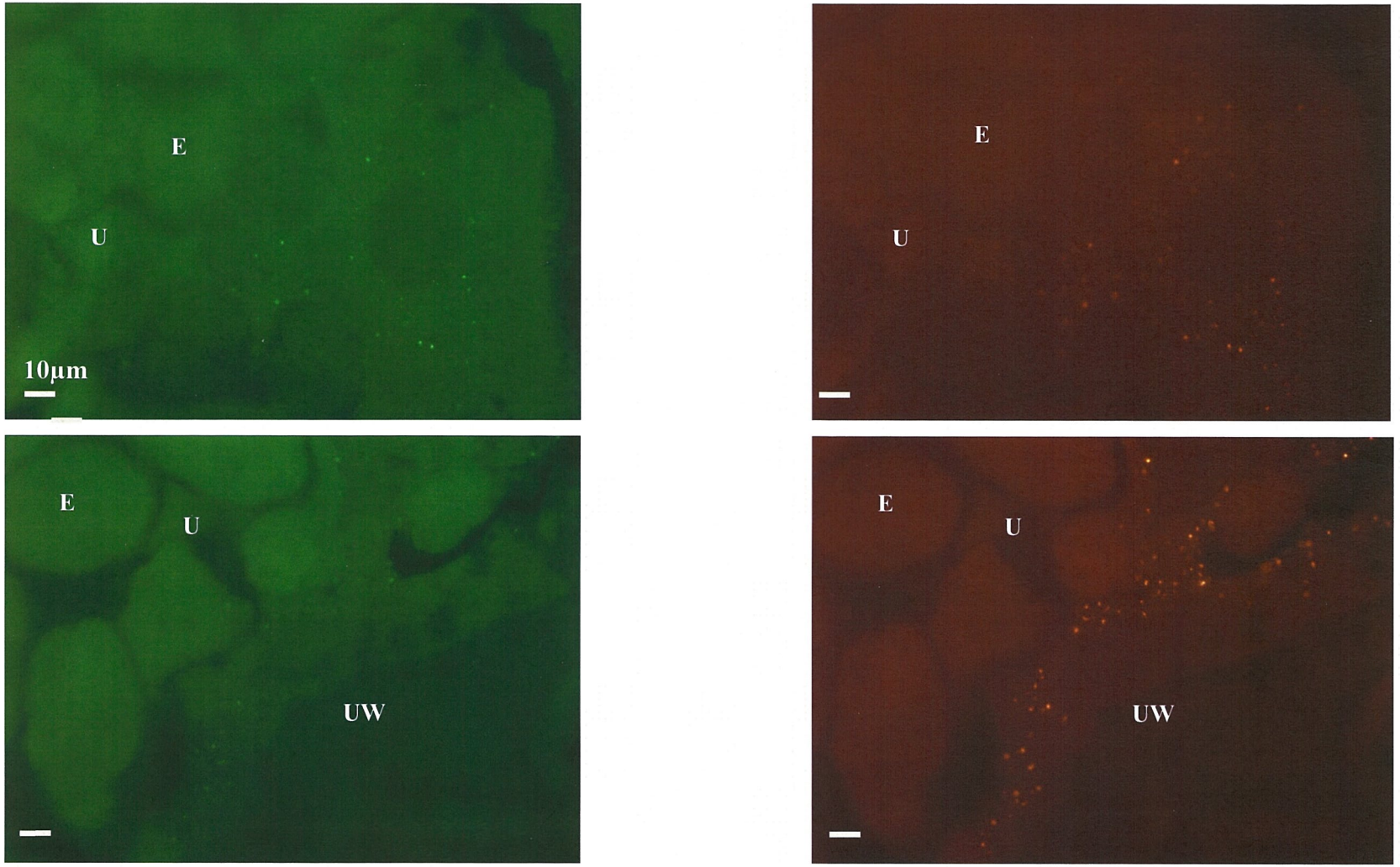

Fig. 6.6. Bacteria in the uteri of female *H. contortus*. Bacteria were targeted by the EUB338 (FITC labelled, left) and Strc493 (Cy3 labelled, right) probes. The EUB338 probe targets all bacteria and the Strc493 probe targets most *Streptococcus* sp. and some *Lactococcus* sp. E: egg; U: uterus; UW: wall of the uterus.

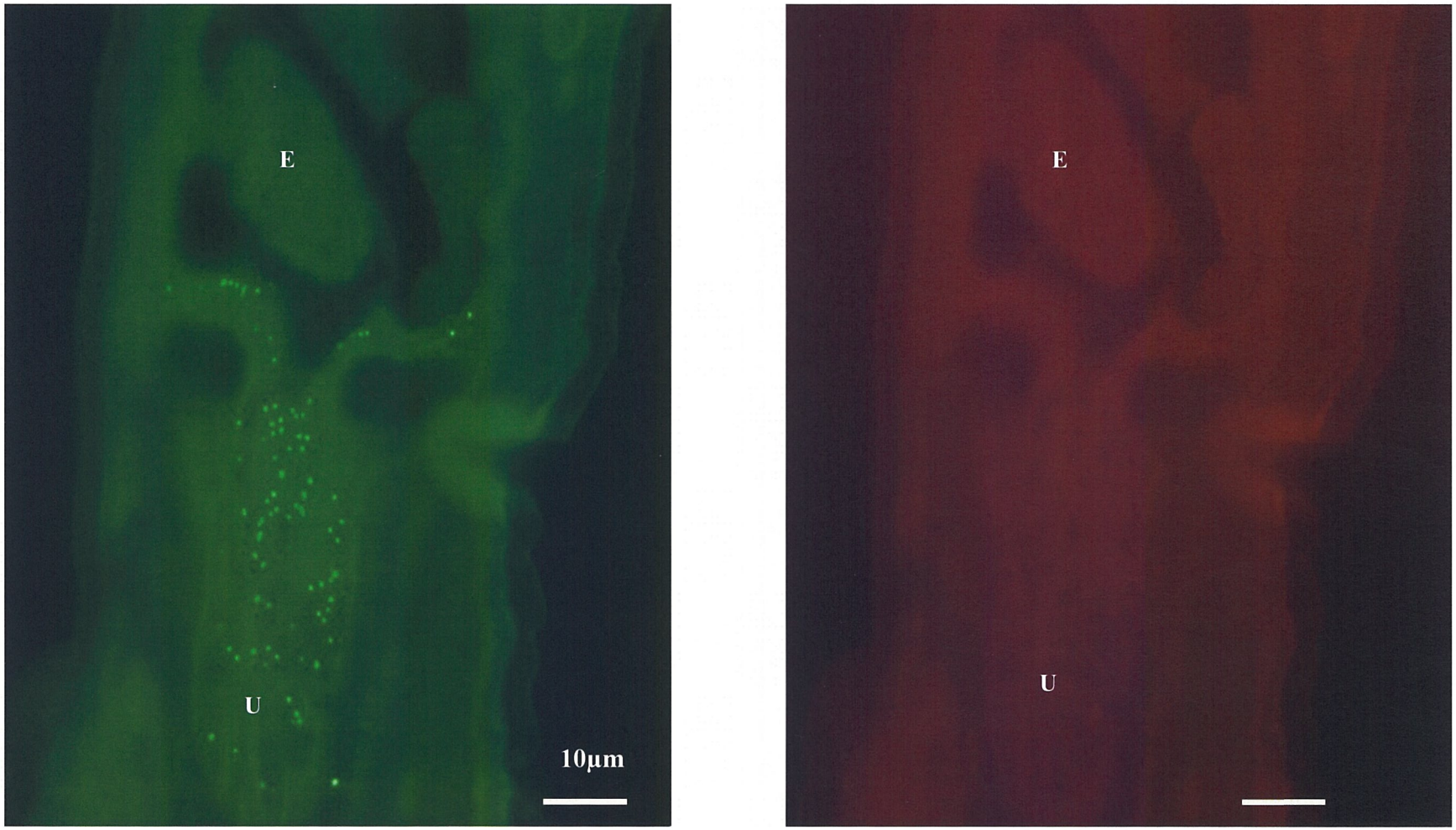

Fig. 6.7. Bacteria in the uterus of a female *H. contortus*. Bacteria were targeted by EUB338 (FITC labelled, left) but not by Lab158 (Cy3 labelled, right). The EUB338 probe targets all bacteria and the Lab158 probe targets most lactic acid bacteria. E: egg; U: uterus.

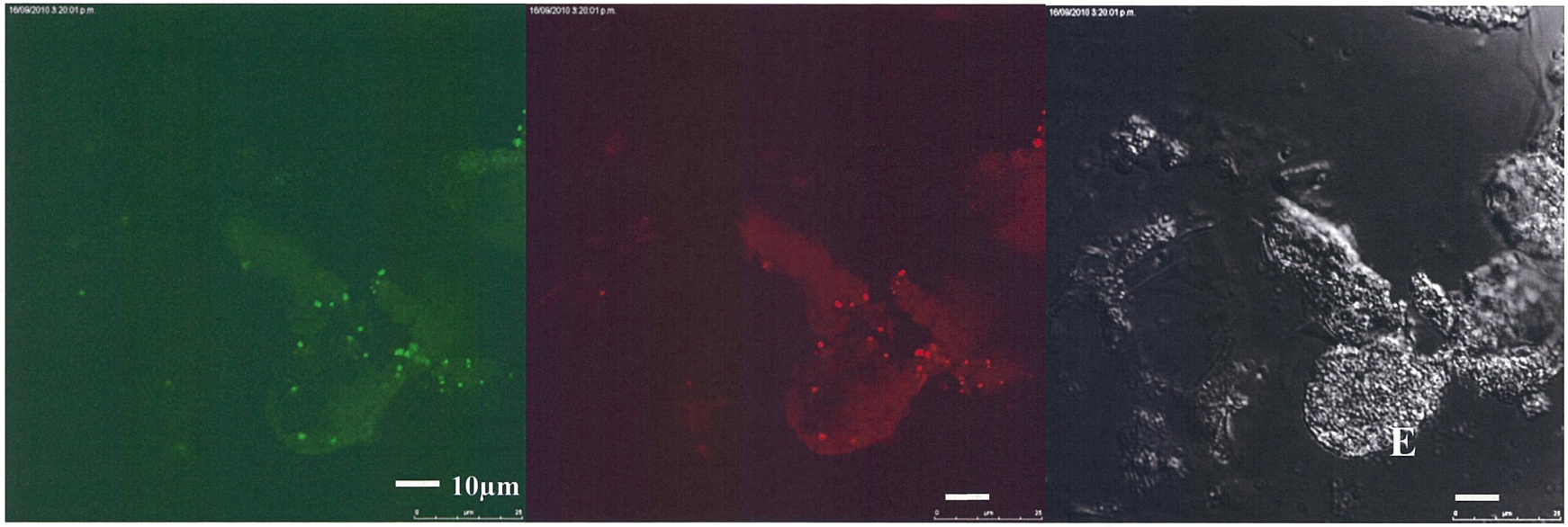

Fig. 6.8. Bacteria in *H. contortus* eggs collected from sheep faeces. Bacteria were targeted by the EUB338 (Cy3 labelled, middle) and Lab158 (FITC labelled, left) probes. Right: phase contrast image. E: egg with developing embryo. The contrast of these images has been enhanced for printing.

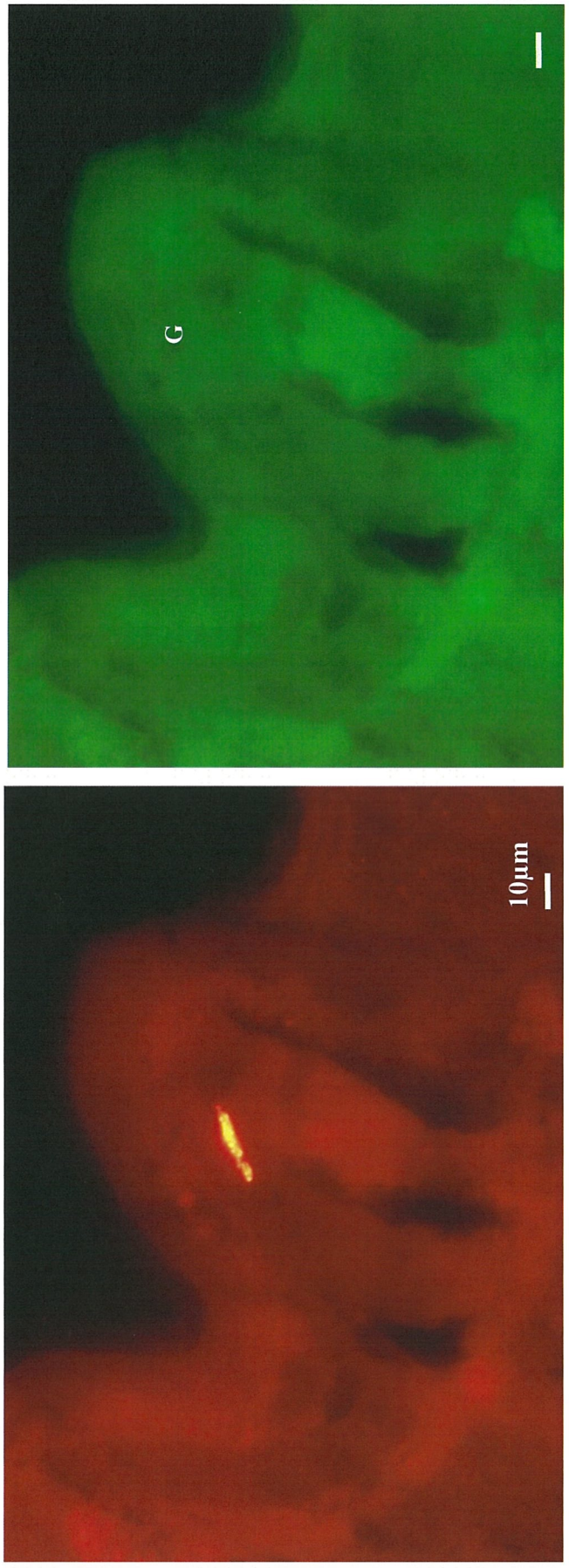

Fig. 6.9. Bacteria in the gut of a female *H. contortus*. Bacteria were targeted by EUB338 (Cy3 labelled, left), but not by Strc493 (FITC labelled, right). G: gut.

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Fig. 6.10. Bacteria in the gut of a female *H. contortus*. Bacteria (white arrow heads) were targeted by EUB338 (FITC labelled, left) and some of them by Strc493 (Cy3 labelled, right). G: gut. The contrast of these images has been enhanced for printing.

6.3.2.1.2. In the uterus but outside the eggs

The Strc493 probe, which targets most *Streptococcus* sp. and some *Lactococcus* sp., hybridised with the bacteria residing outside the eggs, but within the uterus. The worm anatomical structures were identified in phase contrast images of the same sections. All of these bacteria could be hybridised with the Strc493 probe, as there was no discrepancy in the numbers of EUB338 and Strc493 signals (Fig. 6.6). These bacteria did not hybridise with the lactic acid bacterial group specific probe (Lab158 probe) (Fig. 6.7). The three class-specific Proteobacteria probes did not target these bacteria.

6.3.2.2. Bacteria in laid eggs

The Lab158 probe, which targets some of the lactic acid bacteria (Table 6.3), hybridised with the bacteria within eggs laid *in vitro* and from faeces. The numbers and pattern of the signals from EUB338 and Lab158 were identical, therefore eggs did not contain bacteria other than those which could be targeted by Lab158 (Fig. 6.8). Neither the Strc493 probe nor the three class-specific Proteobacteria probes targeted bacteria in laid eggs.

6.3.2.3. Gut bacteria

Bacteria in the male and female gut were hybridised by the EUB338 probe (Fig. 6.9), although in many of the worm sections there were no FISH signals in the gut. This occurred whether or not the worms had been recovered from agar or directly collected from the abomasal mucosa.

The Strc493 probe, which targets most *Streptococcus* sp. and some *Lactococcus* sp., hybridised with the gut bacteria in 3 worm sections (Fig. 6.10), whereas more often (>20 sections out of 70) the EUB338 probe, but not the Strc493 probe, hybridised with gut bacteria. Neither the Lab158 nor Proteobacterial class specific probes targetted the bacteria in the worm gut.

Table 6.6. Summary of the specificities of the probes used in fluorescence *in situ* hybridisation and the locations of the bacteria identified in *H. contortus*.

FISH probes	Specificity	Gut bacteria	Bacteria within uterus	Bacteria within the eggs
EUB338	Most bacteria	✓	✓	✓
Lab158	Lactic acid bacteria	✗	✗	✓
Wgp	*Weissella* sp.	✗	✗	✓
S-G-Wei-0121-a-S-20	*Weissella* sp.	✗	✗	✓
Strc493	Most *Streptococcus* sp. and some *Lactococcus* sp.	✓ and ✗	✓	✗
Beta1	Betaproteobacteria	✗	✗	✗
SteMal_439	*Stenotrophomonas maltophilia*	✗	✗	✗
ALF73a	Some Alphaproteobacteria	✗	✗	✗

✓ detected ✗ not detected

6.3.2.4. Bacteria in L3

There were no positive FISH bacterial signals observed in L3 sections. The background fluorescence (in no probe control) from material adjacent to the L3 and their cuticles were so strong that true FISH signals could not be distinguished from false positives.

6.4. DISCUSSION

Bacteria were identified in three locations within adult worms: inside eggs, within the uterus and in the gut (summarised in Table 6.6). The bacteria in the eggs were targeted by both the lactic acid- and *Weissella* species-specific probes, whilst those in the uterus were targeted by the probe specific to most *Streptococcus* spp. and some *Lactococcus* spp. Close relatives of *Weissella* spp. were found within eggs, including those with morulae, in female worms and in eggs collected from the faeces and laid *in vitro*; this could indicate the lactic acid bacteria are maternal transmitted.

6.4.1. Probe specificities and optimal hybridisation stringencies

Optimisation of hybridisation stringency of the bacterial group-, class-, species-specific probes against reference bacterial cultures showed that, at optimal hybridisation stringency, all probes, except Strc493, were very specific for the target bacterial species. Strc493 hybridised with only one of the non-target species tested (*Leuconostoc citreum*). In all experiments, inclusion of no probe and nonsense (Non-EUB338) probe controls confirmed that signals observed with the EUB338 and bacterial group-, class- and species-specific probes were not autofluorescence or non-specific binding of the probes respectively.

6.4.2. Proteobacteria

Although bacterial sequences belonging to Alpha-, Beta- and Gammaproteobacteria were identified in all three life cycle stages of *H. contortus* in clone library and DGGE band sequences, the class-specific probes (ALF73a, Beta1, and SteMal_439) did not identify any of these bacteria in *H. contortus*. These sequences retrieved from *H. contortus* could either be from bacteria externally associated with the worm or located in the worm gut and removed during sample processing. Bacteria belonging to phylum Proteobacteria are ubiquitous and present in soil, faeces (Kersters et al., 2001) and the rumen (Kim et al., 2011), so it is not surprising to find these bacterial sequences in clone libraries from *H. contortus*.

6.4.3. Gut bacteria

The presence of gut bacteria were confirmed from their hybridisation by the eubacterial probe (EUB338) in adult worms. Their identity could not be determined, as apart from the Strc493 probe, none of the bacterial class-specific or species specific probes used in this study hybridised with the gut bacteria. In a few sections, but not others, both the EUB338 and Strc493 probes hybridised with gut bacteria. This suggests that some of the gut bacteria can be identified by the Strc493 probe, which hybridises with most *Streptococcus* sp. and some *Lactococcus* sp. The gut in many of the worm sections contained no bacteria which could be the result of the agar recovery process. Worms may have taken the agar in through the mouth and continuing gut motility caused emptying of gut contents out through the anus.

6.4.4. Bacteria in eggs

Eggs in the female worms contained bacteria which were very close relatives of *Weissella confusa*. These bacteria could be hybridised by the lactic acid bacterial group-specific probe Lab158, as well as by *Weissella*

sp. specific probes (Wgp and S-G-Wei-0121-a-S-20). These bacterial species continued to be seen within the egg after laying by female worms and could be detected by Lab158 in the eggs collected in faeces. The *Weissella* species- specific probes (Wgp and S-G-Wei-0121-a-S-20) were not tested on laid eggs. Not all the bacteria residing in the eggs could be targeted by *Weissella* species-specific probes, because there were more EUB338 signals than that from Wgp and S-G-Wei-0121-a-S-20 (Figs 6.3-6.4), whereas Lab158 could target all the bacteria within the eggs. These results suggest the possibility of co-existence of closely related bacterial species which belong to the lactic acid bacterial group.

The bacteria had a scattered distribution throughout the *H. contortus* egg ooplasm, as there were either individual or small clusters of signals. This was evident when the bacteria were viewed at different focal planes in the confocal microscopic images (Fig. 6.1). The morphology of these bacteria was either coccoid or diplococcoid (Figs 6.2-6.5), which is consistent with TEM images of the bacteria in the eggs.

The possibility of maternal transmission of these bacteria is indicated by the Lab158 and *Weissella sp.* specific probes (Wgp and S-G-Wei-0121-a-S-20) targeting bacteria within eggs in female worms and in laid eggs. In addition, sequences belonging to the same bacterial species were identified in nearly full length (~1400bp) 16S rRNA gene sequences and short sequences (~190bp) from DGGE bands of all three life-cycle stages. Despite similar bacterial sequences being identified in L3, and male and female worms sharing identical DGGE band patterns, these bacteria were not able to be visualised in L3 or male worms by FISH. This may be due to dormancy of bacteria in L3 and male worms. In L3 sections, background fluorescence was so strong that true FISH signals could not be distinguished from false positives. The location of bacteria in sections of L3 and male worms remains unclear.

6.4.5. Bacteria in the uterus

The coccoid or diplococcoid bacteria within the uterus are close relatives of either *Streptococcus* sp. or *Lactococcus* sp., as they were targeted by the Strc493 probe. The Strc493 probe was not able to be combined with other species-specific probes because of different optimal hybridisation stringency conditions. The bacteria detected in the uterus were not the same as those present in eggs, as in separate FISH experiments, none of the probes Lab158, Wgp and S-G-Wei-0121-a-S-20 hybridised with bacteria within the uterus. These bacteria were not present throughout the length of the uterus, but mainly seen along the wall of the uterus and between developing eggs in the distal parts of the uterus.

Chapter 7

General Discussion

Internal parasitism is a major cause of production losses in sheep-producing countries, such as Australia and New Zealand, and has been estimated to cost several hundreds of millions of dollars annually (Leathwick et al., 2001; Vlassoff et al., 2001). The main internal parasites in New Zealand are roundworms, of which there are 29 species in New Zealand livestock (West et al., 2009). Farmers continue to depend heavily on anthelmintics for effective control of internal parasites, however, resistance to these chemicals has been on the rise in New Zealand since 1980 (Pomroy, 2006). Other control measures are becoming increasingly important such as grazing management, breeding parasite resistant sheep, biological control using fungi and the use of natural compounds. A novel approach could be biocontrol using bacteria pathogenic to nematodes, such as *Bacillus thuringiensis*, which has been used to control insects (Siegel, 2001) and has shown promising results against nematode parasites (Kotze et al., 2005; O'Grady et al., 2007; Linares et al., 2008).

This project is the first research to identify the usual resident bacteria associated with *H. contortus*, with the long-term goal of manipulating them to control sheep gastrointestinal parasites. There is a precedent for this approach, in the use of antibiotics to control filariasis in humans by targeting their symbiotic *Wolbachia* (Hoerauf et al., 2003; Hoerauf and Pfarr, 2007). Prior to the present work, bacteria associated with *H. contortus* and other gastrointestinal parasites of livestock were studied only by bacterial culture (Lacharme-Lora et al., 2009; Treonis, pers. com.). Bacteria have now been identified in the gut and reproductive tract of *H. contortus* using genetic fingerprinting methods, FISH and TEM, but their roles in worm biology are yet to be established. The gut bacteria may be essential to the host, by providing nutrients such as haem, vitamins and cholesterol, or the bacteria in the reproductive tract may be

endosymbionts that manipulate reproduction of the nematode. Potentially, any of these bacteria may become targets to control the parasite.

7.1. Studying the community of bacteria associated with *Haemonchus contortus*

The first approach was the microscopic examination of sections of adult worms for the presence of bacteria. Previously, bacteria were observed in the gut of L3 (Reinhardt, personal communication). In the present study, bacteria were seen in the gut, uterus and eggs of adult worms using electron and light microscopy. The presence of bacteria in unexpected locations, such as the uterus and within eggs, suggests they could play important roles in worm biology. These bacteria may have evolved to a different life-style from those of free living relatives and developed an intimate relationship with the host worm.

Gut bacteria are present in almost all animals and their roles vary from mutualistic symbionts to fatal pathogens. *H. contortus* gut bacteria were both Gram-negative and Gram-positive and had a variety of morphotypes. Only Gram-positive bacteria were present in the uterus. This is the first time that Gram-postive bacteria have been reported in the nematode reproductive tract. Bacteria in the *H. contortus* eggs were of a different morphotype from those in the uterus, but it could not be determined whether they were Gram-postive or Gram-negative. Subsequently, by using DNA fingerprinting these bacteria were determined to be Gram-positive lactic acid bacteria, which are unlike other endosymbionts of nematodes. The endosymbiotic *Wolbachia* in filarial nematodes (Fenollar et al., 2003) and the *Candidatus* in the plant parasitic nematodes *Xiphinema americanum*, *X. rivesi* and *X. brevicollum* (Coomans et al., 2000; Vandekerckhove et al., 2000) are Gram-negative Proteobacteria.

Detailed information about bacteria can be obtained by genetic characterisation using molecular biological methods, rather than attempting to culture them, because many endosymbiotic bacteria are not able to be cultured (O'Neill et al., 1992; Stouthamer et al., 1993). Therefore, genetic fingerprinting techniques were chosen to analyse the bacterial communities associated with *H.*

Table 7.1. Archael primers used to amplify bacterial sequences.

Primer	Sequence	Reference
E334F	CCAGACTCCTAC GGGAGGCAGC	Rudi et al. (1997)
E926R	CCGICIATTIITTTI AGTTT	Watanabe et al. (2001)

contortus and compare them with their environments. DNA was extracted and bacterial sequences were amplified using universal bacterial primers in PCR assays. The Archael primers E334F and E926R (Table 7.1) were used to amplify archael bacterial sequences, but no PCR products were obtained from any of the three life cycle stages (data not presented). The PCR products were analysed by DGGE and bacterial sequences were used to construct phylogenetic trees. Based on the cumulative information of PCR-DGGE and phylogenetic analysis, group-class- and species-specific FISH probes were selected and particularly used to locate the bacteria within the reproductive tract, where there appeared to be two different morphotypes, one in the uterus and other in the eggs.

7.2. Comparison of bacterial communities of parasites and their environments

The bacteria associated with each parasite life-cycle stage could be transmitted horizontally from their respective environments or acquired at one of their life-cycle stages and retained during development (L3 to adult worms). Alternatively, the bacteria could be vertically transmitted within the parasites and be independent of their environment; this would be consistent with bacteria having been seen in eggs by TEM. Bacterial communities of eggs, adult worms and L3 and their respective environments were compared using PCR-DGGE analyses. All three life-cycle stages had similar DGGE patterns, suggesting that they may have similar bacterial communities, which differ from their environments. Bacterial sequences obtained from excised bands common to all three life-cycle stages were related to those of *Weissella* sp. and *Leuconostoc* sp.

Adult worms appeared to have a less diverse bacterial community than the abomasal contents; their bacterial community does not simply reflect abomasal contents. A direct comparison of the bacterial population of faeces with those of L3 and faecal eggs were not possible, as parasites and faecal material require different DNA extraction methods. The DNA extraction method optimised for parasite samples resulted in poor band separation of faecal samples in PCR-DGGE gels. Little information is available about ruminant faecal bacterial diversity but faecal bacterial diversity would be expected to be much greater than in L3 and eggs, based on the large numbers of bacterial species identified in

faeces and large intestinal contents of other mammals (Leser et al., 2002; Matsuki et al., 2002; Guo et al., 2008).

Proteobacterial sequences in the clone libraries are likely to be derived from sheep gut bacteria, or their DNA, attached to the surface of parasites. Proteobacteria are not a dominant bacterial phylum of rumen microbes (Kim et al., 2011). These bacteria are aerobic and *H. contortus* also needs aerobic conditions, so these bacterial species may be intimately associated with the parasites and contaminate the worm surface. It is unlikely that PCR amplification of parasite surface or environmental bacterial DNA can be avoided; however, bacteria were not seen on the cuticle by FISH or light microscopy. Cleaning adult worms with 4% sodium hypochlorite reduced the bacterial diversity (Fig. 3.7), probably by removing loosely adherent surface bacteria. Exsheathing L3 and washing with sodium hypochlorite proved effective in reducing bacteria on the cuticle (Fig. 3.6). Other bacterial sequences from both L3 and adult worms mainly belonged to the Gammaproteobacteria, whereas only adult worms contained sequences affiliated with the phylum Bacteriodetes, typical foregut bacteria of ruminants.

Live helminths actively remove cells (Badley et al., 1987; Spiegel and McClure, 1995), chemicals (López de mendoza et al., 2000), antibodies (Smith et al., 1981; Politz and Philipp, 1992; López de mendoza et al., 1999) and lectins (Page et al., 1992; Spiegel and McClure, 1995) attached to their surface by continuously replacing their cuticles. Bacteria could not be seen on the surface of the worms, using either LM or FISH. Therefore, it is unlikely that there are permanent bacterial communities on the surface of *H. contortus*, whereas the free living marine nematodes carry sulphur-oxidising ectosymbionts which provide nutrients to their host (Ott et al., 1991; Polz et al., 1992).

7.3. Gut bacteria

Gut bacteria were identified in *H. contortus* adult worms by light microscopy, TEM and FISH probes. LM images clearly showed that the gut contained Gram-positive and Gram-negative bacteria. In TEM images, these bacteria had multiple morphotypes and were present in the gut lumen and not attached to the

microvilli or in specialised cells known as bacteriocytes, which contain bacteria in insects (Moran et al., 1993; Douglas, 1998; Shigenobu et al., 2000; Akman et al., 2002). The EUB338 probe, which targets most bacteria, hybridised with the gut bacteria, but only some of these were targeted by Strc493, which targets most *Streptococcus* sp. and some *Lactococcus* sp. Neither the lactic acid bacterial group- nor the *Weissella* species-specific probes targeted any bacteria in the gut.

The gut of adult worms may be colonised either by bacteria in abomasal contents or bacterial species unique to the worms. Interestingly, using the universal bacterial primer pair 27f and 1492r, the most frequently identified bacterial species belonging to the phylum Proteobacteria in adult and L3 was *Mesorhizobium* sp. These bacteria are agriculturally important soil and rhizosphere bacteria (Kaneko et al., 2000) and may have been become associated with the free living stages (L1 or L2) of the parasite, which feed on bacteria. These bacteria may colonise the larval gut and remain in the dormant, non-feeding L3 stage that is able to survive for a long period in harsh environmental conditions. During this dormant stage, gut bacteria may provide nutritional support to their host and subsequently survive in L4 and adult worms.

Bacterial sequences belonging to the phylum Proteobacteria were identified in all three life-cycle stages of the parasite. Protebacteria are present in many environments (Kersters et al., 2001), including the invertebrate (Zouache et al., 2011) and vertebrate (Leser et al., 2002) gut. In this study, these bacteria were not located in *H. contortus* using the class-specific Proteobacterial FISH probes, suggesting they were present either on the worm surface or in the gut and removed during agar recovery and preparation for FISH. Although not detected by FISH probes, even a small amount of DNA from these bacteria could have been detected by PCR, especially if the primer pair preferentially amplified bacterial sequences from the phylum Proteobacteria over the phylum Firmicutes.

7.4. Bacteria in the uterus

The numerous bacteria in the uterus of female *H. contortus* appear to be close relatives of either *Lactococcus* sp. or *Streptococcus* sp., as they were targeted by

the FISH probe Strc493 and also shown to be Gram-positive bacteria in LM images. In TEM images, they were a single morphotype smaller than those within the eggs. Despite their presence in large numbers, particularly in the distal part of the uterus, the adult female worms appeared to be healthy (normal in size, active while they live) and carried normal eggs within them. Therefore these bacterial species seemed to be non-pathogenic to their host and the role of these bacteria is unknown.

The possible routes of entry of those bacteria in the uterus could either from the environment (abomasum) or transmitted by male worms during mating, after which they become resident of the uterus. Alternatively, they could be maternally transmitted on the outside of the eggs. Unusual ways of maternal transmission are known in insects, such as the stinkbug (*Megacopta punctatissima),* which harbours extracellular symbiotic Gammaproteobacteria in the midgut cavity (Hosokawa et al., 2005). These bacteria are deposited with the eggs on plants in "symbiont capsules", are eaten by the newly developing nymphs and colonise the midgut of the insect (Nikoh et al., 2011). Likewise, in *H. contortus*, the bacteria in the uterus could be transmitted on the eggshells, acquired by developing larvae and later colonise the uterus during worm development.

The bacteria found in the uterus of the *H. contortus* appear not to be pathogenic to the worms. The worms may simply be bacterial vectors. Some *Streptococcus* sp. are opportunistic pathogens, while others are commensal bacteria in animals and humans (Lacharme-Lora et al., 2009). The human gastrointestinal pathogen *Salmonella enteritidis* (Bäumler et al., 2000; Reporter et al., 2000; 2003) is well known to be vertically transmitted via chicken eggs (Timoney et al., 1989; Shivaprasad et al., 1990), as well as horizontally by nest boxes, hatchery trucks or hatchery environments (Goren et al., 1988; Cox et al., 1990). Penetration of *Salmonella enteritidis* into the chicken eggs could take place in the chicken reproductive track in the ovary (Baskerville et al., 1992) or in oviduct (Timoney et al., 1989). The chicken egg shells also have been known to carry *Salmonella enteritidis* (Cox et al., 1990; 1991). The bacteria in the uterus of *H. contortus* may be transmitted similar manner to *Salmonella enteritidis.*

7.5. Bacteria in eggs

Bacteria closely related to *Weissella confusa* were identified in PCR-DGGE short sequences and clone libraries of nearly full length sequences from all three life-cycle stages. Bacteria were seen in eggs in female worms in TEM images and were targeted by lactic acid bacterial group- and *Weissella* species-specific probes. The bacteria were dispersed throughout the eggs at different focal planes in confocal microscopy (Fig. 6.1). DNA fingerprinting and visualisation of these bacteria in eggs strongly suggest they are maternally transmitted endosymbionts.

Other bacterial species were also associated with eggs and are probably carried on the egg surface. Bacterial sequences belonging to the genus *Clostridium* were prominent in eggs collected from faeces. This is not surprising, as *Clostridium* sp. are mammalian gut residents (Finegold et al., 1983; Leser et al., 2002; Eckburg et al., 2005; Rajilić-Stojanović et al., 2007) and could contaminate faecal eggs. Bacterial sequences belonging to *Sternotrophomonas* sp. were dominant in the clone library of eggs laid *in vitro*. These bacterial species associated with eggs did not appear to have a major influence on the PCR-DGGE analysis, but possible preferential amplification of these bacterial sequences influenced the composition of the clone library.

Primer biases are common, such as preferential amplification (Polz and Cavanaugh, 1998; von Wintzinerode et al., 1997), the lack of amplification of certain templates (Skillman et al., 2006; Pei et al., 2008) and the variability of the efficiency of PCR amplification in different environmental samples (Juottonen et al., 2006). The primer pair 27f and 1492r may have preferentially amplified bacterial sequences belonging to the phylum Proteobacteria in adult worms, the genus *Clostridium* in eggs collected from faeces and *Sternotrophomonas* sp. in eggs laid *in vitro*. This issue was overcome by construction of a clone library using 27f and the phylum Firmicutes-specific primer1040firmR.

7.6. *Weissella* and *Leuconostoc*

Finding bacteria closely related to *Weissella* and *Leuconostoc* sp in nematode parasites of sheep is unexpected. Naturally occurring *Weissella* and *Leuconostoc*

sp. belong to the same family in the lactic acid bacterial group. In a comparative 16S rRNA analysis, *Weissella* was proposed as a new genus of *Leuconostoc*-like organisms (Collins et al., 1993). Currently, the genus *Weissella* contains 16 validly described species (List of Prokaryotic Names with Standing in Nomenclature, www.bacterio.cict.fr, 26-08-2012). The ecological niches of both genera vary from fresh vegetable, fermented foods (Dellaglio et al., 1984) to meat products (Collins et al., 1993). *Weissella confusa* is considered to be a non-pathogenic organism which can be isolated from food and clinical samples (Bjorkroth et al., 2002).

Weissella confusa may be an opportunistic pathogen transmitted by the nematodes. It was identified and then characterised phenotypically and genetically (16S rRNA sequences) from a person suffering from severe infective valvular endocarditis (Shin et al., 2007). Other reports are of systemic infection in a mona monkey (Vela et al., 2003) and fatal endocarditis in human patients (Flaherty et al., 2003). Similarly, the *Weissella* sp. associated with *H. contortus* could be an opportunistic pathogen to other species, although, there appear to be no reports of *Weissella confusa* causing disease in sheep or other livestock. *Weissella ciberia* was among the bacteria isolated from clinical samples from a dog suffering from otitis (Bjorkroth et al., 2002). At this stage, it is not possible to classify the *Weissella* sp. associated with *H. contortus* as a pathogen or a mutualistic symbiont to the worm.

7.7. Symbionts of *Haemonchus contortus*

In this study, symbionts (organisms closely associated either internally or externally with a host organism) have been found in the reproductive tract as well as in the gut of *H. contortus*. The gut bacteria were not analysed in detail as the major focus of the research was the genetic characterisation of bacterial symbionts in the reproductive tract. Those in the reproductive tract were of two different morphotypes and their sequences matched to unrelated type species in the phylum Firmicutes. These bacteria appeared to have established close relationships with the laboratory strain of *H. contortus*, as they were seen in worms over the whole duration of the study and parasites from many infected sheep. The bacteria found in the uterus are not classic maternally transmitted

endosymbionts, as they were not present within the eggs, but could possibly be transmitted on the eggshell. Those bacteria in the eggs, that are closely related to *Weissella confusa* could be vertically transmitted symbionts, which may manipulate worm biology similar to bacteria in filarial (Taylor and Hoerauf, 1999; Hoerauf and Pfarr, 2007) and plant parasitic nematodes (Coomans et al., 2000; Vandekerckhove et al., 2000).

The lactic acid bacterial group-specific probe (Lab158) was able to target all the bacteria in the eggs, whereas neither of the *Weissella* species-specific probes hybridised with all of these bacteria. This suggests the co-existence of different but closely related bacteria within eggs. During adaptation to endosymbiosis, the size of symbiotic bacterial genomes decreases, as non-essential genes are lost, while essential genes are retained in the new environment (Moya et al., 2008). The bacterial populations in the *H. contortus* eggs could be at the transitional stage between free-living and endosymbiotic bacteria, so that gene sequences are evolving in some of the bacteria in the population. The *Weissella* species-specific probes hybridised with the highly variable regions, which may be evolving and varying slightly within the bacterial population. In contrast, Lab158 targets a conserved region in the 16S rRNA gene of most of the lactic acid bacterial group; this region may not be evolving at this time. Some of the *Weissella confusa* sequences identified in *H. contortus* clone libraries did not contain the sequence that could be targeted by the *Weissella* species-specific probes. This would explain why some of the bacteria in the eggs were not detected by these probes, whereas all of the *Weissella* and *Leuconostoc* sequences identified in *H. contortus* clone library had the target region for Lab158.

Although, the exact roles of these bacteria in worm biology are not known, it appears that they are truly associated with the worm reproductive system. Bacterial species found in the reproductive tract are not restricted to an organ called a bacteriome, but dispersed throughout the tissue. This result suggests that new groups or types of potential symbionts are associated with *H. contortus*.

7.8. Future work

This first study of bacteria associated with ruminant gastrointestinal parasites has identified the presence of possible symbionts in *H. contortus* nematodes, but leaves many questions unanswered. It would be useful to identify bacteria in the gut and determine their contribution to the nutrition of their host.

The presence of *Weissella*-like organisms in eggs is an interesting finding in this project, particularly if they are shown in the future to manipulate worm reproductive biology (Section 1.2.2). *Wolbachia* are essential for filarial development (Taylor and Hoerauf, 1999; Hoerauf and Pfarr, 2007), whereas in arthropods endosymbionts skew the male to female ratio towards dominance by females (Stouthamer et al., 1999). During the *H. contortus* adult worm recovery process from the abomasum, it was observed that female worms outnumbered male worms, which could be related to the presence of bacteria in eggs. It may be possible in the future to control nematode parasites of sheep by manipulating their bacterial endosymbionts

In the reproductive tract, bacteria were located in eggs (*Weissella* sp.) and the uterus (*Streptoccocus* sp. and/or *Lactococcus* sp.). Neither of these bacterial species could be seen in L3 or adult male worms using FISH probes, so it is unknown where the bacteria are located in any of the larval stages and the routes the bacteria take to reach the eggs in female worms. Whole genome sequencing of these bacteria should clarify whether they are free living organisms being transmitted by nematodes or becoming obligate endosymbionts. Comparing their genomes with those of their free living relatives would provide an opportunity to study the evolution of free living organism to endosymbiont (Shigenobu et al., 2000; McCutcheon and Moran, 2007).

This study was carried out on a parasite strain which was originally obtained from the field and maintained in the laboratory for 20 years. Any practical applications of this research would depend on these bacteria being present in field strains of *H. contortus*. There may be similar symbionts in other gastrointestinal nematodes parasites which could be exploited as a mean of parasite control.

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Appendix 1: SAMPLES COLLECTED FROM EXPERIMENTAL SHEEP

		Sheep																								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Adults	Both sexes																										
	Female																										
	Male																										
Larvae																											
Eggs	Faecal eggs																										
	In vitro laid eggs																										
Clone Library	27f and 1492r														A ME L	A		A L			A ME FE L	A	A	A ME FE L	A ME FE L	A ME FE L	A
Abomasal contents																											
Parasites used for FISH																A	A			A ME FE L	A ME FE L	A	A	A			
Worms used for LM																											
Worms used for EM																											

Samples used for optimisation of DNA extraction, PCR conditions and PCR DGGE, samples used after optimisation of DNA extraction method, PCR conditions and DGGE conditions, samples were not taken from these sheep and worms used for FISH, EM and LM.
A: adult worms. ME: in vitro laid eggs, FE: eggs collected from faeces and L: L3.

Appendix 2: PARASITOLOGY

2.1. Faecal egg flotation

1. 2g of faeces were ground in 10ml saturated saline solution and transferred into a narrow necked 50ml bottle.
2. The bottle was filled to the top with saturated saline solution and covered with a cover slip and left for 4-5min
3. The cover slip was placed on a microscope slide and checked under the microscope for attached parasite eggs.

2.2. Infection of sheep with L3

1. Infective L3 were regularly passaged through parasite-free sheep to maintain cultures of pure strains of *H. contortus* or to recover adult worms.
2. Existing worm burdens were removed from sheep by drenching with a broad spectrum anthelmintic (Matrix, Merial Ancare, Auckland, New Zealand) on arrival (2ml/5kg) and again on the following day (1ml/5kg). Faecal floatation was performed after 2 weeks to ensure that sheep were free of nematode infection.
3. The motility of stored L3 was confirmed microscopically to be >95%. Sheep were each infected with 10,000 L3 *H. contortus* orally using a syringe. Infection was confirmed after 3 weeks by the presence of nematode eggs in a faecal flotation test.

2.3. Larval culture

Faeces were collected in faecal bags from Day 21 post infection onward.

2.3.1. Sieve method (A)

1. Faeces were mixed with vermiculite, moistened with water in a tray and incubated in a 22-24°C room for 10 days.
2. The mixture was moistened when necessary and turned over in the tray 3-4 times during the culture period.

3. L3 were recovered by a Baermann technique (Step E, Fig. 3.1). The faeces/vermicultive mixture was placed in sieves lined with tissue paper. Each sieve was submerged in a bowl of RO water and left for 24h. On the following day, the sieve was removed, the water containing L3 was poured through a 20μm sieve and the L3 and residual faeces were collected.
4. L3 were washed from the 20μm sieve into a kitchen sieve, lined with a single layer of tissue, sitting on a funnel attached to rubber tube with the end clamped. The water level in the funnel was adjusted to touch the bottom of the sieve. The Baermann apparatus was left overnight.
5. The following day, L3 which had settled at the bottom of the rubber tube were collected on a 20μm sieve and L3 were washed with copious amounts of RO water. L3 on the sieve surface were washed off into a clean 250ml beaker. If the L3 were mixed with traces of faecal slurry, steps 4-5 were repeated.

2.3.2. Tray method (B)

1. Faecal pellets were emptied into medium or large trays and evenly distributed by shaking. The pellets were irrigated with tap water using a squeeze bottle until the pellets were shiny. The tray was gently shaken and the inside of the tray was washed with water in a squeeze bottle.
2. This step was repeated daily for 7-10 days, until the L3 formed a beige–pink ring just above the level of the faeces.
3. The Baermannising apparatus consisted of a large tray containing an inner mesh-bottomed tray covered with a wet single layer of paper towel. The faecal material was washed thoroughly into the apparatus. More water was added to cover completely the faecal pellets on the mesh-bottomed tray. A second mesh-bottomed tray was placed on top and covered with a lid or another tray. The apparatus was left overnight.
4. The lid and two mesh-bottomed trays were lifted up together, placed on the lower tray and left for 1h to drain the water off the faecal material. The water was poured into a 5 litre glass beaker and the tray was rinsed thoroughly. The beaker was left at 10°C overnight to settle the L3 on the bottom.

5. The upper half of the water was decanted off and the beaker was topped up with tap water and again L3 were left overnight to settle. This step was repeated 2-3 times or till the water became clear.
6. L3 were then recovered as described above in steps 4-5 of method A (Appendix 2.3.1).

2.4. Recovery of adult worms

1. The ligated abomasum was removed from the donor animal immediately after death.
2. The abomasum was opened and the contents emptied on to a tray and the luminal surface of the mucosa was washed with warm 0.9% saline.
3. The washings were mixed with the abomasal contents and decanted into a graduated beaker and the volume was determined.
4. A 3% agar solution was made on the same day by dissolving 30g of agar (Bacto Agar, DIFCO Laboratories, MD, USA) in 0.9% saline and heating for 8-10min in a microwave oven until dissolved. The agar solution was allowed to cool to 37-40°C.
5. Abomasal washings were mixed 2:1 with warm 3% agar in a graduated beaker and thoroughly mixed together. This mixture was poured to a 1cm depth into trays and allowed to set.
6. Warm saline was poured on to the agar blocks to a height of 1-2cm. The trays were kept in a constant temperature room at 37°C for 10-15min.
7. The worms migrated out of the agar blocks into the saline and formed clumps. Clumps of worms were collected into saline for further cleaning and DNA extraction.

2.5. *In vitro* laid eggs

1. Female worms recovered from agar blocks (Appendix 2.4) were incubated overnight at 37°C in PBS in 15-20 Eppendorf centrifuge tubes (1.5ml) (approximately 15 female worms per tube).
2. The following day, the adult worms were removed and eggs collected from the bottom of the tubes were pooled and kept overnight at 4°C to settle.

3. Eggs were washed on a 20μm sterile sieve 4-5 times alternately with 4% sodium hypochlorite and ultrapure water. Finally, copious amount of ultrapure water was used to remove any trace amount of sodium hypochlorite
4. The eggs were aseptically transferred to a sterile centrifuge tube for DNA extraction.

2.6. Eggs extracted from faeces

1. Faeces were collected from infected sheep using faecal bags on Day 21 post infection. A faecal slurry was made in a small bucket by soaking faeces in water (0.5kg faeces in 1-2litres of tap water) for 2h and then faeces were mashed using a potato masher.
2. The faecal slurry was washed sequentially through a kitchen sieve, a 106μm mesh sieve, then a 20μm sieve, which retained the eggs. This last sieve was washed thoroughly with tap water until the effluent water was clear.
3. The particulate material retained on the top of the mesh was transferred into a 20cm diameter glass Petri dish by squirting with 10-20ml of tap water.
4. Saturated salt solution was added to the Petri dish to about 5mm from the top and a 14.5cm glass petri dish was placed upside down on it without making any air bubbles on top of the faecal/salt solution. The dish was left for 5min.
5. The 14.5cm glass petri dish on top of the faecal/salt was carefully lifted (the eggs will be attached to the surface). Using a squeeze bottle containing tap water, the eggs were washed from the under-surface of the dish through a 106μm sieve placed in a tray. The effluent containing the eggs was retained.
6. The petri dish lid was wiped thoroughly and steps 4-5 were repeated 2 times.
7. All three effluents were combined and poured through a 20μm sieve to collect the eggs. The eggs were rinsed thoroughly with tap water.
8. The 20μm sieve was reversed and the eggs washed off with tap water into a 50ml Falcon tube. The eggs were pelleted by centrifugation at 1000*g* for 2min.
9. The supernatant was discarded and the pellet was re-suspended in 50ml tap water and centrifuged at 1000*g* for 2min.
10. If the eggs contained any visible faecal material, steps 3-9 were repeated.

11. The supernatant was discarded and the egg pellet was re-suspended in 10ml MQ water.

Appendix 3: MICROSCOPY

3.1. Neutral buffered formalin

All chemical were purchase from (Ajax Finechem, Auckland, New Zealand)

To make 10litres, the following were mixed:

1. $NaH_2PO_4.2H_2O$	45g
2. Na_2HPO_4	65g
3. Formalin	1litre
4. Water	9litres

3.2. Gram Twort staining

3.2.1 Twort stain

1. 1% neutral red (BDH, Poole, England) in 9ml absolute alcohol
2. 0.2% fast green (CI42053) (BDH) in 1 ml absolute alcohol

9ml of 1% neutral red and 1ml of 0.2% fast green were added to 30ml of distilled water.

3.2.1 Gram Twort staining

1. Sections were de-waxed using a Leica Autostainer XL (Leica).
2. De-waxed sections were placed on a rack and covered with filtered 1% crystal violet (BDH) for 3min.
3. Slides were washed briefly (3-5sec) in water to remove excess crystal violet and then covered with Gram's iodine (BDH) for 3min.
4. Sections were decolourised with 100% acetone until no more colour comes out of the section (5-10sec).
5. Slides were washed in tap water for 30sec, covered with working Twort stain for 5min and then rinsed with tap water
6. Sections were differentiated in 2% acetic acid in ethanol for 10-20sec, dehydrated, cleared and mounted.

3.3. Nematode processing for TEM

1. Fresh worms were collected from abomasal contents, individually placed on glass slides and allowed to dry in a fume hood.
2. Worms were sliced with a clean surgical blade into 6-8mm lengths and placed for 3-5 days in a primary fixative consisting of 3% glutaraldehyde and 2% formaldehyde in 0.1M phosphate buffer ($Na_2HPO_4.12H_2O$ and KH_2PO_4), pH 7.2.
3. Specimens were washed with phosphate buffer 3 times at room temperature, then kept in 1% OsO_4 in phosphate buffer for 30 to 60min at room temperature.
4. Specimens were washed with phosphate buffer 3 times at room temperature
5. The specimens were dehydrated through a graded acetone series of 25%, 50%, 75%, 95% and 2X 100%. They were left for 10-15 min in each solution, except the last 100% acetone in which they were left for 1h.
6. Specimens were infiltrated with an acetone/resin mixture, 50/50 [v/v] on a stirrer overnight, then changed to fresh 100% resin and kept overnight on a stirrer. The following day, this step was repeated for another 8h on a stirrer.
7. Specimens were then embedded in fresh resin in silicone rubber moulds and cured at 60°C for 48h.
8. 0.5-1μm thick sections were cut for light microscopy using a diamond knife on a water surface.
9. The sections were picked up with a wire loop and placed on a water drop on a clean glass slide. The slide was heated to 90-95°C to flatten and stick the sections on to the glass slide.
10. Sections were stained on a hot plate (90-95°C) with 0.05% toludine blue in 0.1M phosphate buffer for 10-15sec.
11. Sections were washed and dried. A drop of immersion oil was added as a mounting solution and the section covered with a cover slip.
12. Sections were viewed in a light microscopy and an area of interest was chosen.
13. The area of interest was trimmed to 0.5mm square. 100nm thin sections were cut on an ultra-microtome using a diamond knife.
14. Sections were picked up on copper grids and double stained with saturated uranyl acetate in 50% ethanol in water (4min), followed by lead citrate (4min).

Appendix 4: DENATURING GRADIENT GEL ELECTROPHORESIS

All chemical were obtained from Sigma (St. Louis, USA) unless stated otherwise

4.1. Solutions

4.1.1. 50X Tris-acetate-EDTA (TAE buffer)

242g tris-acetate, 57.1ml glacial acetic acid and 100ml 0.5M EDTA (pH 8.0) were made up to 1 litre with MQ water. The solution was autoclaved and stored at room temperature.

4.1.2. Dcode dye solution

2% bromophenol blue	0.05g
2% xylene cyanol	0.05g
1X TAE buffer	10ml

4.1.3. 2X Gel loading dye

2% bromophenol	0.25ml
2% xylene cyanol	0.25 ml
100% glycerol	7ml
MQ water	2.5ml

4.1.4. Denaturing gradient solutions for a 6% polyacrylamide DGGE gel

Reagents	30% denaturing solution	45% denaturing solution	55% denaturing solution
Urea	3.15g	4.725g	5.77g
Formamide	3ml	4.5ml	5.5ml
40% acrylamide/bis	3.75ml	3.75ml	3.75ml
50X TAE	0.5ml	0.5ml	0.5ml
MQ water	14.6ml	11.54ml	9.48ml
Total volume	25ml	25ml	25ml

4.2. Gel casting

1. A set of glass plates, a gasket and spacers were thoroughly cleaned with 70% ethanol.
2. The plate was assembled with the gasket and spacers. The other plate was placed on top and both plates were held together with clamps.
3. 95µl of 10% ammonium per sulphate (APS) and 55µl of N, N, N, N'-tetra-methyl-ethylenediamine (TMED) (BIO-RAD, CA, USA) solutions were added to 25ml of each of the denaturing solutions.
4. 15ml of the low and high solutions were added to the respective columns of the gradient maker and the pump was run at a constant rate.
5. The mixture of gradient solutions was carefully pumped into the plates through a needle (21G) connected via a tube to the pump and gradient maker.
6. The comb was inserted between the two plates.
7. The gel was allowed to polymerise for 40min and then the comb was removed. The gel was used immediately or stored at 4°C for later use.
8. Excess acrylamide was washed with 1X TAE buffer into a biohazard waste bag before putting the gel in the tank.

4.3. Sample loading and electrophoresis

1. 25.5 litre of 1X TAE buffer was loaded into the electrophoresis tank.
2. The temperature was set to 60°C and the buffer allowed to heat.
3. The plates were clamped to the inner core assembly (another pair of glass plates was used to form the upper buffer chamber, if only one side of the gel were used).
4. Wells were thoroughly washed with 1X TAE buffer to remove excess urea.
5. A minimum of 300ng of PCR product, mixed with an equal volume of loading dye, was loaded into each well. The wells at the each side of the gel were not loaded to avoid smiling bands.
6. The inner core assembly was submerged in the electrophoresis tank, and the recirculation pipe was attached to fill the inner chamber with buffer.
7. The voltage and time were set for electrophoresis. After the electrophoresis, the plate assembly was disassembled and the gel was carefully detached from the plate.

4.4. Staining and de-staining the gel

1. The gel was briefly (1min) washed with 500ml MQ water and the water was drained off carefully.
2. The gel was immersed into 600ml of MQ water containing 3µl SYBR GOLD (Invitrogen) in a tray and left on a shaker in a dark room for 20min.
3. The MQ water was drained off carefully without breaking the gel. The gel was again immersed into 500ml of MQ water and kept overnight in a dark room to de-stain.

Table 5.1App. Summary of initial phylogenetic affiliations of bacterial sequences obtained from *H. contortus* adult worms, L3, eggs extracted from faeces (HEF) and laid *in vitro* (HEM). ~1400bp and ~1000bp were amplified using universal and Firmicutes-specific primers respectively (URB- uncultured rumen bacteria). The phylogenetic affiliations were obtained by comparing bacterial sequences from *H. contortus* with those in the GenBank database using the BLASTn option in the NCBI website.

Bacteria					Adult worms		L3		HEF		HEM	
Phylum	**Class**	**Order**	**Family**	**Genus**	**Sequence size (bp)**							
					~1400	**~1000bp**	**~1400**	**~1000**	**~1400**	**~1000**	**~1400**	**~1000**
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Lactococcus*	8				2		2	
				Streptococcus	3	1	1	1	1	7		
			Leuconostocaceae	*Leuconostoc*	2	5	1	2		1	3	6
				Weissella	3	2	4	2		4	5	6
			Lactobacillaceae	*Lactobacillus*				1				
		Bacillales	Staphylococcaceae	*Staphylococcus*							4	
	Clostridia	Clostridiales	Clostridiaceae	*Clostridium* III	2				3			
			Clostridiaceae	*Clostridium* XI					12			
			Veillonellaceae	*Veillonella*							1	
			Catabacteriaceae	*Catabacter*	2							
			Lachnospiraceae	*Butyrivibrio*	5				2			
		Total			**25**	**8**	**6**	**6**	**20**	**12**	**15**	**12**
Tenericutes	Mollicutes	Entomoplasmatales	Spiroplasmataceae	*Spiroplasma*					1			
		Total			**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**
Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	*Mesorhizobium*	15		15					
			Rhizobiaceae	*Rhizobium*	3							
			Hyphomicrobiaceae	*Devosia*			1					
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Achromobacter*			1					
				Bordetella			1					
				Pusillimonas			1					
			Comamonadaceae	*Acidovorax*							1	
				Comamonas			5					
				Delftia	1							
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Escherichia*			1		2		6	
		Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	2				8			
		Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*	2				2		12	
		Total			**23**	**0**	**25**		**12**		**19**	
Bacteroidetes	Bacteroidetes	Bacteroidales	Prevotellaceae	*Prevotella*	1							
				URB	7							
		Total			**8**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Total Sequences					**57**	**8**	**31**	**6**	**32**	**12**	**35**	**12**

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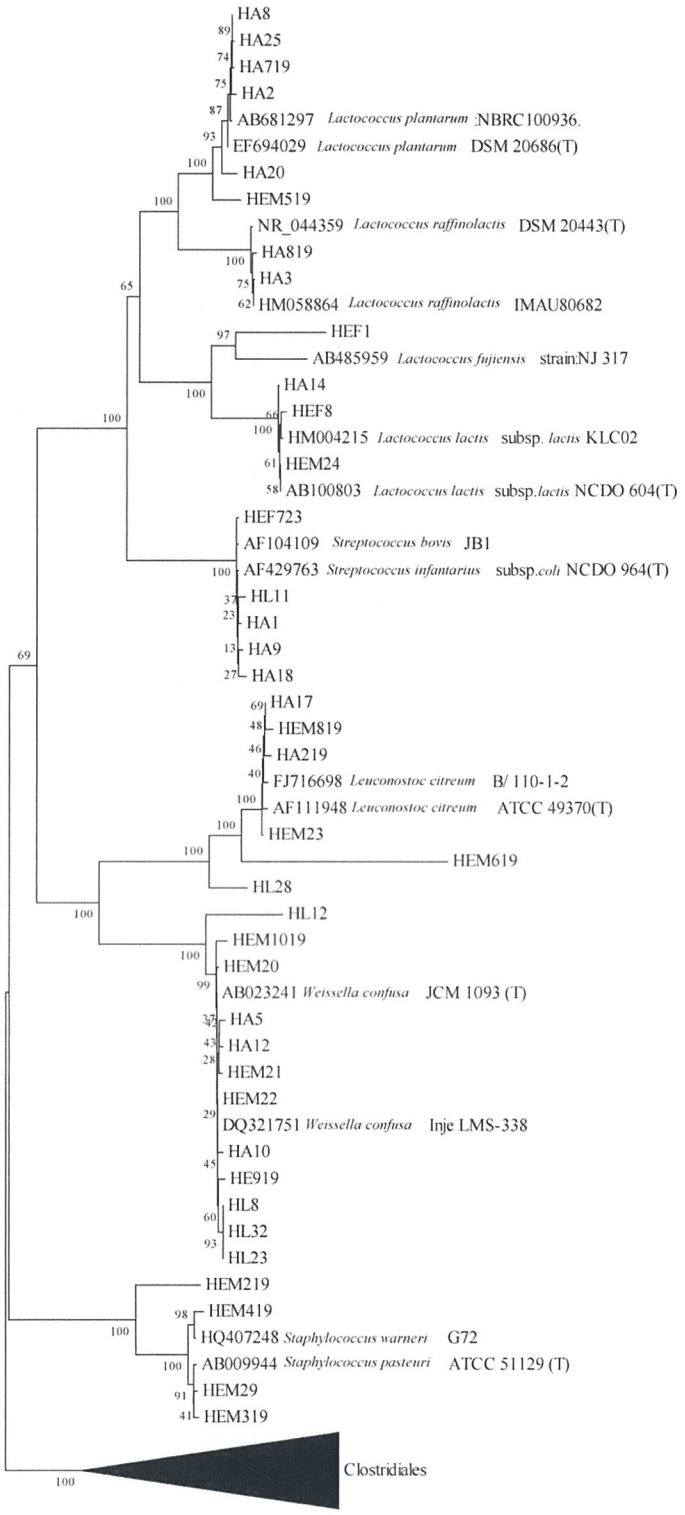

Fig. 5.1App. Phylogenetic tree based upon the neighbour joining method from bacterial 16S rRNA gene sequences obtained from *H.contortus* using the primer set 27f and 1492r and reference 16S rRNA gene sequences. GenBank accession numbers of reference sequences are given before the reference cultures, (T) designates a type strain. Bootstrap values are shown at each node (percent of 500 replicates). Sequences belonging to order Clostridiales were compressed and represented as a triangle in the dendrogram. HA: adult worms; HL: L3; HEF: eggs collected from faeces; HEM: laid *in vitro* eggs.

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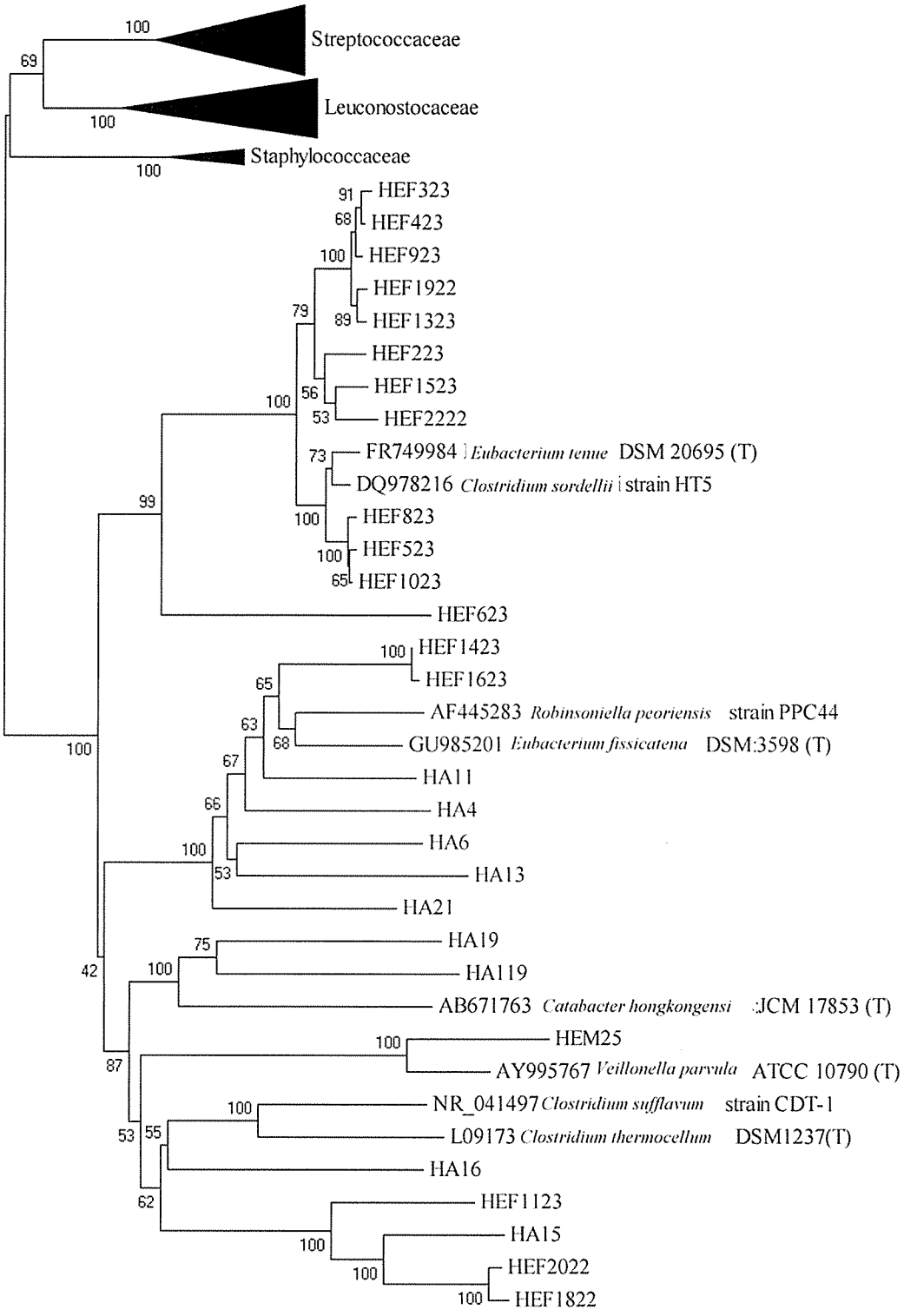

Fig. 5.2App. Phylogenetic tree based upon the neighbour joining method from bacterial 16S rRNA gene sequences obtained from *H.contortus* using the primer set 27f and 1492r and reference 16S rRNA gene sequences. GenBank accession numbers of reference sequences are given before the reference cultures, (T) designates a type strain. Bootstrap values are shown at each node (percent of 500 replicates). Sequences belonging to families Leuconostocaceae, Streptococcaceae and Staphylococcaceae were compressed and represented as triangles in this dendrogram. HA: adult worms; HL: L3; HEF: eggs collected from faeces; HEM: laid *in vitro* eggs.

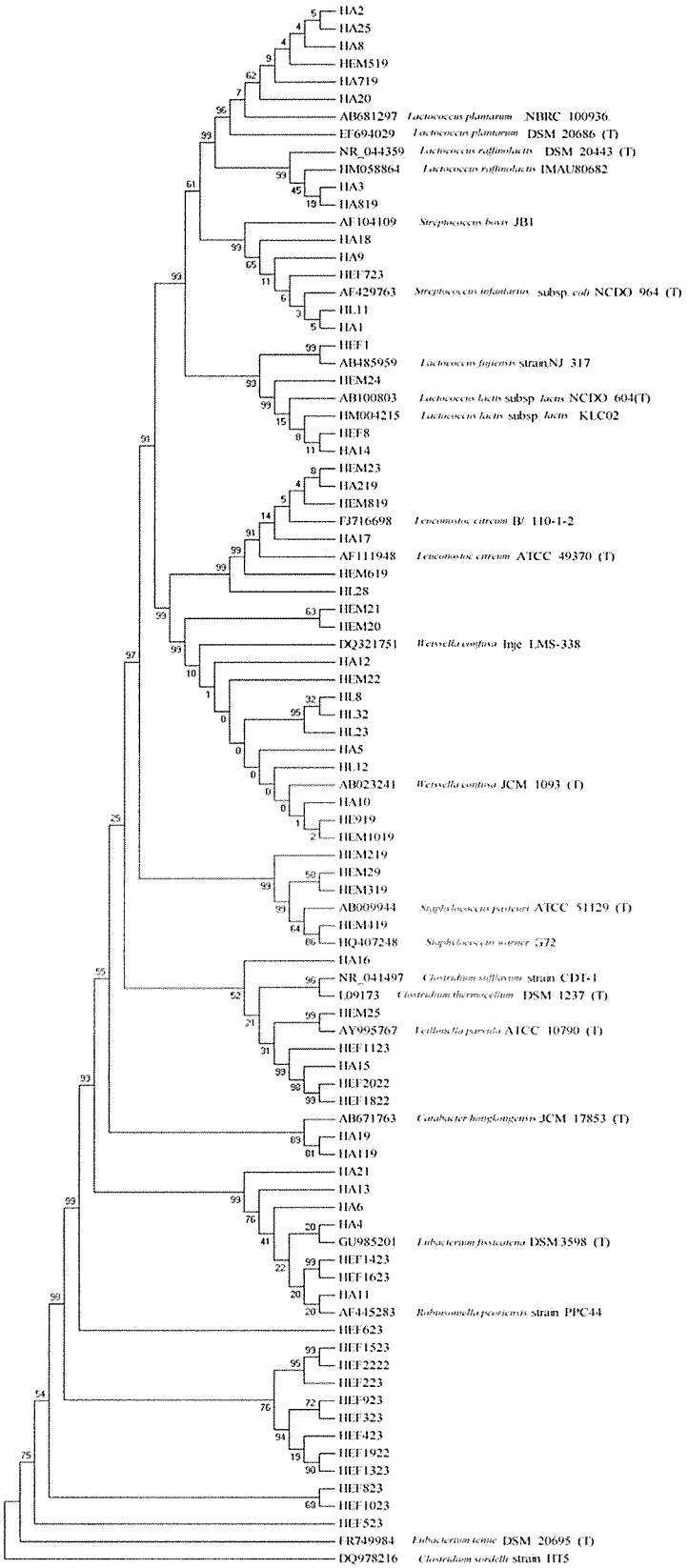

Fig. 5.3App. Phylogenetic tree based upon the parsimony method from bacterial 16S rRNA gene sequences obtained from *H. contortus* using the primer set 27f and 1492r and reference 16S rRNA gene sequences. GenBank accession numbers of reference sequences are given before the reference cultures, (T) designates a type strain. HA: adult worms; HL: L3; HEF: eggs collected from faeces; HEM: laid *in vitro* eggs.

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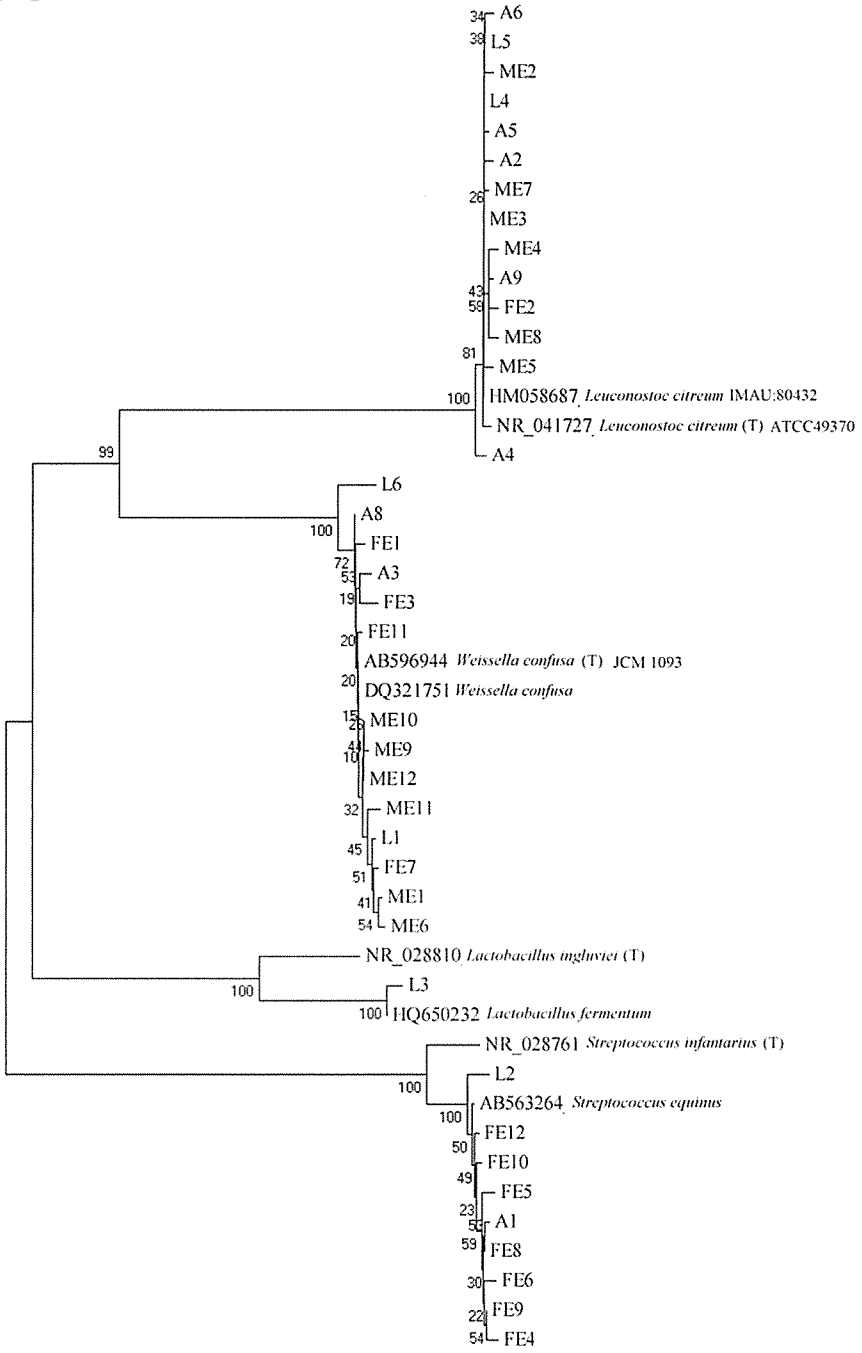

Fig. 5.4App. Phylogenetic tree based upon the neighbour joining method from bacterial 16S rRNA gene sequences obtained from *H. contortus* using the primer set 27f and 1040firmR and reference 16S rRNA gene sequences. GenBank accession numbers of reference sequences are given before the reference cultures, (T) designates a type strain. Bootstrap values are shown at each node (percent of 500 replicates). A: adult worms; L:L3; FE: eggs collected from faeces; ME: laid *in vitro* eggs.

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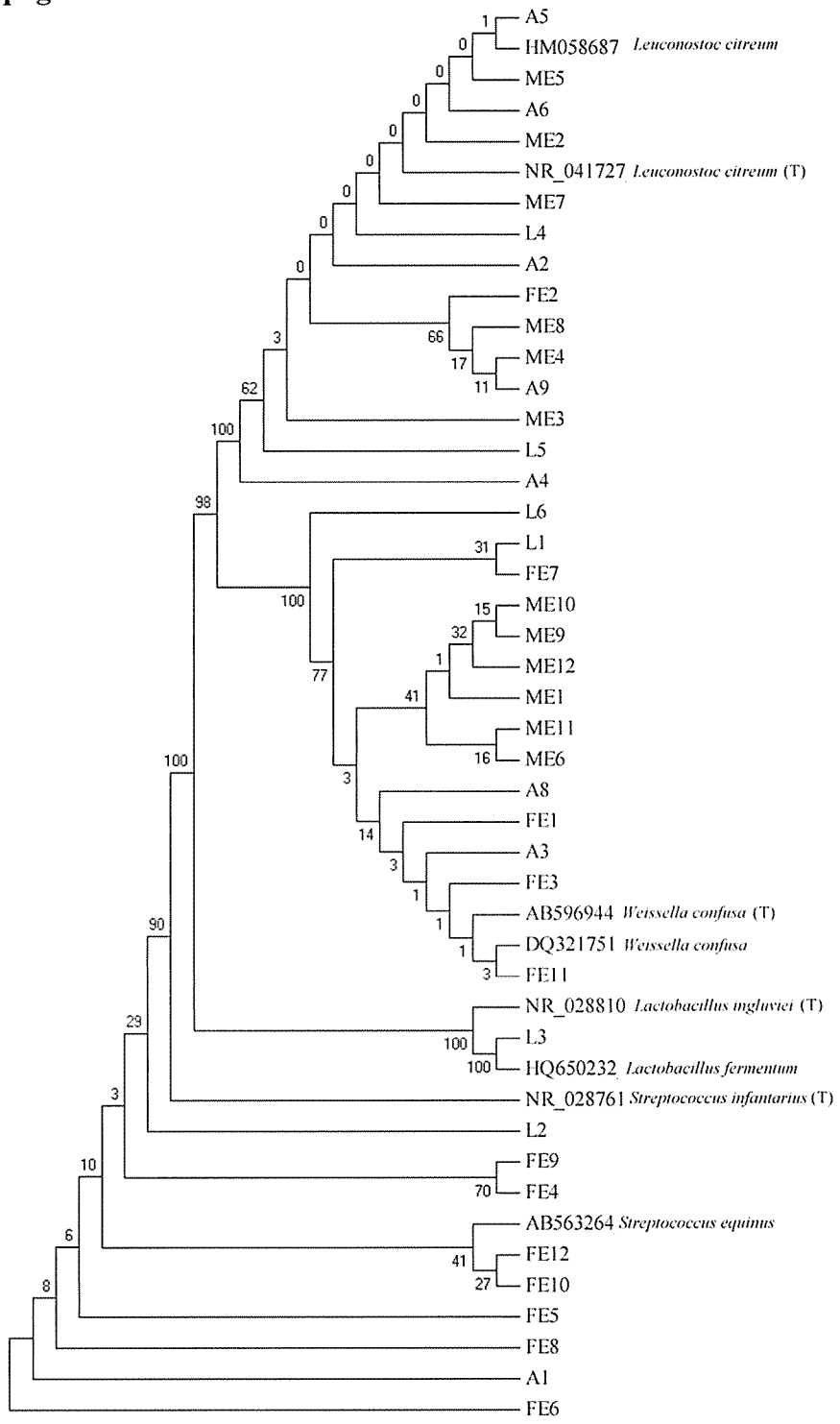

Fig. 5.5App. Phylogenetic tree based upon the parsimony method from bacterial 16S rRNA gene sequences obtained from *H. contortus* using the primer set 27f and 1040firmR and reference 16S rRNA gene sequences. GenBank accession numbers of reference sequences are given before the reference cultures, (T) designates a type strain. Bootstrap values are shown at each node (500 replicates). A: adult worms; L:L3; FE: eggs collected from faeces; ME: laid *in vitro* eggs.

Appendix 5: RESULTS OF PHYLOGENETIC ANALYSES

A summary of the initial BLASTn search results of bacterial sequences obtained from *H. contortus* is given in Table 5.1App.

Phylogenetic trees of bacterial sequences obtained from *H. contortus* using the neighbour joining and parsimony methods are shown in Figs 5.1 - 5.5App.

Table 6.1App. Formamide volumes for hybridisation buffer.

%Formamide	Formamide x (µl)	MQ water y (µl)
0	0	1598
5	100	1498
10	200	1398
15	300	1298
20	400	1198
25	500	1098
30	600	998
35	700	898
40	800	798
45	900	698
50	1000	598

Appendix 6: FLUORESCENCE *IN SITU* HYBRIDISATION

All chemical were obtained from Sigma (St. Louis, MO, USA) unless stated otherwise

6.1. Solutions

6.1.1. 4% Paraformaldehyde (PFA) solution

12g paraformaldehyde
195ml MQ water
2M NaOH
99ml 3X PBS

1. PFA was added to the MQ water and heated to 60°C on a hot plate in the fume cupboard.
2. Drops of 2M NaOH were added to the solution until it became clear. The container then was removed from the hotplate and PBS was added and cooled to room temperature. The pH of the solution was adjusted to 7.2.
3. PFA was filter sterilised using a 0.2μm filter (Sartorium Stedium, Goettingen, Germany). Aliquots of solutions were frozen at -20°C.

6.1.2. Hybridisation buffers

Hybridisation buffers were freshly prepared in 2ml microcentrifuge tubes (one tube of 2ml of buffer per slide) by adding in the following order:

360μl of 5M NaCl (final concentration 0.9M)
40μl of 1M tris-HCl (final concentration 20mM, pH 7.2)
*x*μl of 100% formamide (Table 6.1App)
*y*μl of autoclaved MQ water (Table 6.1App)
2μl of 10% SDS

Table 6.2App. NaCl concentrations of washing buffers according to formamide concentration in hybridisation buffer.

Percentage of hybridisation buffer	5M NaCl Volume z (µl)	Final NaCl concentration of wash buffer (M)
0	9000	0.900
5	6300	0.630
10	4500	0.450
15	3180	0.318
20	2150	0.215
25	1490	0.149
30	1020	0.102
35	720	0.070
40	460	0.046
45	300	0.030
50	180	0.018

6.1.3. Washing buffers

50ml of washing buffers, corresponding to each hybridisation buffer, was prepared in 50ml polypropylene tubes in the following order:

*z*µl of 5M NaCl (Table 6.2App)

1ml of 1M tris-HCl (final concentration 20mM, pH 7.2)

Autoclaved MQ water up to 50mL

50µl of 10% SDS (final concentration 0.01%)

6.2. Hybridisation of reference bacterial cultures

For probe evaluation, first a range of formamide concentrations was used in 10% increments from 0-40%. The optimal formamide concentration was refined by using smaller increments of formamide concentration. Along with the test probe, a nonsense probe (nonspecific binding) and no-probe control (autofluorescence) were included.

1. The hybridisation oven was pre-warmed to 46°C. Hybridisation buffers were freshly prepared in a 2ml microcentrifuge tube (Appendix 6.1.2).
2. 8µl of hybridisation buffer was added to each well containing sample on the slide.
3. A paper towel was folded into rectangle slightly larger than the slide. The paper towel was placed into a 50ml polypropylene tube; the remaining hybridisation buffer was poured onto the paper towel to prevent evaporation of buffer from the wells during hybridisation.
4. 8µl of hybridisation buffer was pipetted into each well. 0.5µl (50ng/µl) of each probe was added and mixed carefully with a filtered pipette tip without touching the cells. The slide was placed in the 50ml polypropylene tube containing the moistened paper towel. The cap was applied loosely and the tube placed horizontally in the hybridisation oven and incubated at 46°C for 3h.
5. During the hybridisation, 50ml of each washing buffer was freshly prepared corresponding to the hybridisation buffer (Appendix 6.1.3). Washing buffers were pre-warmed to 48°C in a water bath or oven.

6. Following hybridisation, using a pipette, wells were immediately rinsed with the corresponding washing buffer (warmed to 48°C) into the hybridisation tube. The slide was carefully removed from the hybridisation tube, placed into the tube containing the corresponding washing buffer and kept at 48°C for 10-15min. The slide was removed from the washing buffer and rinsed briefly in a beaker of ice-cold distilled water, and thoroughly dried (rapid transfer of slides during these steps prevents cooling which can lead to nonspecific probe binding).
7. The wells on the slide were covered with a thin film of Vectashield® anti-fading mounting medium for fluorescence (Vector Laboratories). A large cover slip was placed over the slide to cover all wells. The cover slip was gently pressed down to remove excess mounting medium and to stabilise the cover slip. The edges of the cover slip were sealed with nail polish.
8. The slides were observed using an epifluorescence (OLYMPUS BX61) or a confocal laser scanning microscope (Leica TCS SP5 DM 6000B), starting with the lowest formamide concentration and working upwards.
9. No-probe wells were observed in all available channels for autofluorescence of the sample. The nonsense probe wells were observed in the appropriate channels for probe fluorescence to confirm that no non-specific binding occurred.
10. The wells were observed in the appropriate channels, positive fluorescence signals were confirmed and the formamide concentration noted. The optimal stringency for the test probe was taken as the highest formamide concentration before specific hybridisation signal was decreased.

6.3. FISH on nematode samples

1. The hybridisation oven was pre-warmed to 46°C.
2. All slides were placed on a hot block at 100°C for few seconds (3-5sec) to melt the paraffin. Once the paraffin melted, all slides were immersed into xylene for 15 min and then transferred to 100 % ethanol and left for 15 min. These steps were repeated twice.

3. Hybridisation buffers were freshly prepared in 2ml microcentrifuge tubes (one tube of 2ml of buffer per slide) by adding in the following order:

    360µl of 5M NaCl (final concentration 0.9M)

    40µl of 1M tris-HCl (final concentration 20mM, pH 7.2)

    *x*µl of 100% formamide (Table 6.1App)

    *y*µl of autoclaved MQ water (Table 6.1App)

    2µl of 10% SDS

4. 50ml of washing buffers, corresponding to each hybridisation buffer, was prepared in 50ml polypropylene tubes in the following order:

    zµl of 5M NaCl (Table 6.2App)

    1ml of 1M tris-HCl (final concentration 20mM, pH 7.2)

    autoclaved MQ water up to 50mL

    50µl of 10% SDS (final concentration 0.01%)

    360µl of 5M NaCl (final concentration 0.9M)

5. A wetted paper towel with hybridisation buffer was placed in a medium size or large sealable hybridisation box. Circles were drawn using a Liquid Blocker-Super PAP pen (Daido Sangyo) around the nematode sections. Sufficient 8µl aliquots of hybridisation buffer were added to cover the whole nematode section on the slide.
6. For each 8µl of hybridisation buffer, 0.5µl of probe solution (50ng/µl) was added and mixed carefully with a pipette tip (avoiding touching the surface of the slide with the pipette tip as this will disturb the attached worm tissues). The lid was placed on the box without tightly closing. All washing buffer tubes were pre-heated to 48°C in the hybridisation oven.
7. Following hybridisation, the slide was rinsed with 48°C washing buffer into the hybridisation box, using a pipette. The slide was then placed into the pre-warmed washing buffer tube, and kept at 48°C for 10-15min. The slide was removed from the washing buffer, rinsed briefly in a beaker of ice-cold distilled water and thoroughly dried (rapid transfer of slides during these steps prevents cooling which can lead to nonspecific probe binding).
8. Thin layer of anti-fading mounting medium was applied to the worm tissue and covered with a large cover slip. The coverslip was pressed down gently to remove excess medium and to stabilise the cover slip. The edges of the cover slip were sealed with nail polish.

9. Slides were viewed under an epifluorescence or confocal laser scanning microscope.

Table 7.1App. Lab158 probe penetrability into target and non-target bacterial species, treated and not treated with lysozyme prior to hybridisation. Target species are shown in green and non-target species are shown in red. Penetrability was assessed from the true signal intensity.

Formamide concentration	0		10		20	
Bacterial species	**Untreated**	**Treated**	**Untreated**	**Treated**	**Untreated**	**Treated**
Leuconostoc citrium	+	++	+	+++	+	+++
Streptococcus mutans	(+)	(+)	-	-	-	-
Weissella confusa	+	++	+	+++	+	+++

+++: Very strong positive; ++: Strong positive; +: Positive; (+): Background signals; - : No signals.

Table 7.2App. Verification of probe specificity and determination of optimal hybridisation stringency of the Lab158 probe, using reference bacterial cultures. Target species are shown in green and non-target species are shown in red. Results shown for hybridisation buffers containing 25-40% formamide.

Bac. sp / Form.con	*Lactobacillus acidophilus*	*Enterococcus faecalis*	*Streptococcus mutans*	*Leuconostoc citrium*	*Lactococcus lactis*	*Weissella confusa*	*Weissella paramesenteroides*
25	+++	++	-	+	-	+	-
30	++	+	-	+ < (+)	-	+ & (+)	-
35	(+)	(+) > +	-	(+)	-	(+)	-
40	-	-	-	-	-	(+)	-

+++: Very strong positive; ++: Strong positive; +: Positive; (+): Background signals; ((+)): Strong background and -: No signals. Abbreviations: Bac.sp: Bacterial species; Form.con: Formamide concentration

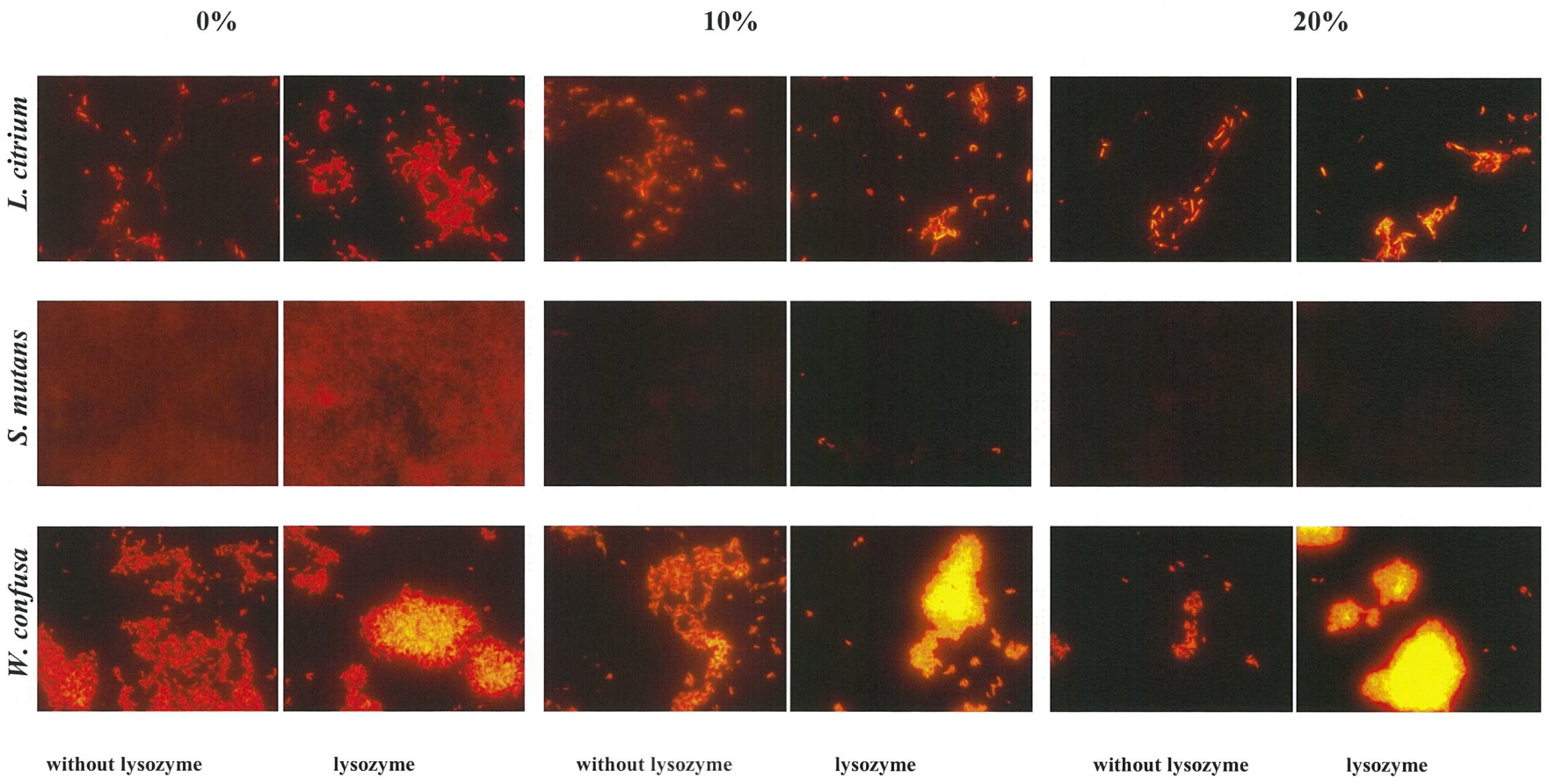

Fig. 7.1App. Penetrability of the Lab158 (Cy3 labelled) probe with and without lysozyme treatment before the hybridisation. Reference bacterial cultures were PFA fixed and hybridisations were performed at 0 to 40% formamide concentrations. Images are shown for 0, 10 and 20% formamide concentrations. FISH experiment included target (*Weissella confusa* and *Leuconostoc citreum*) and non-target (*Streptococcus mutans*) bacterial species.

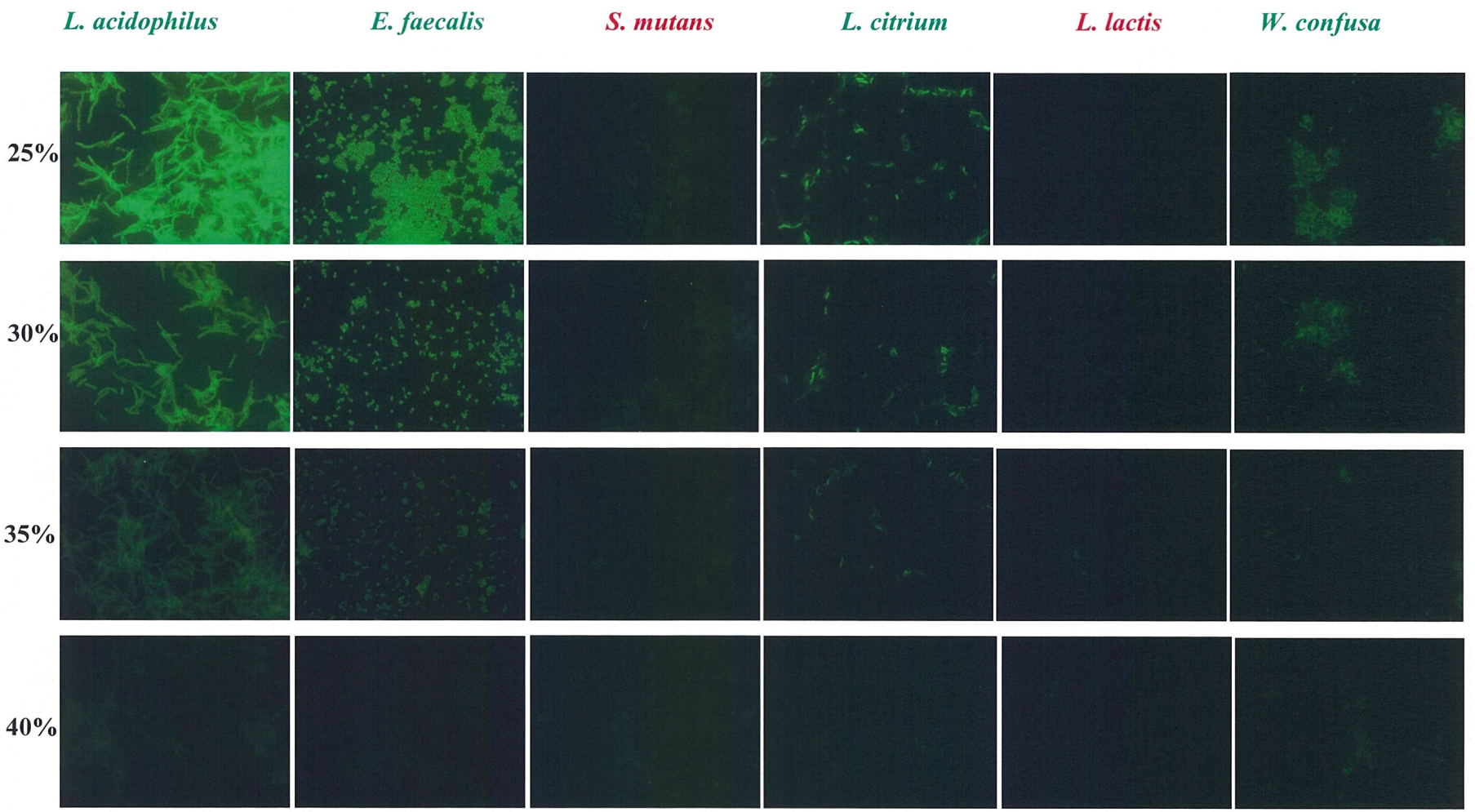

Fig. 7.2App. Verification of probe specificity and determination of optimal hybridisation stringency of the Lab158 (FITC labelled) probe. The reference bacterial cultures were PFA fixed and hybridisations were performed in 0-40% formamide. Illustrated are 25-40% formamide. FISH were carried out on target (green) and non-target (red) bacterial species. Images were from *Weissella. paramesenteroides* (all negative) not shown

Appendix 7: RESULTS OF OPTIMISATION OF PROBE HYBRIDISATION STRINGENCY AGAINST REFERENCE BACTERIAL CULTURES

7.1. Lactic acid bacterial specific probe Lab158

The penetrability of the Lab158 probe was increased by lysozyme treatment of bacterial cells prior to hybridisation. The result was given here only for Lab158 (target species *Weissella confusa* and *Leuconostoc citreum*). The probe did not hybridise with non-target species (*Streptococcus mutans*). The Lab158 probe was very specific for both *Leuconostoc citreum* and *Weissella confusa*, whereas there was a small number of signals from lysozyme-treated *Streptococcus mutans* in 10% formamide, but not wells containing other formamide concentrations. These signals may have come from *Leuconostoc citreum* cells, which were washed in from the adjacent well (Table 7.1App. and Fig.7.1App.).

For Lab158 probe, the optimal hybridisation stringency was seen in 25% formamide in hybridisation buffer (Table 7.2App. and Fig. 7.2App.). No signals were observed from the non-target bacterial species (*Streptococcus mutans*, *Lactococcus lactis* and *Weissella paramesenteroides*) in 20-35% formamide. There was significant loss of signal intensity from target bacterial species in 30% formamide compared with 25% formamide. The no probe control slide showed that there was no auto-fluorescence from any bacterial species. No signals were observed for the nonsense probe control.

Table 7.3App. Verification of probe specificity and determination of optimal hybridisation stringency of the S-G-Wei-0121-a-S-20 probe using reference bacterial cultures. Target species are shown in green and non-target species are shown in red. The hybridisation buffers contained 0-40% formamide.

Form.con / Bac. sp	0	10	20	30	40
Weissella confusa	+++	+++	+++	+++	+++
Streptococcus mutans	(+)	-	-	-	-

+++: Very strong positive; (+): Background signals; -: No signals. Abbreviations: Bac.sp: Bacterial species; Form.con: Formamide concentration.

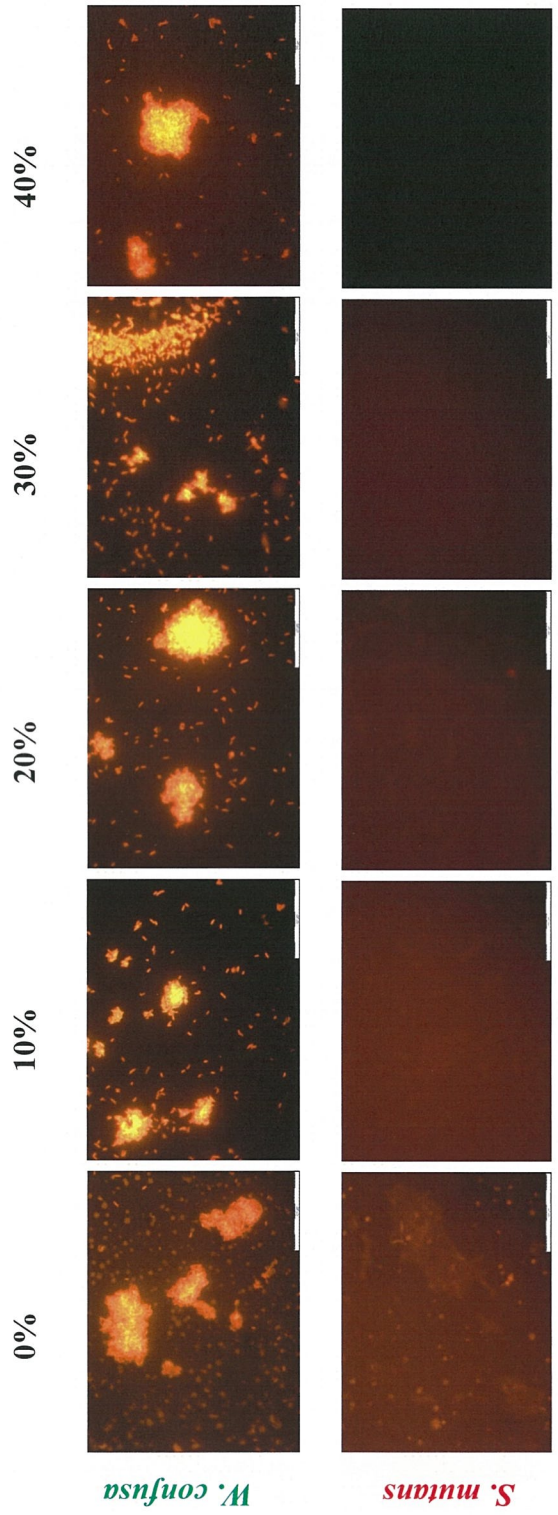

Fig. 7.3App. Verification of probe specificity and determination of optimal hybridisation stringency of the S-G-Wei-0121-a-S-20 (Cy3 labelled) probe. The reference bacterial cultures were PFA fixed and hybridisations were performed in 0-40% formamide. FISH were carried out on target (green) and non-target (red) bacterial species.

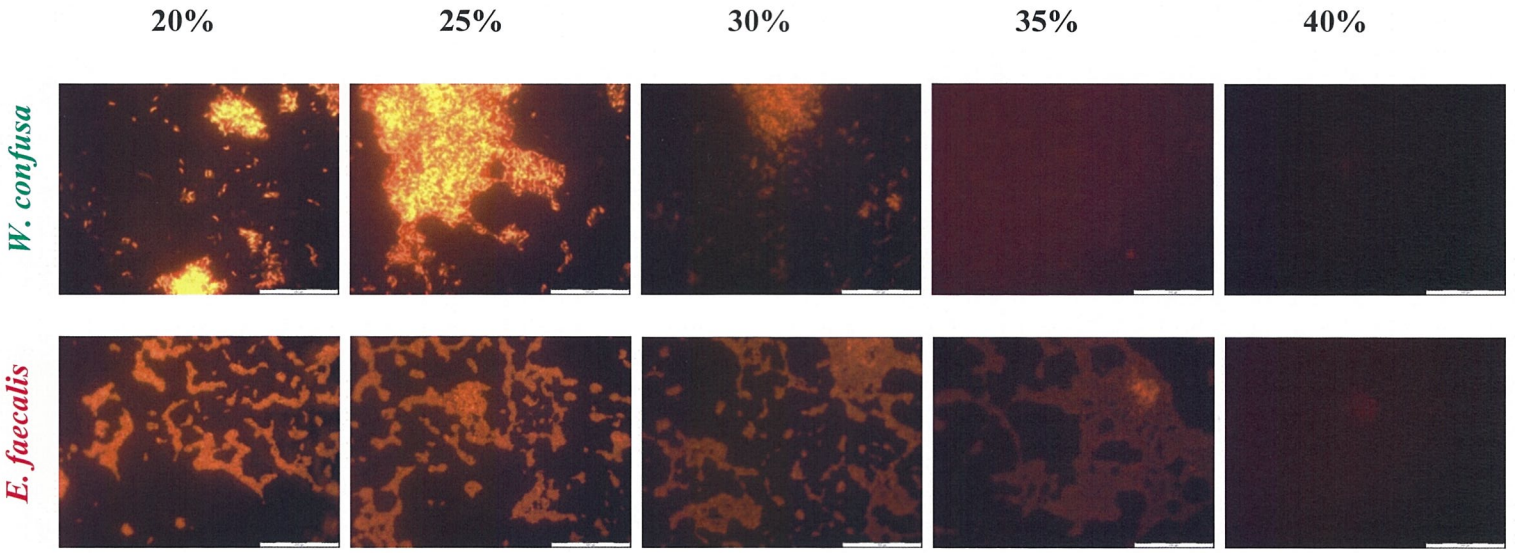

Fig. 7.4App. Verification of probe specificity and determination of optimal hybridisation stringency of the Wgp (Cy3 labelled) probe. The reference bacterial cultures were PFA fixed and hybridisations were performed in 0-40% formamide. Illustrated are 20-40% formamide. FISH were carried out on target (green) and non-target (red) bacterial species.

7.2. *Weissella sp.* specific probes (S-G-Wei-0121-a-S-20 and Wgp)

The penetrability of the Wgp and S-G-Wei-0121-a-S-20 -0121-a-S-20 probes was increased by lysozyme treatment of bacterial cells prior to hybridisation (data not shown).

The S-G-Wei-0121-a-S-20 probe hybridised at all formamide concentrations from 0-40%, with *Weissella* sp., but did not hybridise with non-target organisms (Table 7.3App and Fig. 7.3App). For the Wgp probe, there was significant signal loss in 30% formamide and no signals were seen at higher concentrations (Fig. 7.4App. and Table 7.4App.). The optimal stringency for Wgp was determined to occur in 25% formamide. No signals were seen with the nonsense probe (Non-EUB338) control, nor were there auto-fluorescence signals from any bacterial samples (no-probe control).

Table 7.5App. Verification of probe specificity and determination of optimal hybridisation stringency of the Strc493 probe, using reference bacterial cultures. Target species are shown in green and non-target species are shown in red. Results shown for hybridisation buffers containing 25-40% formamide.

Bac.sp / Form.con	*Lactobacillus acidophilus*	*Enterococcus faecalis*	*Streptococcus mutans*	*Leuconostoc citrium*	*Lactococcus lactis*	*Weissella confusa*	*Weissella paramesenteroides*
25	((+))	-	+ < (+)	+	+	-	-
30	((+))	-	+ < (+)	+ > (+)	++	-	-
35	-	-	(+)	+	++	-	-
40	-	-	(+)	+ < (+)	+ & (+)	-	-

+++: Very strong positive; ++: Strong positive; +: Positive; (+): Background signals ((+)): Strong background signals and -: No signals. Abbreviations: Bac.sp: Bacterial species; Form.con: Formamide concentration.

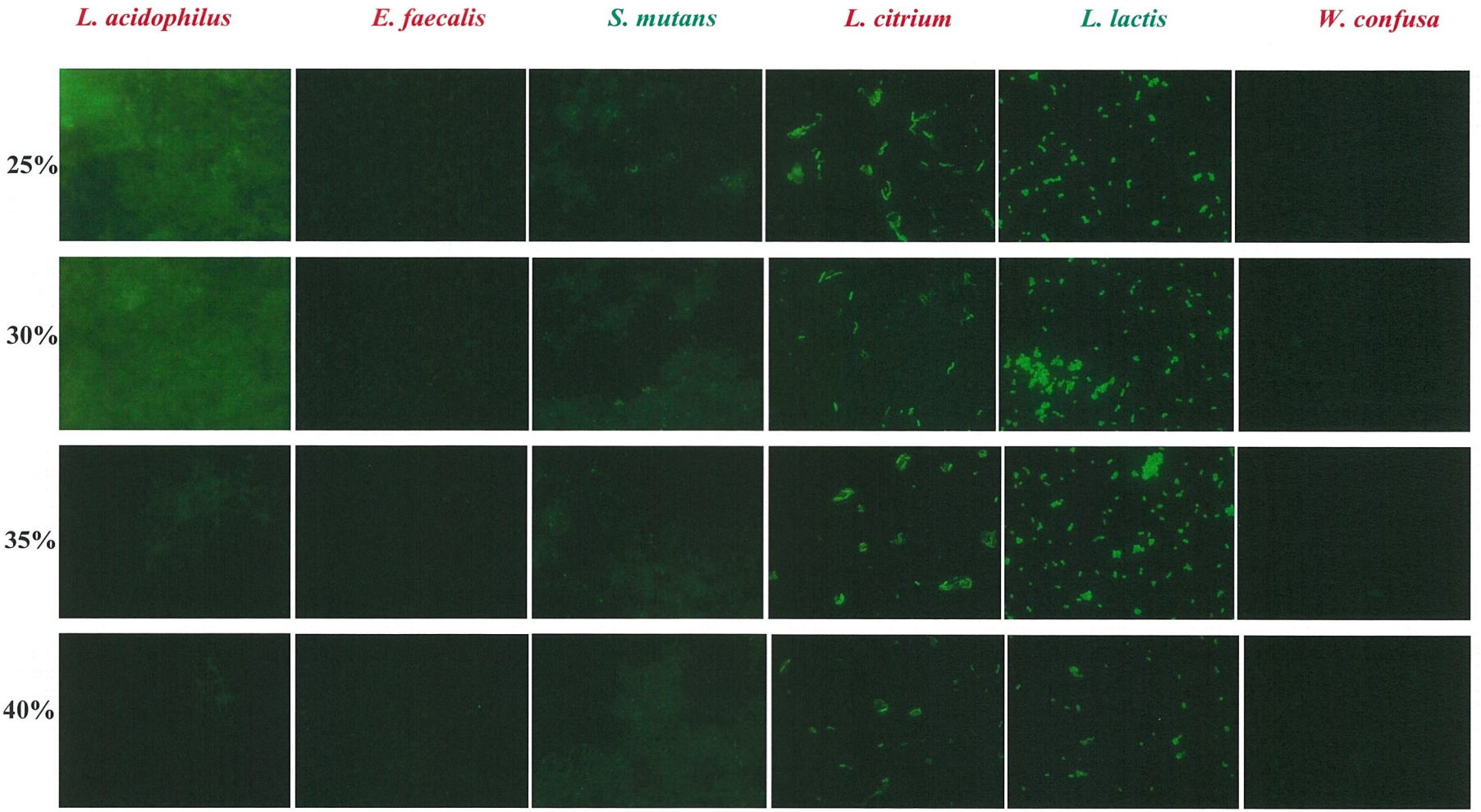

Fig. 7.5App. Verification of probe specificity and determination of optimal hybridisation stringency of the Strc493 (FITC labelled) probe. The reference bacterial cultures were PFA fixed and hybridisations were performed in 0-40% formamide. Illustrated are 25-40% formamide. FISH were carried out on target (green) and non-target (red) bacterial species. Images from *Weissella paramesenteroides* (all negative) *n*ot shown

7.3. Strc493

The penetrability of the Strc493 probe was increased by lysozyme treatment of bacterial cells prior to hybridisation (data not shown).

The optimal stringency for the Strc493 probe occurred at 35% formamide (Table 7.5App. and Fig. 7.5App.). There was significant loss of signal intensity from the target bacterial species (*Streptococcus mutans*, and *Lactococcus lactis*) between 35% and 40% formamide. Signals were seen from *Leuconostoc citreum* one of the non-target species, up to 35% formamide concentration. No signals were seen with the nonsense probe (Non-EUB338) control nor were there auto-fluorescence signals from any bacterial samples (no-probe control).

Table 7.6App. Verification of probe specificity and determination of optimal hybridisation stringency of the class-specific phylum Proteobacterial probes using reference bacterial cultures. The hybridisation buffers contained 0-30% formamide.

Bac.sp / Form.con	*Aminobacter niigataensis* (Alpha)	*Comamonas testosteroni* (Beta)	*Pseudomonas beteli* (Gamma)
0	+	++	++
10	+ < (+)	+ & (+)	+
20	(+)	(+)	(+)
30	((+))	((+))	-
Fine optimisation of formamide concentration			
Bac.sp / Form.con	***Aminobacter niigataensis* (Alpha)**	***Comamonas testosteroni*(Beta)**	***Pseudomonas beteli* (Gamma)**
0	+	++	++
5	+ < (+)	+	+
10	(+)	+	+
15	NA	(+)	(+)
20	NA	(+)	(+)

++: Strong positive, +: Positive, (+): Background signals, ((+)): Strong background signals – : No signals and NA: not applicable. Abbreviations: Bac.sp: Bacterial species; Form.con: Formamide concentration

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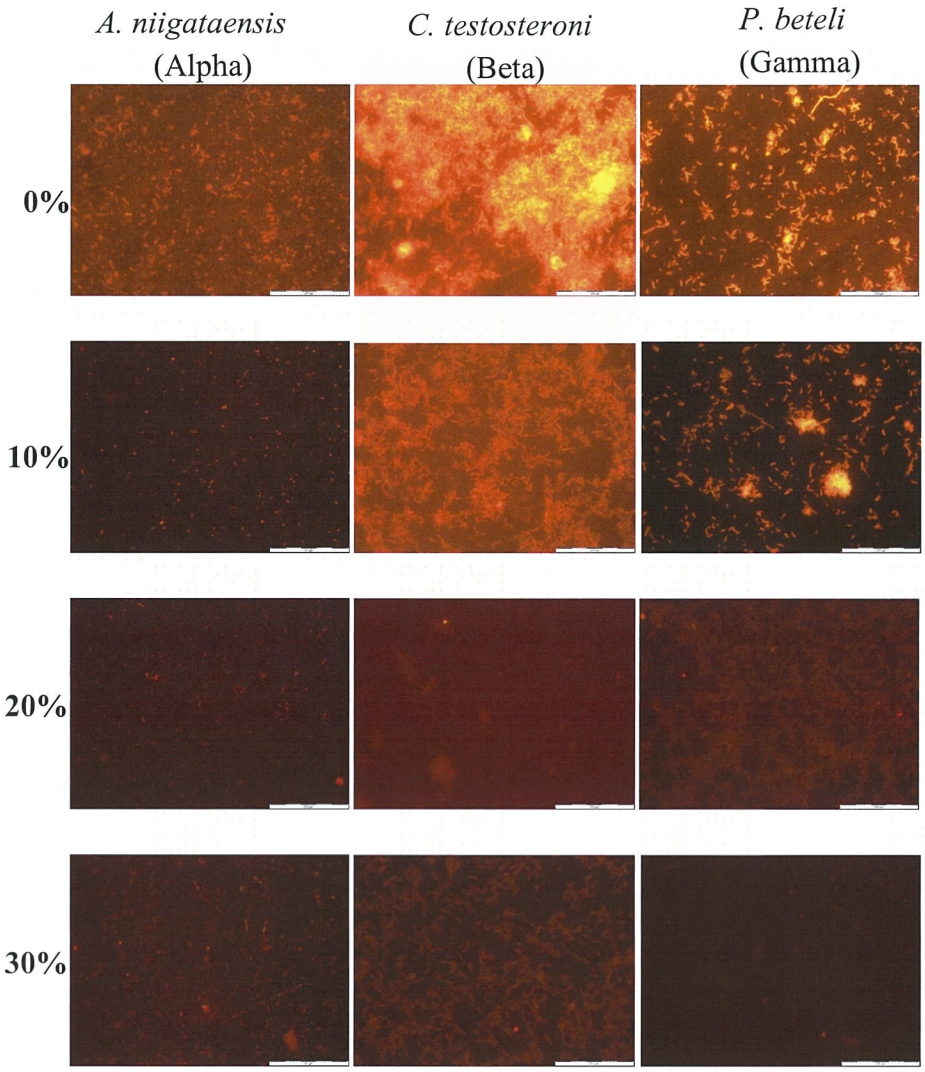

Fig. 7.6App. Optimisation of hybridisation stringency and verification of specificity of the ALFa1, Beta1 and SteMa1_439 (CY3 labelled) probes. The reference bacterial cultures were PFA fixed and hybridisations were performed in 0-30% formamide. The fine optimisation was performed in 0, 5, 10, 15 and 20% formamide (picture are not shown). FISH were carried out on target and non-target bacterial species.

7.4. Proteobacterial probes

Initial optimisation of hybridisation stringency for all 3 probes using 0-30% showed that the fluorescence signals from the target organisms were lost in 20% and higher formamide concentrations (Table 7.6App. and Fig. 7.6App.). For Alphaproteobacteria (*Aminobacter niigataensis*), signals were significantly reduced at 10% formamide and fine optimisation showed that the optimum hybridisation stringency for ALF73a probe occurred in 5% formamide. For Beta- (*Comamonas testosteroni*) and Gammaproteobacteria (*Pseudomonas beteli*), there was significant reduction in signal intensity between 10 and 20% formamide concentrations and fine optimisation showed that the optimum hybridisation stringency for Beta1 and SteMa1_493 occurred in 10% formamide. No signals were observed from the non-target bacterial species, nonsense probe control and no probe control cells.

Appendix 8: BACTERIAL SEQUENCES OBTAINED FROM THE DGGE BANDS AND ACCESSION NUMBERS OF THEIR CLOSEST MATCH FROM THE NCBI DATABASE

Number	Closest matched bacterial sequences	Accession number
1	*Enterococcus italicus* strain E110	JX267123
2	*Lactococcus lactis* subsp. cremoris SK11 strain SK11	NR_074949
3	*Streptococcus gallolyticus* UCN34 strain UCN34	NR_074849
4	*Leuconostoc citreum* strain ML-67	JX258812
5	*Weissella cibaria* strain PPG-W2-Talpur	JX861203
6	*Weissella confusa* strain PPG-IW-Talpur	JX861202
7	*Lactobacillus delbrueckii* subsp. bulgaricus strain ATCC11842	NR_075019
8	*Clostridium perfringens* strain AN 110/95	DQ196133
9	*Catonella* sp. oral taxon 164 clone BR063	GU406942
10	Butyrate-producing bacterium GM2/1	AY305315
11	*Veillonella magna* strain E037	JX267068
12	*Acinetobacter* sp. WJ07	HM045831
13	*Pseudomonas baetica* strain Hb-10	KC139413
14	*Stenotrophomonas maltophilia* strain DR220	KC122707
15	*Ewingella americana* strain NSBx.16(JWB2)	JF439580
16	*Delftia* sp.strain C 1-4	NR_074626
17	*Acidovorax* sp. STEP_aq_283	JQ595544
18	*Comamonas* sp. 01xTSA12A_H10	HM113649
19	*Prevotellaceae* bacterium WR041	AB298732
20	Uncultured rumen bacterium clone L7B_C10	EU381733
21	Uncultured soil bacterium isolate DGGE gel band 18	DQ792764
22	*Chryseobacterium stagni* strain Cb2	JN706760