ASSESSMENT OF PET DOGS AS A RESERVOIR OF ANTIBIOTIC RESISTANT BACTERIA

by

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M.V.Sc., Nagpur Veterinary College, Maharashtra, India, 2005

AN ABSTRACT OF A DISSERTATION

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Department of Diagnostic Medicine/Pathobiology
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Abstract

Transfer of bacteria, including antibiotic resistant strains between companion animals and people is likely due to close physical contacts. However, surveillance programs on prevalence of antibiotic resistance are focused mainly on food animals and very little is known about the role of companion animals in the development and spread of antibiotic resistant bacteria. For this study, enterococci were chosen as model organism due to intrinsic and acquired antibiotic resistance and several virulence traits that make them the 3rd most important nosocomial pathogens. In addition, increased fecal shedding of antibiotic resistant bacteria from stressed animals has been reported from studies on food animals. To determine whether the gut microbiota of pet animals serves as a reservoir of clinically important enterococci, 360 enterococcal isolates from two groups: healthy group and pyoderma (stressed) group with 9 dogs in each were identified and screened for resistance to 10 antibiotics and 4 virulence traits. The transferability of resistance determinants and clonality of selected isolates were assessed by horizontal gene transfer assays and pulsed-field gel electrophoresis, respectively. In addition, overall diversity of bacteria as well as antibiotic and metal resistance genes in feces of healthy dogs was assessed by tag-encoded parallel pyrosequencing and microarray analysis, respectively.

The most prevalent enterococcal species identified was *E. faecalis*: healthy group (70.5%); pyoderma group (44.0%). In the pyoderma group, antibiotic resistance and virulence traits (*esp*, *gelE*) were more frequent than in the healthy group; however, the overall prevalence of antibiotic resistant strains was low (< 37%) in both groups. The most prevalent resistance genes were *tet*(M)and *tet*(S). The antibiotic resistance traits were transferable *in-vitro* in *E.*

faecalis (tetracycline, erythromycin, doxycycline) and *E. faecium* (tetracycline). Genotyping revealed less diverse *E. faecalis* community in pyoderma infected dogs.

Pyrosequencing (~7,500 sequences per dog) revealed *Firmicutes* as the dominant phylum and most common genera included *Turicibacter*, *Lactobacillus*, *Ruminococcus*, *Clostridium*, and *Fusobacterium*. Two phyla *Lentisphaerae* (<1%) and *Fibrobacteres* (<1%) are reported for the first time from healthy dogs. Microarray data revealed the presence of several tetracycline, erythromycin, aminoglycoside, and copper resistance genes; however, most of these originated from one animal with history of chronic skin infection two year prior to our sampling.

Higher prevalence of antimicrobial resistance in pyoderma infected dogs may be related to stress; however, this requires further investigation. In conclusion, based on our data, healthy and pyoderma infected dogs do not represent an important reservoir of clinically significant antibiotic resistant microbiota.

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Dedication

I dedicate my doctoral work to my family and Goddess Vana Durga,

KADAMPUZHA DEVI

Abbreviations

MRSA	Methicillin resistant *Staphylococcus aureus*
VRE	Vancomycin resistant enterococci
VREF	Vancomycin resistant *Enterococcus faecalis*
VREFe	Vancomycin resistant *Enterococcus faecium*
CDC	Centers for Disease Control
WHO	World Health Organization
ENS	Enteric nervous system
GIT	Gastro intestinal tract
QRDR	Quinolone resistance determining region
E. coli	*Escherichia coli*
DNA	Deoxyribonucleic acid
$MgCl_2$	Magnesium chloride
dNTP	Deoxyribonucleotide triphosphate
ddH_2O	Double distilled water
TBE	Tris-borate-ethylenediamine tetraacetic acid
NaCl	Sodium chloride
EDTA	Ethylenediamine tetraacetic acid
RNase	Ribonuclease A
SDS	Sodium dodecyl sulfate
TE	Tris ethylenediamine tetraacetic acid
ATCC	American type culture collection
BHI	Brain heart infusion
GLIMMIX	Generalized linear mixed model
cyl	Cytolysin
esp	Enterococcal surface protein
asa1	Aggregation substance
gel	Gelatinase
NaOH	Sodium hydroxide
rRNA	Ribosomal ribonucleic acid
MUSCLE	Multiple sequence alignment with high frequency and high throughput
OTU	Operational taxonomic unit
ACE	Abundance based coverage estimator
Chao1	Richness estimator
RDP	Ribosomal database project
HCL	Hydrochloric acid
BSA	Bovine serum albumin

SSC	Saline-sodium citrate
cat	chloramphenicol acetyltransferase encoding resistant determinant
ere	Gene encoding erythromycin esterase type II
erm	Erythromycin ribosomal methylation
mef	Macrolide efflux
lin	Lincosamide inactivation nucleotidylation
carA	Carbomycin resistance conferring gene of type A
sat	Streptogramin acetyltransferase
vat	Viriginamycin, factor A acetylation
par	Fluroquinolone resistance determinant encoding topoisomerase IV
bla	Beta-lactamase
ccr	Cassette chromosome recombinase
pen	Penicillin
dfr	Dihydrofolate reductase
sul	Sulphanilamide
tet	Tetracycline
aad	Aminoglycoside adenyltransferase
aac	Aminoglycoside acetyltransferase
ant	Aminoglycoside nucleotidyltransferase
aph	Aminoglycoside phosphotransferase
van	Vancomycin
flo	Florphenicol
cfr	Chloramphenicol-florphenicol resistance
ole	Oleandomycin
vga	Virginiamycin A-like antibiotics
srm	Spiramycin
tlc	Tylosin
lmr	Lincomycin resistance
lnu	Lincosamide *O*-nucleotidyltransferase
vgb	Virginiamycin B-like antibiotics
msr	Macrolide-streptogramin resistance
mph	Macrolide phosphotransferase
pbp	Penicillin binding protein
mec	Methicillin resistant determinant
arr	Rifampin ADP ribosyltransferase
ble	Bleomycin resistance gene
ars	Arsenic resistance system
cad	Cadmium
cus	Copper sensing
czc	Cobalt, zinc, cadmium

cop	Copper
cnr	Cobalt and nickel resistance
tcr	Transferable copper-resistance
qar	Quaternary ammonium compounds
BnALMT	*Brassica napus* aluminium activated malate transporter
yba	Aluminium resistance determinant
cys	Cystein synthase
ncr	Nickel-cobalt resistance
yoh	Resistant-cobalt-nickel
cue	Copper efflux
pco	Plasmid-borne copper resistance

Chapter 1

Literature review

1.1 Introduction

Multi-drug resistance is an emerging problem in human pathogens, including zoonotic pathogens (Tenover *et al*., 1996; Akkina *et al*., 1999). Antimicrobial agents are routinely used to treat and prevent diseases in human and veterinary practices. Overuse and misuse of antibiotics provides tremendous selection, perhaps contributing spread of resistant clones, and acquisition of resistance determinants from resistant bacteria (Levy, 2002). Examples of common antibiotic resistant bacteria include MRSA (Methicillin resistant *Staphylococcus aureus*), MR-CoNS (Methicillin resistant coagulase negative *Staphylococcus aureus*), vancomycin resistant enterococci (VRE) and beta-lactam and macrolide resistant *S. pneumoniae*. The problem of antimicrobial resistance has been declared to be one of the top concerns of the U.S. Centers for Disease Control (CDC) (www.cdc.gov/ncidod/aip/research/ar.html).

In the United States, the annual healthcare cost associated with the treatment of antibiotic resistant infections exceeds $4 billion/year (Stacey *et al*., 2003). A 2008 study that was carried out in a Chicago teaching hospital estimated the cost associated with treatment of antibiotic resistant infections in 188 patients accounted for between 13.35-18.75 million dollars (Roberts *et al*., 2009). This economic burden is associated with increased severity of illness due to treatment failure and long term hospitalization. Longer hospital stays caused increased healthcare costs and more exposure to antibiotics. There has increased severity of illness and mortality rate is also high.

Inappropriate use of antibiotics for therapeutic and prophylactic purposes is considered a significant contributor to emergence of antibiotic resistance in zoonotic pathogens (McGowan JE Jr. 1983) such as MRSA, VRE, and extended spectrum β-lactamase producing *Escherichia coli*. Commensal bacteria have become reservoirs of antibiotic resistance genes (Marshall, 2009). For example: Penicillin resistant *Staphylococcus aureus* which emerged after the introduction of penicillin, later became resistant to methicillin and then third generation penicillins. Similarly, there are other commensal bacteria such as *Enterococcus* species and *Escherichia coli* present in the intestinal tract of animals and humans that have emerged as antibiotic resistant pathogens globally and pose a threat to public health. Studies performed by Calva *et al*.,(1996) and Levy *et al*.,(1988) revealed a high frequency of antibiotic resistance among the fecal microbiota in humans (Salyers *et al*., 2004). Further, commensals can act as a source of horizontal transfer of resistance genes to pathogens. Similarly, clonal spread (Kuhn *et al*., 2000; van den Bogaard and Stobberingh, 2000) and transfer of resistant genes from animal bacteria to human bacteria (van den Bogaard and Stobberingh, 2000) is a concern associated with antimicrobial resistance among commensal bacteria.

Resistance gene transfer between commensals and pathogens depends on several factors such as total number of donors and recipients, nutrition, selective pressure and transfer mechanisms. The gut gene pool is large, harboring diverse population of microbes and thus providing a suitable environment for antibiotic resistance gene transfer (Marshall, 2009). The level of resistance among gut commensals such as *Enterococcus* spp. is considered a good indicator of antibiotic resistance (Capriolo *et al*., 2000).

During the recent decades, enterococci have gained considerable attention among public health officials because of their increasing antimicrobial resistance and as important nosocomial

pathogens. Enterococci are associated with a variety of infections such as bacteremia, urinary tract infections (UTI) and surgical wound infections (Murray, 1990) in humans. They are intrinsically resistant to antibiotics used in hospitals such as cephalosporins, lincosamides, nalidixic acid, and low level of clindamycin and aminoglycosides (Murray, 1990), which is believed to contribute to their ability to cause infection. Additionally, they readily acquire high level of resistance to antibiotics, including penicillin, high level aminoglycosides, vancomycin, chloramphenicol, erythromycin, high level clindamycin, tetracycline, and fluoroquinolones (Murray, 1990; Chow *et al*., 2000). These resistant bacteria or their resistance determinants may be transmitted from an animal reservoir to humans (Witte, 1998).

A major factor associated with the dissemination of resistant determinants is selection pressure exerted by the use of antibiotics, selecting resistant bacteria by killing the susceptible ones. The removal of selection pressure will not eliminate the resistance genes from this bacterial population (Salyers and Amabile-Cuevas, 1997). This increase in the fitness cost in the absence of any antibiotic selection pressure allows rapid spread of antimicrobial resistant strains by replacing the susceptible ones (Enne *et al*., 2004; Luo *et al*., 2005).

Besides selective pressure by the antibiotics, there are other factors such as "stress in animal" that can play a role in the prevalence of resistant bacteria in the gut (Moro *et al*., 2000; Moro *et al*., 1998; Molitoris *et al*., 1987). All bacteria including commensals, obligate or opportunistic pathogens within the host are subjected to stressful conditions. For example enteric bacteria have to overcome the effects of gastric acid (with varying pH depending on the diet of the individual), bile and organic acids, competing gut commensals (for binding the receptor sites and for nutrition), and host immune responses. Animals subjected to stressors such as infection, transportation, change in the environment, etc, can release stress hormones via the enteric

nervous system. Evidence indicates that these stress hormones enhance the bacterial growth and expression of virulence determinants in enteric pathogens (Lyte and Ernst, 1992; Lyte *et al.*, 1996) and affect intestinal functions such as decreasing gastric acidity (Bailey, 2010).

Systemic surveillance and timely reporting of antibiotic resistance among bacteria has been developed by National antimicrobial resistance monitoring system for food animals and humans. However, the data from companion animals with regard to trends in the antibiotic resistant bacteria and risk assessments are absent or scarce. Pet animal numbers have substantially increased and based on market research statistics, the dog population accounts for 72 million in USA (http://www.avma.org/reference/marketstats/sourcebook.asp). Direct contact with pet animals is considered a source of pathogenic bacteria for humans (Simoons-Sumit *et al.*, 2000). The risk factors responsible for the transmission of resistant bacteria to humans includes direct contact (petting, licking, physical injuries, etc.) or through the domestic environment (contamination of food, furnishings, etc.) (Guardabassi *et al.*, 2004). Moreover, most of the antimicrobials used in small animal veterinary practice are used in human medicine as well and are regarded as critically important for human use (Heuer *et al.*, 2005). Once the bacteria from the pet animals develop resistance to antibiotics used for human treatment, transmission of these resistant bacteria may become a public health concern.

1.2 Zoonotic pathogens

Zoonosis is defined as a disease or infection that is communicable between animals and man under natural conitions (WHO/FAO, 1959) and can affect humans or animals. The majority of the infections in humans are caused by the zoonotic pathogens. For instances, classification of 1709 human infectious agents such as viruses, bacteria, prions, rickettsia, fungi, protozoa and helminthes revealed that 55% of them were zoonotic pathogens (Taylor and Woodhouse, 2000).

During the past three decades about 30 new human pathogens were detected and 75% of them were considered zoonotic (WHOb, 2006). Several factors contribute to the emergence of zoonotic pathogens: close physical contact between animals and man, advanced transportation, ecological and environmental changes and bioterroism (Murphy, 1998). In developing countries, growing populations of humans and animals provide suitable conditions for the microorganisms to adapt to human hosts (Bhatia and Narain, 2010).

Zoonotic diseases impact the global economy. The World Bank estimated that a severe avian influenza pandemic could cause reduction in the cost of world's gross domestic product by $40 trillion to US $1.25 trillion (The World bank, 2008). Another example of global economic loss due to rabies in humans that is transmitted from dogs, which account for US$ 1 billion on rabies prevention programs (WHOa, 2007). From 2008-2010 the animals tested positive for rabies in Kansas State includes skunk, bovine, equine, fox, feline, bat, raccoon, sheep, goat, coyote, and bobcat (http://www.vet.k-state.edu/depts/dmp/service/rabies/2010/KS10_map.gif). For example, zoonotic pathogens include viruses (rabies, avian influenza, Japanese encephalitis), bacteria (salmonellosis, anthrax, leptospirosis), and parasites (toxoplasmosis, taeniasis, hydatidosis) with domestic and wild animals acting as a reservoir. Development of antimicrobial resistance in these zoonotic pathogens and the transmission of the antibiotic resistant zoonotic pathogens from animals to humans is a public health concern. For example, MRSA of pig origin was detected in the severly diseased human patients (van Loot *et al.*, 2007)

1.3 Antimicrobial resistance

Antimicrobial resistant bacteria has the ability to survive within the labeled drug dose because the systemic drug concentration of the agent will be lower than the MIC of the causative agent with normal drug dosage and/or fall in the range or where specific microbial resistance

mechanisms are likely and clinical efficacy has not been reliable in treatment studies (CLSI/NCCLS M31A2).

Antibiotic resistance traits were present in the environment before the introduction of antibiotics for therapeutic use but were present only in the antibiotic producing microorganism (Levy, 1992). In order to overcome the inhibitory effect of these antibiotics, the nearby bacterial species might have developed resistance mechanism or acquired the resistance genes from their neighboring antibiotic producing bacteria. Moreover, conjugative plasmids in bacteria collected during the pre-antibiotic era were lacking the resistance determinants (Hughes *et al*., 1983).

With the introduction of new drugs, resistant strains started evolving. The association between antibiotic use and its impact on resistance development has been discussed extensively (Kummerrer, 2004; Shea, 2004; Seveno *et al*., 2002; Barbosa *et al*., 2000).

Therapeutic use of antibiotics in the treatment of human and animal infections eliminates not only the infectious organism but also the non-disease producing beneficial commensal bacteria, thus altering the natural microbiota and creating an imbalance between the susceptible and resistant strains. This disruption in the microbial ecology may lead to emergence of distinct populations of bacteria that are drug resistant variants of the same bacterial population that were already present or a different population of resistant bacteria (Levy, 1997). Once they acquire resistance, they reproduce giving rise to large numbers of resistant progeny in the presence of the antibiotic.

There are multiple mechanisms by which a bacterium can be resistant to any drug. Resistance to the antimicrobials can be intrinsic or acquired (Guardabassi *et al*., 2006). Intrinsic resistance can be due to the lack of cellular mechanism required for the drug to have activity. Acquired resistance is mainly due to chromosomal mutation or through horizontal gene transfer.

Chromosomal mutations can lead to structural alterations such as active efflux pump (tetracyclines); enzymatic inactivation of antibiotics (beta-lactamase); alteration in cell wall impermeability (chloramphenicol); alteration in target sites (aminoglycosides); low drug affinity; or increased production of metabolites (sulfa drugs). The second mechanism, horizontal gene transfer can occur by three different ways: transduction, transformation and conjugation (Sorensen *et al*., 2005). Transduction is the process of phage mediated plasmid DNA transfer from one bacterium to another. Through transformation, naked DNA is transferred between the bacterial cells. Conjugational DNA transfer is mediated by F pilus and driven by the conjugative plasmids, transposons or integrative conjugative element from a donor to recipient cell. These resistant microbes can spread genes for resistance to other bacteria (Boerlin *et al*., 2007). Thus bacteria have diverse mechanisms to transmit resistance traits to other members of the same or to other species.

After acquiring antimicrobial resistance genes, resistance transfer can occur irrespective of presence of antibiotics in the environment (Kruse *et al*., 1994). A bacterium can be resistant to multiple antibiotics due to the presence of different resistant determinants on the same genetic element or same genetic element conferring resistance to different antibiotics (Anderson *et al*., 2010).

1.4 Antimicrobial resistance in zoonotic pathogens

The development of antimicrobial drug resistance among zoonotic bacteria has emerged as a major public health concern and tend to be associated with the use and overuse of antimicrobial agents in human and veterinary practices.

1.4.1 Food animals

It has been reported that the use of antibiotics for the nontherapeutic purpose of growth promotion accounts for a significant fraction of total antibiotics used in United States (Mellon *et al.*, 2001; National research council, committee on drug use in food animals, 1999). Antibiotics for growth promotion are fed to cattle, swine and poultry. There are reports where antibiotic resistant bacteria are found in associated animal food products and also in the farm workers. The environment is also considered as a potential pathway of resistant bacteria to humans where animal wastes are applied to agricultural fields (Smith et al., 2002). Food animals also act as reservoir hosts for some of the food-borne and zoonotic pathogens such as *Salmonella*, *Campylobacter*, *Yersinia,* Methicillin resistant *Staphylococcus aureus*, Enterococci and enterrohaemorhagic *E. coli*. There is considerable evidence that these antibiotic resistant bacteria of animal origin cause infections in humans (Neu, 1992; Witte, 1998) and are likely to be transferred through food chain.

There are reports of transfer of resistant *Salmonella* spp. from animals to humans. Food animals such as cattle, sheep, goat, pigs and poultry can be carriers of *Salmonella* spp. in their intestinal tract. In the U.S, it is estimated that 95% of human *Salmonella* infections are acquired through food chain (Mead, *et al.*, 1999). Some species of *Salmonella* such as *Salmonella typhimurium* cause serious enteric infections in animals. Therapeutic use of antibiotics in infected animals selects for resistance in these strains. Molecular typing of fluoroquinolone resistant *Salmonella typhimurium* DT 104 strains obtained from infected humans in Denmark was indistinguishable from a swine isolates (Molbak *et al.*, 1999). Similarly, typing of ceftriazone resistant *Salmonella typhimurium* isolated from a diarrhic child revealed that the strain was of cattle origin (Fey *et al.*, 2000)

Campylobacter jejuni and *Campylobacter coli* are commensal bacteria of cattle, poultry and swine and serve as a reservoir for human *Campylobacter* infections. In the U.S, *Campylobacter* is a leading cause of foodborne gastroenteritis (Mead *et al*., 1991). In the Netherlands, enrofloxacin was introduced in 1987 for therapeutic purposes in poultry. Endtz *et al*.(1991) studied the prevalence of antibiotic resistant *Campylobacter jejuni* from human stools from 1982-1989 and observed that the prevalence increased from 0%-11%; suggesting the possible transfer of resistant bacteria from poultry to man via food chain.

Animals such as pigs, rodents, livestock and rabbits act as a reservoir for *Yersinia* (Gutman *et al*., 1993). The outbreak of *Yersinia enterocolitica* infection in U.S invoved several serotypes such as O:8; O:13a,b; O:3 and O:1,2,3 (Bottone, 1997; Stock and Wiedemann, 1999). Susceptibility patterns differ in the biogroups within the serotypes. In serotype O:8, biogroup 1B are resistant to carbenicillin, ticarcillin and cephalothin while 1A are resistant to amoxicillin/clavulonic acid (Preston etal., 1994; Ahmedy et al., 1985); in serogroup O:1,2,3, biotype 3 are resistant to amoxicillin/clavulonic acid and cefoxitin (Pham *et al*., 1991); and serotype O:3 are resistant to carbenicillin, ticarcillin and ampicillin (Pham *et al*., 1995). Perez-Trallero *et al*., (1988) reported the transfer of *Yersinia enterocolitica* from animals to humans with reduced susceptibility to chloramphenicol.

In the Netherlands, Canada, and the Unites States, high prevalence of MRSA is found in humans having direct contact with swine (van Cleef et al., 2010; Golding *et al*., 2010; Smith *et al*., 2009). For indigenous bacteria such as *E. coli* and enterococci, emergence of antibiotic resistance clones has been documented several times (Lewis *et al*., 2007; van der Auwera *et al*., 1996). In humans, *E. coli* resistance to extended spectrum cephalosporins is of major concern due to their involvement in urinary tract infections (Pitout and Laupland, 2008) and pose a threat

to the drugs of last resort such as ceftazidime, cefepime and azetonam (Beovic, 2006). Among the enterococcal isolates in animal feces (pigs and chickens), emergence of glycopeptide resistance was assumed to be linked to avoparcin (glycopeptide feed addative) feeding in those animals (Klare *et al*., 1997). The majority of the glycopeptide resistant enterococcal strains causing outbreaks worldwide belonged to the clonal complex CC17 (*E. faecium*) that are characterized by their resistance to ampicillin (Willems *et al*., 2005; Top *et al*., 2008).

1.4.2 Companion animals

Pet animals also act as a reservoir for several zoonotic pathogens. Pet animals are considered a source for campylobacteriosis in humans, contributing about 6% of infections (Saeed *et al*., 1993). Damborg *et al*.,(2004) examined the clonality in *Campylobacter jejuni* in dogs and humans living in different geographical regions. Pulsed field gel electrophoresis (PFGE) revealed identical clones in dogs and humans, suggesting the clonal lineage in these hosts.

There are various reports documenting dog-human transmission of MRSA (Cefai *et al*., 1994; Simoons-Smit *et al*., 1997). Humans can acquire infection through contact with pets colonized with MRSA (Manian, 2003). Pets can also acquire MRSA from people. Genotyping of MRSA isolates from dogs and cats confirmed the isolates were indistinguishable to the human hospital acquired clones (Leonard and Marky, 2008). *Staphylococcus pseudintermedius*, indigenous flora present on the skin of dogs are reported to be found commonly among veterinary staff treating dogs and owners of dogs with dermatitis and pyoderma infections (Harvey *et al*., 1994; Guardabassi *et al*., 2004). In cases where pyoderma is a chronic and recurrent infection, prolonged antibiotic treatment can lead to development of resistance in *Staphylococcus pseudintermedius*.

Pet animals are considered to be responsible for 1% of reported Salmonellosis infections in humans annually in the U.S. (Stehrgreen and Schantz, 1987). Direct contact with pets, their feces, and commercial pet food treats and supplements are reported to be the possible routes for the transmission of *Salmonella* species from pets to man (Cherry *et al*., 2004; Health Canada, 2006). In the U.S., genotyping of *Salmonella* isolates from an outbreak revealed indistinguishable PFGE profile in *Salmonella typhimurium* DT104 isolates from animal healthcare workers, pet owners and their pets. These strains exhibited penta-resistance phenotype (resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) (Centers for Disease Control and Prevention, 2001)

Similarly, Johnson *et al*.,(2001) compared the clonality and virulence genotype of *E. coli* isolates from canine UTI with human extraintestinal pathogenic *E.coli*. Most of the isolates of canine origin harboured the virulence determinants that were specific for human extraintestinal pathogenic *E.coli*. Pulsotype of *E. coli* isolates from canine UTI clustered with human extraintestinal pathogenic *E.coli* suggesting dog-human transmission of the coliforms.

Willems *et al*.,(2000) used amplified fragment length polymorphism (AFLP) analysis to explore the genetic relationship between vancomycin resistant *Enterococcus faecium* isolated from various sources such as hospitalized patients, nonhospitalized people and different animals (dogs, cats, pigs, veal calves, chickens and turkeys). Isolates from non hospitalized patients (75%) clustered with all pig isolates (100%) while isolates from hospitalized patients (84%), dogs (100%), cats (100%) and veal calf (25%) formed a separate cluster.

These studies indicate an increasing concern for transfer of these resistant zoonotic bacteria from pet animals to humans.

1.5 Epidemiology of antimicrobial drug resistant bacteria in dogs

There are studies reporting antimicrobial resistance in bacterial species recovered from pet animals. Lloyd *et al*.,(1996) reported that coagulase positive *Staphylococcus* recovered from skin, ear and mucosa infections in dogs over 1980-1986 showed increased resistance to penicillin (69-89%), and oxytetracycline (40%). While the isolates collected from 1987-1989 showed a 20% increase in the antimicrobial resistance prevalence for the antibiotics erythromycin and lincomycin and a 15% increase to sulphonamides/trimethoprim.

Normand *et al*.,(2000) studied antimicrobial resistance trends for *Escherichia coli* and *Staphylococcus* species isolated from clinical cases of canine and feline origin in the United Kingdom. These cases included the samples received by a diagnostic laboratory from 1989-1997 for culture and sensitivity testing. They found a significant rise in antibiotic resistant *Escherichia coli* showing resistance to amoxicillin, amoxicillin-clavulanic acid and streptomycin; as well as increasing resistance to erythromycin among the Staphylococcal species. For Staphylococcal spp. a decreasing trend in resistance was observed for ampicillin and penicillin.

A study was conducted in Sweden by Holmes *et al*.,(2002) to compare the resistance profile of *Staphylococcal* spp. from the canine pyoderma cases isolated from the first infection and recurrent infections during 1995-1996. The isolates that were resistant to three or more antibiotics were defined as multidrug resistance. There was a significant increase in resistance for macrolides, lincosamides, fusidic acid and streptomycin from recurrent cases compared with the first infection. Forty five percent of isolates from recurrent cases had multidrug resistance profiles while only 20% of isolates from first infection had multidrug resistance.

Prescott *et al*.,(2002) determined the prevalence of antibiotic resistant, coagulase positive *Staphylococcus aureus* in clinically infected dogs from 1984-1998. A significant increase in

resistance towards cephalothrin, enrofloxacin and gentamicin and decrease in penicillin and ampicillin resistance were observed among these isolates. While no change in trend was observed for clindamycin and erythromycin resistance. This fluctuating trend in the resistance patterns of *Staphylococcus aureus* was correlated to the type of antibiotic used.

Several multidrug resistant gram negative bacteria such as *Actinobacter baumannii* and *Salmonella enterica* have also been identified in hospitalized dogs (Tillotson *et al*., 1997). In Switzerland, the nosocomial pathogen, *Actinobacter baumannii* was isolated from clinically diseased dogs. Genotypic fingerprinting by restriction polymorphisms of ribosomal DNA suggested this pathogen was responsible for the infections (Francey *et al*., 2000).

1.6 Sources for acquisition of antibiotic resistant pathogens

The major source for acquisition of antibiotic resistant pathogens is the use of antibiotics in human and agriculture practices. In human medicine, a patient admitted to the hospital can acquire resistant bacteria from the hospital through various sources such as instruments, and workers hands. Once the patient is discharged, the antibiotic resistant bacteria persist and can act as a source for household transmission of resistant bacteria to other individuals.

Antibiotics in agriculture are used for veterinary practices, feed additives and biocides for crop production. These chemicals can enter the environment through various routes such as disposal of drugs and containers, disposal of waste from drug manufacturing plants, and application of animal waste on the farm as a source of manure (Buchberger, 2007; Utah Department of Health, 2007; Daughton, 2004). Much of the antibiotics fed to the animals are not absorbed and are excreted in the feces as parent compound and its breakdown metabolites (Boxall *et al*., 2004; Feinman and Matheson, 1978). It has been reported that about 25% of orally administered tetracycline is excreted in the feces and 50-60% in urine indeed, 67% of

administered tylosin is excreted via feces (Feinman and Matheson, 1978). Once the animal waste is applied to the land, antibiotics can be carried through surface and ground waters (Krapac *et al*., 2004; Yang and Carlson, 2003). The use of water from these sources for vegetation and human use can transmit these resistant pathogens to humans causing public health concerns. There are also reports of antibiotic drug residues in meat which can also be a threat to human health and companion animals fed on it (Martínez, 2005).

1.7 Stress

Stress is a non specific response of the body to external stimuli such as psychological or physical stimuli that disrupts the internal homeostasis (Bhatia *et al*., 2005). Stress was first described by Seyle in (1936) who used a rat model and observed rats exposed to stressors such as nocuous environmental factors responded with a symptom termed "*General Adaptation Syndrome*" irrespective of the type of stimuli. This symptom is divided into three stages: Exposure to a stressor results in alarm stage that is characterized by hyperfunction of the adrenal glands. The second stage is the "resistance stage" during which the body remains alert and the animal become resistant to that particular stressor and the body continues to function normally. The last stage is the "stage of exhaustion" during which the animal fails to maintain homeostasis resulting in loss of resistance and may result in illness (Seyle, 1937; Seyle, 1946).

1.7.1 Animal stress and bacteria

Stress in animals can be caused by external factors, nutritional or physiological disorders (Siegal, 1995), inadequate nutrition, heat, cold, overcrowding, handling, transportation, unfamiliar environments (visit to a clinic) and ill state (Roatango, 2009). Stress imposed on an animal host can cause impairment in the growth, development, or reproduction (Hochachka &

Somero, 2002) and can suppress the immune system increasing vulnerability to infections (Moberg, 2000).

In response to stressors, the hypothalamic-pituitary-adrenal axis is stimulated leading to secretion of "stress hormones"; catecholamines (epinephrine and norepinephrin), adrenocorticotrophin and glucocorticoids (cortisol and corticosteroid) (Axelrod and Reisine, 1984; Greenberg and Wingfield, 1987). These hormones can be responsible for increased severity and susceptibility of infection associated with stress in humans and animals (Dreau *et al*., 1999; Groves, *et al*., 1973). Studies have revealed that catecholamines administered in healthy subjects caused increased severity of *Clostridium perfringenes* infection in humans and enterohemorrhagic *Escherichia coli* in calves (Lyte, 2004; Vlisidou *et al*., 2004; Williams *et al*., 2006). Norepinephrin treatment also increases virulence in bacteria, including *Salmonella enterica* (Methner *et al*., 2008), *Campylobacter jejuni* (Cogan *et al*., 2007) and *E. coli* O157:H7 (Dowd, 2007). Additionally, stress in swine has a role in colonization and fecal shedding of pathogens such as *E. coli* (Moro *et al*., 1998; Moro *et al*., 2000). Stress in these animals increased the intestinal motility and fecal shedding of resistant *E. coli* harboring the lower GIT. In piglets, stress in reponse to weaning and cold stress has increased antibody titer and fecal shedding of *E. coli* (Jones *et al*., 2001).

The above studies suggest that stress in the animals cause alterations in the GIT functions, expression of virulence genes, increased colonization and fecal shedding of resistant bacteria.

1.7.2 Stress and gastrointestinal tract (GIT)

The enteric nervous system (ENS) is invervated within the wall of the GIT. The ENS programs the different functions of the GIT such as microcirculation, motility and exocrine and

endocrine secretions. The enteric nervous system forms the gut-brain axis with the central nervous system (brain and spinal cord) by connecting through the sympathetic and parasympathethic nervous systems. During physical and psychological stress, neuroendocrine mediators are released from the central nervous system and endocrine system. The hypothalamus releases corticotrophin releasing hormone which stimulates adrenocorticotrophin secretion from the pituitary which in turn stimulates the release of glucocorticoids from the adrenal gland (Burnett, 2005). These neuroendocrine mediators example norepinephrin are released in the gut by the gastrointestinal neurons. About half of the total norepinephrin produced in the body might be in the ENS (Aneman *et al*., 1996).

Several studies done involving *E.coli* suggests that norepinephrine (NE) released by the ENS under stress increases growth, iron acquisition, and motility among the bacteria. NE also causes increased expression of certain virulence factors such as type III secretion, Shiga like toxins and locus of enterocyte effacement (LEE) encoded proteins in enterohemorrhagic *E. coli* O157:H7 (Freestone *et al*., 2003; Lyte *et al*., 1997; Dowd, 2007; Sperandio *et al*., 2003) and K99 pilus adhesion in enterotoxigenic *E. coli* (Lyte 1997). It has been also demonstrated in *Salmonella* that NE increases motility, expression of iron uptake genes (Bearson *et al*., 2008; Bearson *et al*., 2008; Karavolos *et al*., 2008), and stimulates the invasion of porcine jejunal explants (Green *et al*., 2003). Thus NE released in the gut in response to host stress, seems to contribute to the the pathogenicity of enteropathogens.

These mediators are responsible for inducing alterations in gastrointestinal functions (Enck, *et al*., 1989), thus altering the microenvironment of the gut. Stress induces a variety of disturbances in the GIT such as alterations in gut microbiota, changes in intestinal motility, decreased gastric acid secretion (Hayase and Takeuchi, 1986), increased intestinal permeability

(Kiliaan *et al.*,1998) and increased susceptibility to colonic inflammation. Stress can also cause alterations in the normal gut microbiota that prevent pathogen colonization by competitive exclusion and induction of antimicrobial peptides production (defensins and IgA in the animal host which aid inprotecting the host from colonization of pathogen by competitive exclusion and induce production of antimicrobial peptides such as defensins and IgA (Guarner and Malagelada, 2003). Stressor-induced reduction in *Lactobacilli* has been demonstrated in mice during rehousing into new cages (Schaedler and Dubos, 1962). This reduction in the number of lactic acid bacteria may increase the susceptibility of host to enteric infections as these bacteria, eg. *Lactobacillus* species are contributors to microbiota mediated colonization exclusion. Bailey *et al.*,(2006) reported overgrowth of *Enterobacteriaceae* in the small and large intestine and cecum of mice when they were restraining for prolonged period. Similarly, changes in the GIT functions such as decreased gastric acid production will increase the pH in the stomach, which may increase survival and colonization of food-borne pathogens to survive and colonize in the gut. Stress-induced increase in the intestinal permeability leads to greater probability of bacterial translocation in GIT. Gastrointestinal motility is thought to facilitate normal cleansing mechanism of the intestine (Gangrosa, 1977). Decreased motility can result in replication of pathogens and their invasion of host tissue. Therefore, stress induced alteration of the normal intestinal microbiota will reduce the host's innate immune mechanism increasing the potential for proliferation of pathogenic bacteria or creating niches for opportunistic proliferation of normal, commensal species.

Quorum sensing is a cell-to-cell signaling mechanism of bacteria. In this process, each cell produces and releases chemical molecules known as autoinducers. When the density of the bacterial population increases and the level of autoinducers reach a critical threshold

concentration, it leads to alteration in the bacterial gene expression (Miller and Bassler, 2001). Sperandio *et al*.,(2003) observed that the normal GIT microbiota produces the quorum sensing AI-3 signalling cascade in response to host stress hormones, which in turn activates the expression of bacterial genes essential for colonization of the host intestine.

1.7.3 Association between stress and antimicrobial resistance in the gut microbiota

There are some reports documenting stress under transport, overcrowding in pens and rough handling in food animals before slaughter associated changes in the antimicrobial resistance phenotypes in the gut microbiota. Molitoris *et al*., (1987) studied the changes in the antimicrobial resistance prevalence in fecal coliform and fecal streptococci in pigs with a variety of transit and holding time at slaughter houses. Comparison of streptococcal and coliform isolates from the live pigs at slaughter houses and pigs at farms revealed that antimicrobial resistance in streptococcal and coliform isolates were much higher in the slaughter house pigs after short transit time (2 hours) and long holding time. Long holding time (39 hours) of pigs also increased the rate of resistance gene transfer in coliforms. Thus, transit and holding stress in pigs increased the proportions as well as resistance transfer ability of antimicrobial resistant strains of *E. coli* .

Effect of loading and transportation stress on the prevalence of antibiotic resistant fecal Gram negative enteric bacteria in pigs was reported by Langlois and Dawson,(1999). They observed a significant increase in the proportion of these antibiotic resistant bacteria. Further, the mean number of antimicrobial agents these bacteria were resistant to also increased, in association with loading and transportation for 30 min.

Langlois *et al*.,(1988) studied the effect of age and housing location on antibiotic resistance of fecal coliforms from pigs that had no prior contact with antibiotics for 126 months.

Pigs less than seven months of age and the pigs housed in the finishing unit had a higher level of antibiotic resistance as compared to older pigs and the pigs housed in the farrowing house, respectively. Greater than 80% of isolates from pigs under 6 months were resistant to tetracycline, streptomycin and sulfisoxazole. Pigs in the finishing unit harboured fecal coliforms that were significantly more resistant to ampicillin, tetracycline and carbenicillin as compared to pigs in the farrowing pen.

According to Hedges and Linton,(1988), simply moving the animal in and out of their pen changed the antimicrobial resistance frequency. The antimicrobial resistance prevalence in *E. coli* was studied within group of pigs not exposed to antibiotics for 156 months. Withdrawal of subtherapeutic feeding of antibiotics for 13 years showed progressive decrease in the prevalence of antibiotic resistant *E. coli*. However, transportation of these animals caused a significant increase in antibiotic resistant *E.coli* despite the fact that they were still on the nonantibiotic diet (Langlois *et al*., 1986).

Moro *et al*.,(1998) found a 6-27% increase in resistance to ampicillin and tetracycline among *E. coli* from the fecal samples in a swine herd exposed to excessively cold conditions. The same authors investigated the effect of heat stress on the prevalence of antibiotic resistant *E. coli* from the intestinal tract of swine and showed an increased level of antibiotic resistant *E.coli* in pigs kept at +34ºC for 24 hours as compared with isolates from a control group. Stress also increased the incidence of multiple drug resistance in these isolates as compared to their resistance profile prior to stress exposure. They further demonstrated that increased intestinal motility increased the occurrence of resistant *E. coli* in the colon and rectum. These stressed animals had reduced intestinal transit (Moro *et al*., 2000).

In a study by Minton *et al.*,(1983), antibiotic resistant fecal coliforms were isolated during acute phase of enteritis from a 10 month old dog that had never been exposed to antimicrobial agents. After restoration of animal to normal health, the coliform isolates had lost their resistance to several antibiotics.

These studies suggest that stress can increase the prevalence of antibiotic resistance of bacteria in the gut.

1.8 Enterococci, the opportunistic pathogen

Enterococci are gram positive facultative anaerobes occurring singly, in pairs or short chains. The genus *Enterococcus* belongs to the phylum *Firmicutes,* class *Bacilli*, order Lactobacillales and family Enterococcacaceae. These gut commensals are present in the intestine in numbers as high as 10^8 colony forming units per gram of feces but account for less than 1% of total intestinal microbiota (Jett *et al.*, 1994). Enterococci can tolerate a wide range of temperatures (10°C-45°C or up to 60°C for 30 min) (Hancock *et al.*, 2000) or pH of 9.6. They are also tolerant of high salt (6.5%) and bile concentrations (40%). Their resistance to chemical and physical stress aids in survival outside the natural host for long periods (Kuhn *et al.*, 2000). Viable enterococci have been recovered from inanimate objects for up to 4 months (Went *et al.*, 1998).

There are 37 species classified under this genus. *E. faecalis* is the most prevalent enterococci found in the gut of dogs. Other species may include *E. faecium*, *E. avium*, *E. hirae*, *E. cecorum* (Gilmore, 2002). These organisms are responsible for a variety of nosocomial infections including urinary tract infections, bacteraemia, endocarditis, neonatal sepsis, intra abdominal and pelvic infections and meningitis (Marothi *et al.*, 2005). In humans *E. faecalis* and *E. faecium* are the most common pathogens, 60% and 40%, respectively (Hidron *et al.*, 2008;

Top *et al*., 2007). *E. faecium* exhibit resistance to more antibiotics (Kawalee *et al*., 2001; Harbarth *et al*., 2002; Tokars *et al*., 2001; Mundy *et al*., 2000).

1.8.1 Antibiotic resistance in enterococci

Enterococci are commensal inhabitants of the GIT of humans and animals. Although a variety of enterococcal species are present in healthy individuals, enterococci are also opportunistic pathogens causing infection under certain conditions such as an impaired immune response. They are one of the leading cause of nosocomial infections in humans (Huycke *et al*., 1998) ranking as the second most common agent of blood, third in catheter associated urinary tract infections, tenth in ventilator associated pneumonia, third in surgical site infections (Hildron *et al*., 2008; Wisplinghoff *et al*., 2004) and the second leading cause of endocarditis in U.S. (Huycke *et al*., 1998). VRE is a serious problem from public health point of view as they are capable of transferring vancomycin resistant *vanA* to other bacteria such as *Staphylococcus aureus* (Weigel *et al*., 2003). They have been observed to spread their resistance genes to other species including *Listeria monocytogenes*, and *Bacillus* species (Kuhn *et al*., 2000; Leclercq *et al*., 1996). Thus antibiotic resistant enterococci are a serious threat to public health from the perspective of treatment failure.

The factors that limit the choice of antimicrobial for the treatment of these organisms include intrinsic resistance to antibiotics and ability to acquire resistance to antibiotics including chloramphenicol, erythromycin, tetracycline and vancomycin primarily through conjugation (Walsh, 2000).

Enterococci are intrinsically resistant to most beta-lactam antibiotics. These cell wall active antibiotics (penicillins, cephalosporins) were used in combination with aminoglycosides (streptomycin, gentamicin, kanamycin) in the treatment of enterococcal infections. In the early

1970's, streptomycin resistance followed by gentamicin resistance was reported in enterococci thus exhibiting resistance to cell wall active antibiotics-aminoglycosides combination (Mollering, 1990). In 1983, Murray and Mederski-Samoraj reported the β-lactamase determinant in *E. faecalis* that was believed to be acquired from *Staphylococcus aureus* and resulted in major concern associated with treatment.

Emergence of vancomycin resistant enterococci provided another therapeutic challenge. Vancomycin is an antibiotic of last resort in the treatment of Gram positive bacterial infections including enterococcal infections. Emergence of vancomycin resistant enterococcal strains and the risk of transmission of resistance genes to the susceptible bacteria poses a serious risk to public health (Pearson, 2002). Presence of VRE in clinical patients results in a 20% increase in treatment failure, and mortality is also increased from 27-52% (Brown *et al*., 2006); Walsh, 2000).

The contribution of enterococci to the problem of antimicrobial resistance is associated with its ability to pass the resistance determinants to other bacteria of the same species or different species by the process of conjugation. Thus, resistance gene transfer to pathogenic species and emergence of new type of resistance is a serious concern associated with these bacteria. Genome sequences have revealed that one-fourth of the total genome of *E. faecalis* V583 is composed of mobile genetic elements (Paulsen *et al*., 2003). About three to five coresident plasmids are commonly found in clinical isolates (Dunny *et al*., 1981; Tomich *et al*., 1979).

The sex pheromone system in *E. faecalis* plays an important role in activating the conjugative system and transfer of pheromone inducible plasmids. There are two types of conjugative plasmids present in enterococci: pheromone responding plasmids and the plasmids

that do not use pheromone signals for conjugal transfer. Pheromone inducible plasmids are conjugative elements present in the donor cells that respond to the pheromone produced by the recipient cells and initiate the mating process. Pheromones are peptides made of 7-8 amino acids and are encoded on the chromosome of most of the *E. faecalis* strains. Pheromones are produced from the donor and recipient cells but the donor synthesizes inhibitor peptides to prevent self induction. The donor produces pheromone binding protein on its cell surface. The pheromone binding protein recognizes the pheromone produced by the recipient cells and this protein along with a chromosomally encoded oligopeptide permease system import the pheromone into the cytoplasm of the donor cell. Transport of pheromone into the donor cell and its binding to a negative regulator of expression of transfer genes stimulates the induction process in the donor cell. Thus there is activation of conjugal gene expression along with cell surface adhesin. This cell surface adhesin mediates cell to cell contact followed by formation of a mating channel between the donor and recipient cell through which the plasmid transfer occurs. After acquisition of the plasmid by the recipient cell, synthesis of cell surface adhesion stops, and expression of inhibitor peptides and negative regulator of expression of conjugation function begins (Chandler *et al*., 2004). Each *E. faecalis* strain possesses only one pheromone plasmid and it will not excrete the corresponding pheromone. However, it can excrete another pheromones corresponding to other pheromones plasmids that it lacks. Thus the strains can accumulate many conjugal plasmids (Wirth, 1994). These plasmids are encoded by antibiotic resistance and virulence traits such as hemolysin and bacteriocin. Thus *E. faecalis* can be a gene pool for other bacteria (Clewell, 1981).

1.8.2 Virulence factors

There are several virulence factors associated with the pathogenecity of these species example host cell attachment (aggregation substance, enterococcocosal surface protein) and cell and tissue damage (cytolysin, gelatinase). Gelatinase, a zinc metalloendopeptidase can hydrolyse host collagen which helps in formation and remodeling of tissue through its extracellular matrix degrading function. It provides nutrients to the bacteria by degrading the host tissue (Gilmore, 2002). Unregulated activity of gelatinase causes several pathological conditions. Reports indicate the involvement of *gelE* in the severity of systemic diseases (Eaton *et al*., 2001).The regulation of the protease, gelatinase occurs through a quorum sensing system encoded by *fsr* (fecal streptococci regulator), a locus similar to staphylococcal *agr* locus (Peng *et al*., 1998). Bacterial population communicates via production of autoinducing peptides, which increase in concentration as the cell density increases. These peptides enter/reenter the cells and causes transcription regulation (Alksne and Projan, 2000; Gobbetti *et al*., 2007). The *fsr* locus is comprised of three genes: *fsrA*, *fsrB*, *fsrC*. In this system, GBAP (gelatinase biosynthesis activating pheromone), an autoinducer peptide is synthesized by *fsrB*. The *gelE* gene encoding gelatinase is located downstream of *fsrC*. GBAP interacts with *fsrC*, a signal transducer and cause phosphorylation of *fsrA*, the response regulator. This is followed by the autoregulation of *gelE* gene (Gilmore, 2002).

Cytolysin and aggregation substance are encoded on conjugative elements. Cytolysin causes lysis of erythrocytes and is encoded on pheromone responsive plasmids near the genes for aggregation substance or within pathogenecity islands in close proximity to the *esp* gene encoding enterococcal surface protein (Shanker *et al*., 2002). Expression of cytolysin is regulated by a quorum sensing system. Aggregation substance, a surface glycoprotein induces the

clumping of bacterial cells. Along with other virulence factors such as enterococcal surface protein, aggregation substance mediates the adhesion of bacteria on the host cell surface. When the cell density increases to a level required for quorum sensing, it results in autoinduction of cytolysin (Gilmore, 2002).

Aggregation substance is a pheromone responsive surface protein produced by *E. faecalis.* During the process of conjugation, aggregation substance is produced by the donor cell in response to pheromone produced by the recipient cell. This facilitates close cell to cell contact between donor and recipient cell resulting in the clumping and transfer of conjugative plasmids between the cells (Jett, 1994). In addition to conjugal transfer mechanism, it also helps in the binding of bacteria to the host cell (Kreft *et al*., 1992). Sussmuth *et al*.,(2000) has shown that aggregation substance helps the bacterium to invade the host immune system by opsonin independent binding, adhesion, and internalization of *E. faecalis* by macrophages. In the same study, the authors also showed that aggregation substance positive strains were able to escape from phagocytosis by inhibiting the respiratory burst.

Enterococcocal surface protein (*esp*) is associated with the bacterial cell wall. The close proximity of *esp* to cytolysin operon which is regulated by a quorum sensing mechanism, helps in the biofilm formation due to the accumulation of large number of bacteria. *Esp* also contributes to cell adhesion, colonization, and evasion of immune system (Moreno *et al*., 2006).

1.9 Epidemiology of antibiotic resistant (AMR) enterococci in dogs

Enterococci are a leading cause of nosocomial infections in humans (Richards *et al*., 2000) including urinary tract infections, hepatobiliary sepsis, endocarditis, surgical wound infection, bacteremia and neonatel sepsis (Poh *et al*., 2006). In the U.S., the therapeutic cost associated with treating ~800,000cases/year of enterococcal infections is estimated to be $0.5

billion (Tendolkar *et al*., 2003). The following reviews the studies done on antibiotic resistant enterococci isolated from dogs from different parts of the world.

1.9.1 United States

Simjee *et al*., (2002) studied AMR in clinical enterococcal isolates from dogs. Urine samples were collected from dogs with UTI at Michigan State University Veterinary Teaching Hospital. A total of 35 enterococcal isolates were recovered and evaluated for susceptibility to gentamicin, vancomycin, erythromycin, penicillin and tetracycline antibiotics. The isolates belonged to four different species: *E. faecium* (n=13), *E. faecalis* (n=7), *E. gallinarum* (n=11), *E. casseliflavus* (n=4). Most isolates were resistant to three or more antibiotics. One *E. faecium* isolate showed high level resistance to gentamicin, vancomycin, tetracycline and penicillin. Molecular and PFGE analysis revealed the presence of the transposon *Tn1546* carrying *vanA* gene, which has been reported to be found only in human vancomycin resistant isolates in the U.S.

In a recent report, 420 enterococcal isolates were recovered from nasal, teeth, rectal, belly and hindquarters of clinically healthy (155) dogs and (121) cats that came to veterinary clinics in Athens, GA, (Jackson *et al*., 2009). A total of 80% of dogs and 60% of cats were positive for enterococcal isolates. *E. faecalis* was the most dominant species in dogs and *E. hirae* from cat samples. Based on the antimicrobial susceptibility testing for 17 antibiotics, the highest level of resistance was observed to ciprofloxacin, chloramphenicol and gentamicin in dog samples while in cat samples resistance was observed for nitrofurantoin. Multidrug resistance was observed to a minimum of 2 and maximum of 8 antibiotics. The same authors studied the mechanism of antimicrobial resistance. Among the tetracycline resistant strains, five *tet* genes were detected with *tet* (M) (60%) being the most prevalent. About 96% of erythromycin resistant strains

harboured *erm (B).* The majority of kanamycin resistant isolates were positive for *aph(3')-IIIa* gene. Gentamicin and streptomycin resistant isolates showed the presence of bifunctional aminoglycoside resistance gene, *aac(6')-Ie-aph(2')* and *ant(6')-Ia*, respectively. PFGE analysis revealed genetic relatedness of AMR enterococcal strains. Dogs and cats from the same geographical area share the same profile, indicating the possibility of exchange of resistance traits and resistant bacteria between dogs and cats. About 80% of the dogs positive for enterococci were boarded at the clinic. The authors concluded that this increased MDR enterococci isolated from different body parts could be a source of infection in humans.

1.9.2 European countries

Denmark

Damborg *et al.*,(2008) monitored the prevalence of antibiotic resistance in indicator bacteria: *E. coli* and *Enterococcus* spp. in healthy dogs. Fecal swabs were collected from 127 dogs and all *E. coli* (n=117) isolates were screened for resistance to 12 antibiotics, and *E. faecalis* (n=51) and *E. faecium* (n=10) isolates were screened for 10 antibiotics. Based on the questionnaire study, a significant association was observed between the antibiotic treatment and resistance in *E. coli*. Ampicillin resistant *E. faecium* was found in two dog fecal swabs. Genotyping of these two isolates by multilocus sequence typing revealed that two isolates belonged to sequence types including those associated with human nosocomial infections and one isolate was related to clonal complex 17, an important nosocomial pathogen.

Belgium

Devriese *et al.*,(1996) examined the prevalence of Vancoycin resistant enterococci in animals including pet animals from 557 different farms/homes. Vancomycin resistant *E. faecium* was isolated from fecal samples of horses, dogs, chickens, and pigs. Eight percent of *E. faecium* from dog samples were positive for vancomycin resistance. It was concluded that pet animals

could have acquired vancomycin resistant *E. faecium* from the feeding of meat products originating from the farm animals fed with a glycopeptides antibiotic avoparcin as growth promoter.

De Graef *et al*.,(2004) determined antimicrobial susceptibility patterns among *E. coli* and *E. faecalis* from healthy individually-owned and kennel dogs. Isolates were collected from dogs of 92 different owners and 8 breeding kennels. Resistance to antibiotics tested was higher in the *E. coli* strains from kennel as compared to individually owned dogs. Among *E. faecalis*, resistance was observed for gentamicin with high level resistance for tetracycline, and macrolides.

The Netherlands

Wagenvoort *et al*.,(2003) conducted a study to determine the prevalence of vancomycin resistant enterococci in sick urban dogs of Parkstead Limburg, Netherlands. No VRE was recovered from rectal swabs of 100 diseased dogs.

Finland

A study by Rantala *et al*.,(2004) evaluated AMR in canine *Staphylococci, E. coli and* Enterococci. The study included two groups: treatment group (n=22) that included dogs with pyoderma infection that received antibiotics and the control group (n=56), which were not exposed to antibiotics for at least for 6 months prior. *E. coli* and *Enterococcus* spp. were isolated from faecal samples and *Staphylococci* from perianal mucosal swab. The total number of colonies isolated for each species was 2 per sample. Susceptibility testing revealed that staphylococcal and *E. coli* isolates from pyoderma dogs were more resistant to sulphamethoprim than the control dogs. Among the enterococcal isolates from both the groups, ampicillin resistance was low (4-7%) and all the enterococcal isolates were resistant to vancomycin.

New Zealand

Manson *et al*.,(2003) reported vancomycin resistant *E. faecalis* (VREF) from a dog with mastitis infection. The isolate harbored *vanA*, *erm* (B), and *tet* (M) genes encoding vancomycin, erythromycin and tetracycline resistance, respectively. Pulsotyping and comparison of this isolate with poultry and human VREF isolates from New Zealand revealed clonal lineage of VREF. The isolate also harbored the trasnposon *Tn 1546.*

Portugal

Poeta *et al*.,(2005) evaluated the mechanism of vancomycin resistance in fecal enterococci from healthy dogs, poultry, humans and wild animals. A multidrug resistant *E. faecium* containing *vanA* was detected from one dog of 71. This isolate showed resistance to vancomycin (MIC>128 mg/L), teicoplanin (MIC-64mg/L), ampicillin, kanamycin, tetracycline, erythromycin and ciprofloxacin. They also identified *vanA* among fecal enterococci from poultry and humans. All isolates from wild animals were negative for the presence of the *vanA* gene.

The same authors evaluated the antimicrobial resistance and mechanism of resistance in fecal enterococci from healthy dogs, poultry, and humans that were not exposed to antibiotics for 4 months prior to sample collection. Among the collection of 440 enterococcal isolates, *E. faecium* (n=227), *E. faecalis* (n=177), *E. hirae* (n=32) and *E. durans* (n=4) were present. Among the isolates from different sources, poultry isolates showed the highest level of resistance followed by pet isolates with intermediate resistance to ampicillin, tetracycline, erythromycin and chloramphenicol. *E. faecium*, *E. faecalis*, *E. hirae* were identified from the canine fecal isolates. When tested for susceptibility to 11 antibiotics, canine isolates showed a lower level of resistance (10%) for most antibiotics except for tetracycline (50%), erythromycin (47%) and kanamycin (18%). *E. faecium* were resistant to more antibiotics as compared to *E. faecalis* and *E. hirae* among canine fecal samples. Tetracycline and erythromycin resistance was higher among *E. faecalis* (Poeta *et al*., 2005).

Morris *et al*.,(2007) studied the susceptibility to antimicrobials among enterococci isolated from dogs with UTI (n=40), otits externa (n=11) and pyoderma (n=4) infections. Eighty four percent of isolates were *E. faecalis* and 10.9% were *E. faecium* while the remaining 5.5% were *E. durans*, *E. gallinarum* and *E. hirae*. Higher level of resistance to aminoglycosides (gentamicin [12.7%]; streptomycin [36.4%]; gentamicin+streptomycin [10.9%] and fluoroquinolones (ciprofloxacin [45.5%]; levofloxacin [41.8%]; moxifloxacin [41.8%]) were observed. All the isolates were susceptible to ampicillin, penicillin and vancomycin

Spain

Torres *et al*.,(2003) and Herrero *et al*.,(2004) reported higher percentage of *vanA* positive enterococcal isolates from companion animals in Spain. Torres *et al.* analyzed VRE from fecal samples of healthy pigs (n=66) and pets (dogs+cats=22). VRE were detected in 4.5% pig samples and 22.7% pet samples. All the *van A* positive isolates harbored *tet* (M) while 6 isolates were positive for *erm* (B) gene and 3 isolates were positive *aac(6')-Ie-aph(2")-Ia* gene.

Herrero *et al*.,(2004) investigated the presence of VRE in 87 diseased dogs in Spain. Among 87 dog fecal samples, they isolated 15 VRE strains, 11 belonging to *E. faecium* and 4 *E. gallinarum*. Most of the vancomycin resistant *Enterococcus faecium* (VREFe) were resistant to multiple antibiotics, example: tetracycline (n=11), erythromycin (n=10) and bacitracin (n=10), chloramphenicol (n=6), ciprofloxacin (n=6), streptomycin (n=5), penicillin (n=2), and quinupristin-dalfopristin (n=1) with only one VREFe isolate susceptible to erythromycin and bacitracin.

Italy

A study was carried out in Italy by Ossiprandi *et al*.,(2008) to determine the prevalence of antibiotic resistance in fecal enterococcal strains from dogs exposed or not exposed to antibiotic treatments. The treatment group included the dogs exposed to at least one antibiotic(s)

dose for 6 months prior to the study. All isolates showed high level of resistance to erythromycin, tetracycline, rifampicin and enrofloxacin. None of their isolates were resistant to vancomycin. There was no significant difference in the resistance profile between both groups except for *E. faecium* from treatment group that showed high resistance to tetracycline antibiotic.

1.10 Summary

Enterococci, the normal gut commensal of many animals, are also present in the GIT of dogs. However, during last few years, their importance is increasing from the medical point of view. The enterococci have emerged as a medically important nosocomial pathogens contributing mortality rates up to 61% (De Fatima Silva Lopes *et al*., 2005). Stress in animals selects for antibiotic resistant bacteria (Langlois *et al*., 1999; Corrier *et al*., 1990) and causes increased shedding of resistant (Moro *et al*., 2000; Moro *et al*., 1998) and pathogenic (Whyte *et al*., 2001; Pfeiffer *et al*., 2009) bacteria in the feces of cattle (Corrier *et al*., 1990; Pfeiffer *et al*., 2009), swine (Moro *et al*., 2000; Moro *et al*., 1998;) and poultry (Whyte *et al*., 2001). However, this has not been investigated in dogs. Dogs are prone to stress due to several causes such as change in environment, mistreatment by owners and others, physical pain, fear, aggression and illness. Resistant bacteria, if shed in the feces of stressed dogs, can be transmitted to the people in contact with them, such as veterinary staff or dog owners and thus in theory could be a source of opportunistic infections.

Published research on the prevalence of antibiotic resistant enterococci from dogs in the U.S. is sparse. The most recent publication documenting the prevalence of antibiotic resistant enterococci in U.S. dogs was by Jackson *et al*.,(2009), where they reported the presence of antimicrobial resistant enterococci from different body parts of healthy animals. They found that *E. faecalis* was the most dominant species in dogs and was isolated more from the rectal samples. However, these data are not sufficient to represent the entire gut, as the sample size in the study was very small (n=86 from rectal samples collected from 155 dogs). Although this study highlighted the risk associated with the transfer of resistant bacteria from pet animals to

humans, it does not provide information about of the prevalence of antibiotic resistant enterococci in the gut of pet animals.

Other studies documenting trends in antibiotic resistant enterococci in dogs have been reported from different parts of the world (Damborg *et al*., 2008; De Graef *et al*., 2004; Wagenvoort *et al*., 2003; Rantala *et al*., 2004). To date, there is only one study in the literature reporting the prevalence of antimicrobial resistant enterococci in dogs with pyoderma (Rantala *et al*., 2004). In this study the authors tested for resistance to ampicillin and vancomycin among the enterococci recovered from the dogs with pyoderma infection and compared with control group, healthy dog isolates. They reported low prevalence of resistance to these antibiotics in enterococcal isolates tested. The findings of this study are expected, as vancomycin is not the drug of choice for treatment of infections in dogs. The explanation for finding low ampicillin resistant enterococcal strains is because this antibiotic is not incorporated in their treatment regimen during the course of study. Moreover, the sample size was small to represent the entire gut microbiota. No veterinary studies have been published determining the effect of pyoderma infection on the antimicrobial resistance and virulence in enterococci of intestinal microbiota of dogs.

1.11 Research objectives

In the present study I hypothesized that stress due to pyoderma infection in dogs can increase the antibiotic resistance in the fecal microbiota and may play a role in the dissemination of antibiotic resistance genes.

The two main research goals are:

1. To assess the role of dogs as a potential reservoir of clinically important enterococci.
2. To investigate a potential correlation between stress from pyoderma and antibiotic resistance and virulence frequency in enterococci from the intestinal tract of dogs.

Specific objectives

1. Determine the prevalence, phenotypic and genotypic characterizations of antibiotic resistance and virulence traits, intra-species resistance gene transfer and clonal population structure of antibiotic resistant enterococcal strains from healthy and pyoderma infected dogs.
2. Assessment of overall bacterial diversity within the intestinal microbiota of healthy dogs.
3. Assessment of overall diversity of antibiotic and metal resistance genes in the feces of healthy dogs

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Chapter 2

Population size, diversity, polyphasic characterization of antimicrobial resistance and virulence traits, horizontal transfer of resistance genes and clonal structure of enterococci from the feces of healthy and dogs with pyoderma

2.1 Abstract

Enterococci, the commensal organism of humans and animals, are well known to be carriers of antibiotic resistance genes and for their contribution to the nosocomial infections in humans. Some studies have shown that changes in the antimicrobial resistance phenotypes in the gut microbiota are associated with the stress conditions in animals such as transportation, overcrowding and rough handling. To determine whether the gut microbiota of healthy and pyoderma infected dogs serves as an antimicrobial resistance reservoir, 180 enterococcal isolates from fecal samples of 9 dogs in each group were cultured, enumerated, identified, and screened for resistance to 10 antibiotics and for 4 virulence traits. None of the animals in the either group received antibiotic treatment for at least 7 months prior our sampling. The intra-species transferability of resistant determinants was investigated by the horizontal gene transfer assay. Clonality of selected *E. faecalis* and *E. faecium* isolates was assessed by pulsed field gel electrophoresis (PFGE).

The most prevalent species were *E. faecalis* (44.0%) and *E. faecium* (38.0%) from pyoderma dogs and *E. faecalis* (70.5%) and *E. hirae* (22.2%) from healthy dogs. Enterococci isolated from pyoderma infected dogs were more resistant to antibiotics than those from healthy dogs. Overall, *E. faecium* were resistant to more antibiotics than any other species. Resistance to

erythromycin was most frequent in the pyoderma group [36.6%] and tetracycline in the healthy group [17.7%]); no isolates were resistant to vancomycin or linezolid. The most prevalent *tet* genes were *tet*(M) and *tet*(S) in both animal groups. Most (≥ 80%) of the erythromycin resistant isolates were negative for *erm*(B) gene. No mutation within codon 83 and codon 87 of *gyr*A was detected in fluoroquinolone resistant strains. The *gelE* and *esp* genes were the most prevalent virulence genes in *E. faecalis* and *E. faecium* in both groups with the exception in *E. faecium* from healthy animals, where no *esp* gene was detected. The antibiotic resistance traits were intra-species transferable among *E. faecalis* (tetracycline, erythromycin, and doxycycline) and *E. faecium* (tetracycline) by *in-vitro* conjugation. PFGE analysis revealed that healthy animals harbored more genetically diverse *E. faecalis* as compared to that of pyoderma dogs. The overall study results show that the prevalence of antibiotic resistant enterococci in pyoderma dogs as well as healthy dogs was low. The association between pyoderma infection and higher antimicrobial resistant enterococci in these dogs may be related to stress due to the infection. Our findings help to better understand the role of pet dogs as a reservoir for antibiotic resistant enterococci and transfer of resistant bacteria and antimicrobial resistance genes to people.

Key words: Dogs, antimicrobial resistant enterococci, pyoderma

2.2 Introduction

Antibiotic resistant pathogens are causing substantial burden to public health by confounding the clinical treatment of patients (Andrighetto *et al*., 2001; Baumgartner *et al*., 2004). Antimicrobial resistant infections cause approximately $4 billion treatment cost in the United States per year (Stacey *et al*., 2003). Antimicrobial resistance has become a serious issue in bacteria of animal and human origin both in terms of clonal spread (Kuhn *et al*., 2000; van den Bogaard and Stobberingh, 2000) and exchange of resistance genes between human and animal bacteria (DuPont and Steele, 1987; Phillips *et al*., 2004). These factors may contribute to increasing challenge to treatment of resistant infections which ultimately may lead to increased morbity and mortality. Hence, the World Health Organization has recommended developing surveillance programs to monitor antimicrobial resistance in humans, food animals and retail foods (WHO, 2001).

The problem of antimicrobial resistance in enteric bacteria has become a major issue in terms of public health (Marano *et al*., 2000; WHO, 2000; Tollefson *et al*., 1999). The commensal bacteria of gastrointestinal tract (GIT) may serve as a reservoir of antimicrobial resistant organisms and resistance genes for pathogenic or zoonotic bacteria (Capriolo *et al*., 2000). One such enteric bacterial group that gained the global attention are enterococci, due to their role as a reservoir for antibiotic resistance genes and for being one of the leading causes for nosocomial infections (Marshall, 2009; Huycke *et al*., 1998). They are regular inhabitants of the intestinal tract of animals and humans. Although a variety of enterococcal species are present in healthy individuals, enterococci are also opportunistic pathogens. Over the past three decades, the overall incidence of enterococcal infections has increased as the second most important cause for urinary

tract infections (Huycke *et al*., 1998), and the third most frequent cause for nosocomial blood infections and endocarditis in the U.S. alone (Wisplinghoff *et al*., 2004; Mc Donald *et al*., 2005). Not only do enterococci carry antimicrobial resistance genes, but they are also well known for their ability to transfer them to bacteria of the same or different genera (Weigel *et al*., 2003). Thus enterococci have emerged as a medically important antimicrobial resistant and virulent pathogen (Tendolkar *et al*., 2003; Upadhyaya *et al*., 2009). Besides the digestive tract, enterococci are also widely distributed in the environment including food, water, soil and plants (Gilmore, 2002; Macovei and Zurek, 2006).

Selection pressure due to antibiotic use is often considered to be the major factor for dissemination and maintenance of antimicrobial resistance in bacteria. However, there are also data showing antimicrobial resistance in animal populations never exposed to antibiotics (Langlois and Dawson, 1999). Animal stress has been implicated as one of the factors responsible for increase of antibiotic resistant enteric bacteria (Langlois and Dawson, 1999; Corrier *et al*., 1990) and increased pathogen shedding (Corrier *et al*., 1990; Hussein *et al*., 2001; Issacson *et al*., 1999; Schwartz, 1991). Animals are prone to stress and that has severe effect on overall physiology, health and productivity of animals (Moberg and Mench, 2000). While most research has focused on people and food animals, the importance of companion animals as a reservoir of bacterial pathogens and antibiotic resistance genes is unknown. According to the statistics from the U.S. pet ownership and demographics sourcebook, in 2006, pet dogs accounted for more than 72 million in U.S. and the average expenditure cost per household per pet was estimated to be $366. (http://www.avma.org/reference/marketstats/sourcebook.asp). Companion animals play an important role in the society. Dogs are used as a therapeutic purpose for elderly and mentally ill people and to relieve stress. Studies have shown that pets are very

sensitive to their owners stress levels. Stress due to temperature, crowding, starvation or other dietary conditions, fear and other extreme emotions can cause disturbances in the gut microbiota (Moon *et al*., 1979; Savage, 1982; Tannock, 1983) and contribute to the increase of antimicrobial resistance (Sorum and Sunde, 2001). Some studies have reported increased shedding of drug resistant intestinal bacteria such as *E. coli* animals stress due to shipping and moving, transportation, overcrowding, and holding in a pen (Moro *et al*., 2000; Moro *et al*., 1998; Molitoris *et al*., 1987; Langlois *et al*., 1986). A higher prevalence of resistance in *E. coli* was found in swine herds not exposed to antimicrobial agents (Langlois *et al*., 1988) after shipping (Molitoris *et al*., 1987) and exposure to extreme cold or heat conditions (Moro *et al*., 1998, 2000). An association between stress in animals and altered GIT has been reported (Kiliaan *et al*., 1998; Gangrosa, 1977). These changes include altered gut microbiota, pH, motility and decreased integrity of intestinal epithelium (Schaedler and Dubos, 1962; Kiliaan *et al*., 1998; Enck, 1989). Studies suggest that such intestinal changes are caused by the increased corticosteroids, the neuroendocrine mediators secreted from central nervous system and endocrine system due to stress imposed on animals (Enck, *et al*., 1989). During the episodes of stress, these hormones are released in the gut by the enteric nervous system and affect the commensal bacteria (Freestone *et al*., 2000; Zhou *et al*., 2004; Dreau *et al*., 1999) as well as pathogens such as *E. coli* (Chen *et al*., 2006; Vlisidou *et al*., 2004; Green *et al*., 2003), *Salmonella* (Chen *et al*., 2006; Green *et al*., 2003), *Campylobacter* (Cogan *et al*., 2007) and *Vibri*o (Nakano *et al*.,2007). The role of these stress hormones in relation to increased growth, motility and virulence of bacteria is also documented (Sperandio *et al*., 2003). Evidence suggests that these stress hormones can cause upregulation of bacterial genes required for colonization and virulence (Bansal *et al*., 2007).

In USA, there are several surveillance programs including National Antimicrobial Resistance Monitoring system, FDA, and CDC that monitor the trends in antibiotic resistance in bacteria of human and food animal origin; however, none of them include companion animals. Considering the fact that dogs share the common environment with people, their close physical contact, and use of same antibiotics for their therapeutic purpose (Guardabassi *et al*., 2004), there is a high potential for transmission of resistant bacteria or mobile genetic elements between pets and humans (Pedersen *et al*., 2007).

Recent evidence suggests that dogs can act as a reservoir for antimicrobial resistant enterococcal strains (Simjee *et al*., 2000). Despite this fact, very few studies are available on the epidemiology of antibiotic resistance in healthy dog's microbiota (Hirsh *et al*., 1980; Monaghan *et al*., 1981; Devriese *et al*., 1996; van Belkum *et al*., 1996). The studies published from countries other than U.S. on diseased or healthy dogs have reported decreased susceptibility to antibiotics among the enterococcal strains and most of these studies focused on investigating the prevalence of vancomycin resistant enterococci.

There are only two scientific reports from the U.S. documenting antibiotic resistant enterococci of the canine fecal origin (Simjee *et al*., 2002; Jackson *et al*., 2009). Because of the difference between theses two studies in terms of study design, methodology, and reports documenting the prevalence of resistant enterococi, estimation of an overall prevalence of antimicrobial resistance in enterococci of canine gut is difficult. Until now, no published data from U.S. has evaluated the prevalence of antimicrobial resistance in enterococci from dogs with pyoderma infection. Additionally, the only study available in the literature demonstrating the prevalence of antibiotic resistant enterococci from pyoderma infected dogs was conducted in Finland (Rantala *et al*., 2004). However, the data available from this study are not representative

of resistance prevalence of enterococci from GIT of dogs due to the small sample size (enterococcal isolates in control group [73] versus pyoderma group [37]). We hypothesized that, stress due to pyoderma, a common bacterial infection in dogs, is correlated with higher antibiotic resistance in the fecal microbiota of dogs and plays a role in the dissemination of antibiotic resistance genes.

The objectives of the present study were i) phenotypic and genotypic characterization of antimicrobial resistance and virulence traits and clonal structure of enterococcal strains isolated from the healthy and pyoderma infected dogs. ii) determine the potential for horizontal gene transfer of antibiotic resistant genes/traits *in-vitro*.

2.3 Materials and methods

2.3.1 Study animals

Two groups of dogs were included in this study: 1) healthy group and 2) pyoderma group

The healthy group included nine clinically healthy household dogs from Manhattan, Kansas. Healthy dogs varied in sex, breed, diet, and age (Table 2.1). According to the history taken from the owners, these animals were not exposed to antibiotics for at least one year prior to sample collection (Table 2.1).

The pyoderma group included nine dogs brought to the Kansas State University, Veterinary Teaching Hospital, Manhattan, Kansas, who were diagnosed and treated for pyoderma. This group also included dogs belonging to different sex, breed, and age. The history of prior antibiotic exposure of theses group of dogs are shown in (Table 2.2). Samples from these dogs were collected on the day of visit and prior to treatment. These dogs were not exposed to antibiotics for at least 7 months before sample collection (Table 2.2).

2.3.2 Fecal sampling and processing

Fecal sample from each dog was obtained either from the rectum by swabbing with sterile cotton- tipped applicator moistened in sterile phosphate buffer saline (PBS) and placed into a sterile tube containing 500 µl of PBS or fresh feces were collected with a sterile glove in a sterile tube. Samples were immediately stored at 4°C and were processed within 24 h after collection.

The details of the primer sets for the PCR used in this study are shown in Table 2.3.

2.3.3 Isolation of enterococci

The following procedures were used to isolate and enumerate enterococci from the canine fecal samples.

2.3.3.1 Selective plating

One gram of fecal sample from the center of the fresh collected feces was mixed and homogenized in 10 ml of PBS (pH 7.2; ICN Biomedicals, Ohio) and dilutions were made up to 10^{-3}. One hundred microliter of each dilution of fecal material was drop plated on m-Enterococcus agar (Becton Dickinson, Massachusetts), and incubated at 37°C for 48 h.

The rectal swabs were processed by properly immersing the swab in the PBS present in the tube followed by spread plating of 100µl of this solution onto m-Enterococcus agar and incubated at 37°C for 48 h.

Twenty presumptive enterococcal colonies with red pigment were picked randomly from the m-Enterococcus agar plate representing each sample and plated onto Trypticase soy agar (Becton Dickinson, Massachusetts), incubated at 37°C for 48 h, and stored at 4°C for further analysis.

2.3.3.2 Bacterial enumeration

Red pigmented colonies from each m-Enterococcus agar plate were counted, and bacterial numbers were expressed as colony forming units per gram (CFU/g) of feces. Mean bacterial count was determined from the triplicate spread plate from the dilution representing each sample.

2.3.3.3 Biochemical test to confirm *Enterococcus* genus

Ninty six microwell plates containing Enterococcossel broth (Becton Dickinson, Massachusetts) (0.2ml) were inoculated with red pigmented enterococcal colonies picked from Trypticase soy agar plates and incubated at 37°C for 24 h for the esculin hydrolysis. Enterococcal species can hydrolyze esculin and results in the development of black colored media. Based on the biochemical reactions each isolate was identified to the genus level.

2.3.4 Species determination

Multiplex PCR was employed for identification of four common species, *E. faecalis*, *E. faecium*, *E. casseliflavus* and *E. gallinarum* (Kuhn *et al*., 2000).

2.3.4.1 DNA preparation

Cell suspension of 3-4 presumptive enterococcal colonies of each isolate were made in 50µl. The boiling method was used to extract DNA from the bacteria (Kariyama *et al*., 2000). One to several colonies from the medium were suspended in 50µl of sterile distilled water with 7.5% Chelex 100 (Bio-Rad laboratories, Hercules, California), heated at 100°C for 15 min in a heating block and centrifuged in a microcentrifuge (6000g for 1 min) and supernatant liquid was collected and stored at -20°C for our multiplex PCR assay.

2.3.4.2 Multiplex PCR

Primers and PCR conditions were chosen according to the guidelines of Kariyama *et al*., (2000) and Dutka-Malen *et al*., (1995). The primers were designed to amplify *ddl* (941 bp), *ddl* (658 bp), $vanC_1$ (822 bp), $vanC_2$ / C_3 (484 bp) and 16S RNA (320 bp) genes for the detection of *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. casseliflavus* and 16S RNA, respectively. The reaction components were as follows: 10x buffer-2.5µl, 25mM $MgCl_2$-0.15µl, 1mM dNTP-2.00µl, 1 unit Taq polymerase-0.20µl, 5 sets primers-5.00µl ddH_2O-12.65µl, template DNA-2.50µl to 25µl/reaction.

The thermal cycling were conducted as follows: denaturation of DNA at 94°C for 5 min followed by 30 cycles of 94°C for 60min, followed by annealing at 54°C for 60 min, elongation at 72°C for 60 min. After 30 amplification cycles, a final extension step was performed at 72°C for 10 min.

The negative control used was *E. mundtii* ATCC-43186.

PCR reaction products were resolved by electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.5µg/ml) in 10X TBE buffer. The DNA size marker was 1500 bp. The gel was visualized on a ultraviolet transilluminator. In case of unclear results, the enterococcal isolates were confirmed by amplification and sequencing of conserved *sodA* gene (superoxide dismutase) approach as described by Poyart *et al*.,(2000).

2.3.5 Antibiotic susceptibility test

2.3.5.1 Phenotypic characterization

Antimicrobial susceptibility testing was performed as recommended by Clinical and Laboratory Standards Institute (Clinical and laboratory standards institute 2000).

Disc diffusion technique for all identified isolates were carried out on Mueller-Hinton agar (Difco) plates with standard antibiotic disks (Becton Dickinson, Franklin Lakes, NJ) using

eight antibiotics, gentamicin (120µg/ml), erythromycin (15µg/ml), tetracycline (30µg/ml), doxycycline (30µg/ml), ampicillin (10µg/ml), ciprofloxacin (5µg/ml), enrofloxacin (15µg/ml), and vancomycin (30µg/ml). The zone of inhibition was measured and compared to the standard zone size predetermined for the enterococcal species and then the isolate was classified as sensitive and resistance. Screening for streptomycin (2000µg/ml) and linezolid (8µg/ml) resistance were performed by using the agar dilution technique in brain heart infusion agar plate. For quality control of disc diffusion and agar dilution techniques, appropriate positive and negative controls were included in this study.

2.3.5.2 Genotypic characterization

All identified isolates that were resistant to tetracycline and erythromycin by disc diffusion technique were screened for *tet* and *erm*(B) genes, respectively. The presence of these resistance genes were tested either by multiplex or single PCR using the gene specific primers and conditions for the amplification of *tet*(A), *tet*(C), *tet*(Q), *tet*(M), *tet*(S), *tet*(K), *tet*(O), *tet*(W) and *erm*(B) genes as described previously (Macovei *et al*., 2006). The group I multiplex PCR was designed to amplify *tet*(A) (210bp), *tet*(C) (418bp), and *tet*(Q) (904bp) genes, and group II covered *tet*(M) (406bp), *tet*(S) (667bp), *tet*(K) (169bp), and *tet*(O) (515bp)genes.

Extraction of bacterial DNA was performed by the boiling method as described earlier. Amplification reaction consisted of 10x buffer-2.5µl, 25mM $MgCl_2$-0.20 µl, 1mM dNTP-2.00µl, 1 unit Taq polymerase-0.30µl, 3 sets of primers-3.00µl (groupI) and 4sets of primers- 4.00µl (groupII), ddH_2O-15.50µl, template DNA-3.00µl to 25µl/reaction. Cycling conditions for group I and II multiplex PCR assay were as follows: denaturation at 94°C for 5 min followed by 24 cycles of 94°C for 1.5 min, followed by annealing at 55°C for 1 min, elongation at 72°C for 1.5 min and final extension step of 10 min at 72°C .

Amplification of *tet*(W) (168bp) and *erm*(B) (639bp) genes were performed by single PCR respectively. The reaction mixture consisted of 25 µl Master Mix with 10x buffer-2.5µl, 25mM $MgCl_2$-0.20 µl, 1mM dNTP-2.00µl, 1 unit Taq polymerase-0.30µl, 1set primers-2.00µl (*tet*(W) or *erm*(B)), ddH_2O-16.50µl, template DNA-3.00µl from freshly boiled cells. Cycling parameters were as follows: a) For *tet*(W) - denaturation at 94°C for 5 min followed by 24 cycles of 94°C for 30 sec, followed by annealing at 64°C for 30 sec, elongation at 72°C for 30 sec and final extension step of 7 min at 72°C. b) For *erm*(B)- denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30 sec, followed by annealing at 54°C for 30 sec, elongation at 72°C for 30 sec and final extension step of 4 min at 72°C.

PCR was used to amplify the regions of *gyrA* gene in quinolone resistant strains isolated in this study (Korten *et al*., 1994) and the nucleotide sequence in the amplified regions were determined by 3730 DNA analyzer for sequencing (Applied biosystems). The sequences containing quinolone resistance determining region (QRDR) were aligned and compared with quinolone susceptible and resistant strains of enterococcal species. Conversion of the nucleotide sequences into corresponding amino acid sequences were performed by using Sequencher 4.7 (Gene Codes Corporation, AnnArbor, MI) (Table 2.7)

2.3.6 Virulence factors

2.3.6.1 Phenotypic characterization

In the gelatinase assay, enterococcal strains were inoculated in Trypticase soy agar supplemented with 3% skim milk. Plates were incubated for 24 hrs at 37°C. Formation of a clearance zone surrounding the colonies was monitored (Gilmore *et al*., 2002).

The assay for hemolytic activity were evaluated by plating the enterococcal strains on Columbia blood agar (Becton Dickinson, Franklin Lakes, NJ) supplemented with 5% human

blood. Strains were streaked on the surface of the plate and incubated at 37°C for 48-72 h. The criteria for cytolysin expression were determined as development of complete clearance zone around the colonies (Gilmore *et al*., 2000). *Enterococcus faecalis* strains, OG1X (pAD) and OG1RF (pAD) were used as positive control and V583 as negative control.

Production of aggregation substance in the presence of sex pheromone was conducted by clumping assay (Dunny *et al*., 1978). For the phenotypic expression of the *asa1* gene, *E. faecalis* JH2-2 were grown for 6 h at 37°C in Todd-Hewitt broth followed by centrifugation at 6,000 rpm for 10 min on a Sorvall RC-5B refrigerated (4°C). The supernatant contains the pheromone that causes induction of pheromone-responsive plasmids. This supernatant was removed and autoclaved for 15 min.One milliliter of the supernatant was added to Todd-Hewitt broth (5 ml) containing 6 h grown enterococcal isolates. After incubating overnight at 37°C on a shaker (150 rpm), isolates that showed clumping (examined by naked eye and under a compound microscope) were considered positive for aggregation substance expression. *E. faecalis* OG1RF (pCF10) was used as a positive control.

2.3.6.2 Genotypic characterization

Multiplex PCR for the detection of the following virulence genes were performed: *gelE*, *asal*, *cylA*, and *esp* coding for gelatinase, aggregation substance, cytolysin and enterococcus surface protein respectively, for all the identified isolates (Vankerckhoven *et al*., 2004). The reaction mixture for targeting these virulence genes consisted of 10x buffer-2.5µl, 25mM $MgCl_2$-0.20 µl, 1mM dNTP-2.00µl, 1 unit Taq polymerase-0.30µl, 4sets Primers-5.00µl, ddH_2O-13.50µl, template DNA-1.5µl from freshly boiled cells for 25 µl master Mix/ reaction. Conditions for the PCR were as follows: denaturation at 94°C for 5 min followed by 29 cycles of

94°C for 1 min, followed by annealing at 54°C for 1 min, elongation at 72°C for 1.5 min and final extension step of 10 min at 72°C.

2.3.7 Pulsed-field gel electrophoresis (PFGE)

PFGE was performed performed in CHEF-MAPPER ™ (BIORAD) at 14°C in 5X TBE as described by Murray *et al*., (1990).

2.3.7.1 Casting PFGE plugs

1.6% SeaKem Gold® agarose (FMC trademark) was prepared in 25ml distilled water and 2.5ml 10% SDS and kept at 56°C waterbath until use.

Following overnight incubation of bacterial suspension in brain heart infusion broth (5ml), samples were adjusted to optical density 1.2nm. The cells were pelleted by centrifugation at 11000rpm for 1.5 min. The pellet was suspended in 500µl of 0.85% NaCl and centrifuged. Finally the pellet was suspended in 200µl of 0.85% NaCl and kept on the hot plate at 50°C for 10 min. Plugs were casted by mixing the cell suspension with 250µl of 1.6% SeaKem Gold® agarose and poured into two plug molds and allowed to solidify for 25 minutes at room temperature.

2.3.7.2 Lysis of cells

Solidified plugs were transferred to the 10 ml lysis buffer (1M Tris-HCl (pH-7.4), 5M NaCl, 0.5M EDTA, 20% Sodium Lauroyl Sarcosine, 10% Brij, 10% Deoxycholate, distilled water, 500µg/ml lysozyme, 20µg/ml RNase A) They were then incubated at 37°C in a water bath with shaking at 100rpm for 4hrs. Then the plugs were removed from the lysis buffer and transferred to a new tube with 10ml ESP buffer (10mM Tris-HCl (pH-7.4), 1mM EDTA, 1%

SDS, 50µg/ml Proteinase K) and incubated at 50°C in a water bath with shaking at 80rpm for 16hrs.

2.3.7.3 Washing plugs

Washing of the plugs was performed one time for 20 minutes followed by four times for 10 minutes. Each time the washing was done in 10ml of fresh TE buffer (0.5M EDTA; 1M Tris HCL (pH-7.4)).

2.3.7.4 Restriction enzyme digestion

One third of the washed plug was cut and equilibrated by placing in 1.5ml microcentrifuge tube containg10µl bufferA and 90µl autoclaved distilled water. After incubation at at room temperature for 10 minutes, the buffer solution was aspirated off. Clevage of the agarose embedded DNA was achieved with 0.5µl *Apa*I in 10µl of buffer and making the volume to 100µl with distilled water and incubating at 37°C waterbath for 4 hours.

2.3.7.5 Loading of plugs

After digestion, the restricted fragments were separated by electrophoresis at 1% SeaKem Gold® agarose (FMC trademark) prepared in 100ml distilled water and 10ml 5X TBE buffer.

After digestion of plugs, the digestion mixture was aspirated off and incubated in 200µl 0.5X TBE buffer at room temperature for 10 minutes. Then the plugs were placed on the 0.75mm gel comb and allowed to remain there at room temperature for 30 minutes. The gel comb was then placed in the casting tray and 100ml 1% SeaKem Gold® agarose (FMC trademark) was poured and allowed to solidify at room temperature for 45 minutes. Following solidification, the comb was removed and the wells were closed with1% SeaKem Gold® agarose (FMC trademark).

2.3.7.6 Electrophoresis parameters

Initial switch time 1 second; final switch time 20 seconds; temperature 14°C; total run time 21 hours; included angle 120**;** voltage 6 V/cm.

2.3.7.7 Gel staining

Gels were stained with 1% ethidium bromide for 30 minutes at room temperature followed by two times 20 minutes destaining in distilled water.

To estabilish the clonal relatedness and diversity among the drug resistant *E. faecium* and *E. faecalis* from healthy and diseased groups, the PFGE pulsotypes were analyzed after importing the gel images into BioNumerics software. Dendrograms were created using Dice similarity coefficient (1% optimization) and unweighted pair group method with arithmetic means (UPGMA) clustering analysis with 1.5% band position tolerance. The *E. faecium* ATCC 19454 was used as reference strain.

2.3.8 Conjugation assay

Conjugation assay was performed for antibiotic resistant *E. faecalis* and *E. faecium*. Selection of antibiotics on the agar plate was done based on the resistance pattern of the donor and recipient. Conjugation frequencies were determined by filter mating and broth mating assays.

Broth mating and filter mating were performed in overnight grown (37°C) cultures of donor and recipient strains as described previously (Dunny *et al*., 1978; Tendolkar *et al*., 2006). The broth mating was performed as follows: overnight grown bacterial cultures in brain heart infusion broth (BHI) (5ml) were mixed in a ratio of 1:10 donor: recipient in 4.5ml of fresh BHI broth. This broth containing donor recipient culture was incubated at 37°C for 4 h.

For filter mating, 1:10 donor: recipient ratio of overnight grown bacterial culture was passed through autoclaved 0.22µm pore size nitrocellulose membrane filter (Whatman International Ltd., Germany). This membrane filter was placed on the BHI agar plate with bacteria side up and incubated at 37°C for 16 h. Following incubation, cells from the filter were harvested by suspending the filter in 1ml BHI broth.

For both the mating procedures, controls, donor and recipient cultures were serially diluted and spread plated on BHI plate containing appropriate selective antibiotics. Colonies were counted after 48 h of incubation at 37°C.

Transfer frequency was determined by number of transconjugants per donor and per recipient cell.

2.3.9 Statistical analysis

For all analyses, the GLIMMIX procedure of SAS® (SAS Institute Inc, 2008) was used with the binomial distribution and logit link function to fit logistic models. Results included F-statistics and corresponding p-values to test fixed effects in each model, as well as the mean diversity percents and 95% confidence intervals. All tests were declared significant at a significance level of 0.05.

First, to examine species diversity (i.e., the percent of isolates out of 20 isolates per dog), separate logistic models were run for each species to compare groups. The experimental design for this analysis was a completely randomized design with group (healthy versus pyoderma dogs) as the treatment factor and dog as the experimental unit. The same analysis was performed for prevalence of *tet* genes or virulence genes (i.e., the percent of isolates with a specific *tet* gene out of the total number of tetracycline resistant isolates per dog; the percent of isolates with a

specific virulence gene out of the total number of *E. faecalis* and *E. faecium* isolates, respectively per dog), separately for each gene but pooling over all isolates.

Second, to examine antibiotic resistance (i.e., the percent of resistant isolates out of the number of isolates observed for each dog), separate logistic models were fitted for each species-group combination. Only the antibiotics that had some resistance for each group-species combination were included in that model. For example, for the combination of group=pyoderma and species=*E. dispar*, only the antibiotics ciprofloxacin, enrofloxacin, erythromicin, and tetracycline were included in the model, as the other six antibiotics were 100% susceptible for this group-species combination. For the species *E. casseliflavus* and *E. canintestine*, there was only one dog per group-species combination and hence those data were not statistically analyzable. The experimental design for this analysis was a randomized complete block design where dog was the random block factor and antibiotic was the treatment factor.

In addition, percent resistance was analyzed in a model that contained both groups and antibiotic as treatment factors. The design for this was a completely randomized design with a split-plot. Group was the whole-plot treatment factor and dog was the whole-plot experimental unit. Antibiotic was the split-plot factor. This analysis was again done separately for the three species *E. faecalis, E. faecium,* and *E. hirae*, which were the only three species to occur in both healthy and pyoderma groups. Each model only contained those antibiotics for which resistance was greater than 0% in both groups.

2.4 Results

2.4.1 Prevalence of enterococci

A total of eighteen dogs were included in this study. Twenty isolates of enterococci were selected from each sample (total isolates from pyoderma group, n=180; total isolates from

healthy group, n=180). The mean ± standard error mean from the fecal samples of healthy (based on 8 samples) and the mean from pyoderma dogs (based on 2 samples) were $6.7 \pm 0.2 \times 10^6$ and 1.2×10^6 respectively (Table 2.4).

Based on the multiplex PCR, *E. faecalis*, *E. faecium*, *E. casseliflavus* and by *sodA* analysis *E. hirae, E. canintestini*, *and E. dispar* were identified. As shown in Figure 2.1, diversity of enterococcal species was evident in fecal samples from both groups. Fecal samples from pyoderma dogs demonstrated greater species diversity than the isolates from healthy group (Figure 2.2, 2.3). Among 180 isolates collected from each group of dogs, four species of enterococci were identified from healthy dogs and six species from pyoderma dogs (Figures 2.2; 2.3). The predominant enterococcal species isolated from the fecal samples of dogs was *E. faecalis.* Other common enterococcal species found in healthy dogs were *E. hirae* and *E. faecium*, while in pyoderma dogs *E. hirae*, *E. faecium* and *E. dispar* were commonly present (Figure 2.2). Rare enterococcal species such as *E. casseliflavus* and *E. canintestini* were also detected in our study; however, these collectively comprised ≤ 5% of total isolates. There was a significant difference in the number of *E. facealis*, *E. faecium* and *E. hirae* isolates from both groups ($P<0.05$). Of the total isolates identified, the percentages of the enterococcal strains from healthy and pyoderma groups respectively were as follows: *E. faecalis* (70.5/44), *E. faecium* (6.1/38), *E. hirae* (22.2/5.5), *E. casseliflavus* (1.6/1.0), *E. canintestini* (0/5.5), and *E. dispar* (0/6.0) (Figure 2.1).

2.4.2 Antibiotic susceptibility test

2.4.2.1 Phenotypic characterization

Enterococcal isolates from the pyoderma dogs expressed higher frequency of resistance to tetracycline, erythromycin, doxycycline, ampicillin, ciprofloxacin, enrofloxacin, gentamicin

and streptomycin antibiotics than the healthy dog isolates (Figure 2.4). Considering all isolates that were tested, isolates from both groups were susceptible towards vancomycin and linezolid, and only 20 out of 180 isolates from the pyoderma group were resistant to ampicillin. Typically, high frequency of resistance to erythromycin and tetracycline were detected among the isolates tested. The percentage of resistant isolates recorded for following antibiotics were as follows: tetracycline (healthy [17.7%]; pyoderma [29%]); erythromycin (healthy [4.4%]; pyoderma [36.6%]); and enrofloxacin (healthy [8.3%]; pyoderma [25.5%]); gentamicin (healthy [5%]; pyoderma [8.3%]); doxycycline (healthy [0.5%]; pyoderma [5%]); ampicillin (healthy [0%]; pyoderma [11%]); ciprofloxacin (healthy [5%]; pyoderma [14.4%]); and streptomycin (healthy [1.6%]; pyoderma [14%]).

At the species level, *E. faecium* were resistant to more antibiotics (8 antibiotics) than any other isolates including *E. faecalis* (7 antibiotics) (Figure 2.5, 2.6). At the species level, *E. faecalis* and *E. faecium* showed no significant difference in their resistance profile between both groups ($P<0.05$). Resistance level to tetracycline, ampicillin, ciprofloxacin, enrofloxacin, gentamicin, doxycycline and streptomycin was higher in *E. faecium* isolates from pyoderma infected dogs as compared to healthy dog isolates (Figure 2.6). Among the *E. faecalis* isolates, resistance to gentamicin, erythromycin, doxycycline, ampicillin and streptomycin was higher as compared to the healthy group isolates. Very low level antimicrobial resistance (≤10 isolates/species/antibiotic tested) was observed in *E. hirae* (Figure 2.7), *E. dispar* (Figure 2.8), *E. canintestini* (Figure 2.9), and *E. casseliflavus* (Figure 2.10) isolates for the different antibiotics used in this study. Among *E. dispar*, and *E. canintestini* strains, all the isolates from healthy group and *E. casseliflavus* from pyoderma infected dogs were susceptible to all the antimicrobials in the test panel.

2.4.2.2 Genotypic characterization

All tetracycline resistant enterococcal isolates were screened to identify the *tet* genes. The *tet* genes were detected in 50% of total enterococcal isolates tested. Screening of tetracycline resistant enterococcal isolates by PCR revealed *tet*(A), *tet*(M), *tet*(S), and *tet*(O). No *tet*(C), *tet*(Q), *tet*(K) and *tet*(W) were detected (Figure 2.11). The prevalence of *tet*(M) and *tet*(O) positive isolates was higher from the pyoderma group as compared to healthy group isolates. The *tet*(A) gene was not detected in pyoderma group isolates, but was observed in 18.7% of isolates from the healthy group (Figure 2.11). Three percent of isolates from the healthy group and nine percent of isolates from the pyoderma group expressed the tetracycline resistance phenotypically but did not carry any of the *tet* genes tested. The overall distribution of *tet* genes in the enterococcal isolates tested are shown in Figure 2.11. Multiple *tet* genes co-existed in 18.7% (6/32) and 2.0% (1/52) of isolates from healthy and pyoderma groups respectively. In these, among the healthy group, one isolate harbored *tet*(A)+*tet*(M); one isolate had *tet*(M))+*tet*(S) genes and five isolates carried *tet*(A)+ *tet*(S); while among the pyoderma group one isolate harbored *tet*(M) +*tet*(S).

The resistance phenotype for erythromycin was confirmed by the detection of *erm*(B) gene. However, only 12.5% (1/8) of healthy group isolates and 19.5 % (13/66) of pyoderma group isolates that were resistant to erythromycin phenotypically, harbored the *erm*(B) gene.

All of the resistant isolates with reduced susceptibility to fluoroquinolones (healthy group [24], pyoderma group [72]) were subjected to *gyrA* gene amplification. Sequencing of the quinolone resistance determining region (QRDR) of the *gyrA* gene positive isolates revealed no amino acid change corresponding to codon 83 and 87 of the enterococcus *gyrA* in the isolates tested (Table 2.7).

2.4.3 Virulence factors

One or more putative virulence genes (*asa1*, *gelE*, *esp*) were detected in 120 (66.6%) healthy group isolates and 92 (51.5%) pyoderma group isolates. *E. hirae* and *E. casseliflavus* from the healthy group and *E. hirae* and *E. dispar* from the pyoderma group, did not harbor any of the virulence determinants tested, while they were found at a very low frequency among *E. canintestine* (6 isolates) from pyoderma group isolates. None of the strains examined showed the presence of *cylA* gene in this study.

The distribution of virulence determinants among *E. faecalis* and *E. faecium* is shown in Figure 2.12. The *gelE* was the most widespread virulence determinant present in 67% of the total enterococcal isolates (*E. faecalis* and *E. faecium*). A higher percentage of *gelE* and *esp* positive isolates were found in *E. faecalis* (*gelE* 92.4%; *esp* 52.0%) and *E. faecium* (*gelE* 16.2%; *esp* 1.47%) isolates from the pyoderma group compared with the isolates from healthy group. Also, in the *E. faecalis* isolates from the pyoderma group, *asa1* positive isolates was lower (53.2%) than in the isolates from healthy group (63.7%).

Gelatinase, haemolysin and aggregation substance production was tested by phenotypic assays. Most of the *gelE* positive *E. faecalis* isolates expressed this gene phenotypically in gelatinase assay (Figure 2.13). Among the *E. faecium* strains, only one strain from the healthy group and none from the pyoderma group was positive for *gelE* and gelatin hydrolysis. However, 13% of *E. faecalis* isolates from both groups and eleven *E. faecium* isolate from the pyoderma group displayed specific *gelE* PCR product but did not degrade casein.

Haemolytic activity was detected in one isolate from the healthy group and five isolates from pyoderma group, but *cylA* gene was not found.

None of the isolates positive for the *asa1* gene showed the production of aggregation substance by clumping assay.

2.4.4 Clonal diversity

E. faecalis and *E. faecium* strains were selected for PFGE analysis. PFGE clusters were based on the similarity cut off of 90% (Cook *et al*., 2002).

2.4.4.1 *E. faecalis* isolates

Out of 206 *E. faecalis* strains, 60 isolates were selected (5 isolates per dog): 35 isolates from the healthy group and 25 isolates from the pyoderma group.

Among the healthy group isolates, six different PFGE patterns [type 'A' (n=5), type 'B'(n=15), type 'C'(n=8), type 'D'(n=1) type 'E'(n=5), type 'F'(n=1)] were observed (Fig 2.14.). The largest PFGE cluster consisted of 15 isolates. The PFGE type 'B' and type 'C' were commonly distributed among different dogs. The similarity indices within each clusters were >92%. Within the isolates from each dog, the fingerprinting patterns had 100% similarity except for one dog (H7) where three different sets of patterns were observed. The minimum similarity between different clusters was 53% (Figure 2.14).

Five major clusters [type 'G'(n=5), type 'H'(n=5), type 'I'(n=6), type 'J'(n=5), type 'K'(n=4)] were formed among the pyoderma group isolates (Figure 2.15.). The largest PFGE cluster consisted of 6 isolates. The minimum similarity between different clusters was 54%. The similarity indices within each clusters were >90%. No clonal relation was detected among the isolates from different dogs except for the PFGE cluster type 'I' where one isolate from P3 and five isolates from P5 were included in the same cluster. Except for P3, all dogs showed homogenous population of *Enterococcus faecalis*.

2.4.4.2 *E. faecium* isolates

All *E. faecium* strains from the healthy group (n=10) and a group of 15 isolates (3 isolates per dog sample) from the pyoderma group were picked randomly to be analyzed by PFGE. PFGE clustering grouped 10 isolates from healthy group into 7 different restriction profiles [type'A' (n=2), type 'B'(n=3), type 'C'(n=1), type'D'(n=1), type 'E'(n=1), type 'F'(n=1)] (Figure 2.16.) and 15 isolates from pyoderma group into 7 types [type 'H'(n=1), type 'I'(n=1), type 'J'(n=2), type 'K'(n=1), type 'L'(n=6), type 'M'(n=1), type 'N'(n=3)] (Figure 2.17).

Among the healthy group isolates, 6 different clusters were found among the isolates collected from one dog (H7), suggesting strain diversity within the animal.

Among the pyoderma group also strain diversity was also observed within the dogs except for P5 and P7 dogs. Three dogs i.e P1 (2 isolates), P3 (2 isolates) and P5 (3 isolates) shared the same PFGE cluster (type 'L').

2.4.5 Conjugation results

We tested if the tetracycline resistant *E. faecalis* and *E. faecium* could transfer their resistance traits. Additionaly, we also performed the mating experiment for the remaining *E. faecalis* and *E. faecium* isolates showing resistance towards other antibiotics included in this study and for this, we randomly collected five isolates each from *E. faecalis* and *E. faecium* for each antibiotic.

Sixty five tetracycline (30 *E. faecalis*; 35 *E. faecium*); 16 erythromycin (6 *E. faecalis*; 10 *E. faecium*); 13 streptomycin (8 *E. faecalis*; 5 *E. faecium*); 7 doxycycline (4 *E. faecalis*; 3 *E. faecium*); 15 gentamicin (10 *E. faecalis*; 5 *E. faecium*); 6 ampicillin (1 *E. faecalis*; 5 *E. faecium*) and 11 enrofloxacin (3 *E. faecalis*; 8 *E. faecium*) resistant donors were tested for the conjugative transfer of resistance traits in filter and broth mating traits (Table 2.5).

Twenty seven tetracycline resistant isolates (8 *E. faecalis*; 19 *E. faecium*); 1 erythromycin resistant *E. faecalis* and 1 doxycycline resistant *E. faecalis* yielded their respectively antibiotic resistant transconjugants. The transfer frequency ranged from 10^{-2} to 10^{-12} transconjugants/ donor and 10^{-5} to 10^{-13} transconjugants / recipient (Table 2.5).

Among the tetracycline resistant isolates, we were able to transfer *tet*(M) and *tet*(S) in *E. faecium*, while *tet*(M) was the only *tet* gene transferred in *E. faecalis*. The transfer frequencies are shown in Table 2.6. Transconjugants positive for *tet* genes were confirmed by the PCR assay.

2.5 Discussion

The development and spread of antimicrobial resistance in clinical practice is a major threat to the public health (Levy *et al*., 2004). The commensal gut bacteria are likely to be the major contributor for the development of resistance in bacterial pathogens (Salyers *et al*., 2004). Gram positive cocci, in the genus *Enterococcus*, the common gut microbiota of humans and animals, are one of the medically important antibiotic resistant nosocomial pathogen (Hildron *et al*., 2008). There are several sources for the acquisition of antibiotic resistant enterococci including care providers, food animals, contaminated meat, waste water and pet animals (Hammerum *et al*., 2010). Studies have shown that stress in the host animals can alter the gastrointestinal microbiota (Moon *et al*., 1979; Savage *et al*., 1982; Tannock *et al*., 1983) and increases the antimicrobial resistance in the intestinal microbiota (Dawson *et al*., 1984; Langlois *et al*., 1986; Molitoris *et al*., 1987). Animals, particularly the pet animals are believed to be a reservoir of antibiotic resistant bacteria (Guardabassi *et al*., 2004). Several studies in Europe and U.S. have reported increased prevalence of resistance to antibiotics in bacterial species such as *Staphylococcus intermedius*, *Escherichia coli* and *Enterococcus* isolated from pet animals

(Guardabassi *et al.*, 2004). One of the major concerns is the potential transmission of antimicrobial resistant pathogens from pet animals to humans.

Relatively sparse data are available from the U.S. with regard to enterococcal prevalence in dogs except for the studies done in dogs with urinary tract infection (UTI) (Simjee *et al.*, 2002) and one study in clinically healthy dogs (Jackson *et al.*, 2009). Simjee *et al.*,(2002) determined the antibiotic susceptibility profile of urinary tract isolates, while Jackson *et al.*, (2009) reported susceptibility profile of enteroccci from nasal, teeth, rectal, belly and hindquarter sites of dog. Simjee *et al.*,(2002) observed high incidence of resistance to gentamicin (37%), penicillin (74%) and tetracycline (77%) in their total 35 enterococcal isolates. *E. faecium* isolates were found to be resistant to more antibiotics than other enterococcal species identified in this study. Molecular analysis of one *E. faecium* isolate with higher level of resistance to gentamicin and vancomycin revealed the presence of two transposons, Tn*1546* and Tn*5281* associated with *vanA* and *aac6'-aph2"* genes, respectively (Simjee *et al.*, 2002). Genotypic analysis of this canine *E. faecium* clone suggests that Tn*1546* associated with *vanA* was acquired from the human vancomycin resistant *E. faecium*. Jackson *et al.*,(2009) isolated enterococci from healthy dogs and found that ≥33% of the isolates were resistant to three antibiotics: lincomycin (58%), flavomycin (33%) and tetracycline (33%); while ≤7% of isolates were resistant to the remaining antibiotics tested: erythromycin (7%), tylosin (7%), penicillin (6%), kanamycin (6%), streptomycin (6%), gentamicin (4.5%), chloramphenicol (4%), ciprofloxacin (2%), nitrofurantoin (1%). None of their isolates were resistant to vancomycin, daptomycin and linezolid. These studies reported the data from different sites of body which is not sufficient to determine the antimicrobial resistance of the gut microbiota in companion animals. Only one study is available reporting the susceptibility profile of enterococci from dogs with pyoderma

infection (Rantala *et al*., 2004). They compared 37 enterococcal isolates from pyoderma infected dogs (collected after an antibiotic treatment) with 73 isolates from the control group. For studying the resistance prevalence in these isolates, they screened for two antimicrobial agents: ampicillin and vancomycin. The frequency of ampicillin resistance was lower (4-7%) with no significant difference between the two groups. The enterococcal isolates from both groups were susceptible to vancomycin. Examining published data, it could be concluded that they examined only few isolates and there is lack of information regarding how many isolates were collected from each fecal sample (Rantala *et al*., 2004).

Our study is the first to address the potential association between pyoderma infection and the increase in antimicrobial drug resistance and virulence of enterococci from the feces of dogs in the U.S. In addition, it also provides detailed susceptibility profile, virulence profile of enterococci and genotypic relationship of these resistant isolates in dogs. Pyoderma is a common bacterial skin infection in dogs most often caused by *Staphylococcus pseudintermedius*. It can be a acute or chronic problem (Pedersen *et al*., 2007) with clinical symptoms characterized by scaling. Chronic infections can cause stress in animals due to several factors including discomfort and pain. Furthermore, some studies in humans have demonstrated that the plasma level of catecholamines was altered in several skin diseases such as psoriasis (Weigl, 2000; Schmid-Ott *et al*., 1999), cystic acne (Schulpis *et al*., 1999), alopecia (Puchalski, 1986) and vitiligo (Zaki *et al*., 2009). Hence, the study was proposed that stress due to pyoderma infection increases antimicrobial resistance and virulence and decreases the clonal diversity of the enterococcal population of dogs.

In this study, samples were collected from clinically healthy dogs and pyoderma infected dogs. Both groups consisted of dogs belonging to different age, sex, breed and owners. In the

healthy group, animals were not exposed to antibiotics for at least one year prior to sample collection. In pyoderma group, 3 dogs were never exposed to antibiotics prior to the collection of the samples for this study; while 6 dogs had prior exposure to antibiotics but none of these 6 dogs received antibiotics for at least seven months prior to sampling. The concentration of enterococci in healthy dogs ranged from 10^5-10^7 CFU/g which is similar to the concentration reported in healthy humans (Jett *et al.*, 1994; Kleessen *et al.*, 2000), birds (Middleton and Ambrose, 2005) and cattle (Anderson *et al.*, 2008). The concentration of enterococci in pyoderma infected dogs could not be determined due to samplying type (rectal swab).

Ossiprandi *et al.*(2004), reported that *E. faecalis* was the most prevalent species in dogs with and without previous antibiotic treatment. A similar result was reported by Jackson *et al.*,(2009) where *E. faecalis* was the dominant species isolated from rectal samples of healthy dogs. Indeed, *E. faecalis* and *E. faecium* are the two species reported to be most frequently isolated from feces of dogs and cats (Rodrigues *et al.*, 2002; Poeta *et al.*, 2006; Jackson *et al.*, 2009). In the present study also *E. faecalis* was the dominant spp. in the healthy dogs. A new species, *E. canintestini* as described by (Naser *et al.*, 2005) was found in the fecal microbiota of one dog from the pyoderma group.

Based on the antibiotic resistance phenotype, erythromycin and tetracycline resistance were encountered most frequently as compared to the other antibiotics tested; and the similar results are reported in previous studies (Poeta *et al.*, 2006; Ossiprand *et al.*, 2008; De graef *et al.*, 2004; Jackson *et al.*, 2009). This relatively high prevalence of tetracycline resistant isolates may explain the wide distribution of tetracycline in the environment. Still the overall prevalence of tetracycline resistance in our dog isolates were low to what was found in cattle and people where

tetracycline resistance levels were more than 40% (Hershberger *et al*., 2005; Anderson *et al*., 2008; Atkinson *et al*., 1997)

Although the tetracycline resistance was ≤ 30%, the level of doxycycline resistance was only ≤5% in our study. Similarly, lower level of doxycycline resistance has also been reported in enterococci isolated from human clinical infections from the U.S. (Low *et al*., 2001; Evans *et al*., 1997). Doxycycline is a semisynthetic tetracycline derivative and was developed to enhance the antibacterial activity of tetracycline. This may be the reason for finding doxycycline to be more effective against our enterococcal isolates than tetracycline.

In the present study, resistance to aminoglycoside antibiotics (gentamicin, streptomycin) was detected in both groups, but at low frequency. Absence of high level gentamicin resistance has been reported previously (Poeta *et al*., 2006). Damborg *et al*.,(2008) studied resistance levels among enterococci isolated from the healthy dogs in Portugal, showing low resistance to gentamicin (6%).

The beta-lactam antibiotic, ampicillin was more effective. Only 11% of isolates were resistant to ampicillin from the pyoderma group while all the isolates from the healthy group were susceptible. All of these ampicillin resistant isolates were *E. faecium* isolated from one dog. Low levels of ampicillin resistance in enterococci from pet animals with pyoderma infection has been previously reported (Rantala *et al*., 2004) which may be because this antibiotic is not usually included in their treatment regimen due to their ineffectiveness against *Staphylococcus pseudintermedius*.

A relatively low percentage (≤ 25%) of quinolone resistant isolates was observed in this study (Figure 2.4). This is higher when compared to the results described by Poeta *et al*., (2006), reporting 8% of ciprofloxacin résistance in enterococcal isolates from pets. Fluoroquinolones are

broad spectrum antibiotics and are often used for treating infections in animals and humans. In the U.S., enrofloxacin, difloxacin and orbifloxacin are liscensed for use in pet animals and are usually used for treating skin/wound, urinary tract and respiratory infections (http://whqlibdoc.who.int/hq/1998/WHO_EMC_ZDI_98.10.pdf).

Linezolid, a drug used as second line of treatment option for human enterococcal infections (Damborg *et al*., 2008) was active against all the enterococcal isolates which is consistent with the results reported by Jackson *et al*., (2009).

No pets were positive for vancomycin resistant enterococci (VRE) in this study which matches with studies of healthy dogs reported from the U.S. (Jackson *et al*., 2009); Portugal (Delgado *et al*., 2007), and Italy (Ossiprandi *et al*., 2008). Our results are in contrast to the observation reported from other studies from Europe where VRE was identified from dogs (Devriese *et al*., 1996). This might be probably due to their association with farm animals fed with glycopeptides, avoparcin as a growth promoter or might be due to ingestion of VRE contaminated raw meat from these animals (Van Belkum *et al*., 1996; Borgen *et al*., 2001).

The resistance mechanism for the observed phenotypes were determined for erythromycin, tetracycline and quinolone antibiotics as these were the only antibiotics with greater than 25% phenotypic prevalence. None of our macrolide resistant enterococcal species other than *E. faecalis* (none in the healthy group; eleven isolates in pyoderma group) and *E. faecium* (one isolate in healthy group; two isolates in pyoderma group) species carried the *erm*(B) gene, thus partly confirming the data of Jackson *et al*.,(2010). They found erythromycin resistance only in *E. faecalis* and *E. faecium* isolates with *erm*(B) gene gene detected in 95.6% resistant isolates. The *erm*(B) gene, the most frequently found macrolide resistance gene among the enterococcal isolates (Poeta *et al*., 2006; Jensen *et al*., 1999) also exhibits macrolide-

lincosamide-streptogramin cross-resistance (Jensen *et al*., 1999). About ≥80% of the macrolide resistant isolates examined in this study were negative for the presence of *erm*(B) gene, suggesting the presence of other erythromycin resistance genes such as *erm*(A), *erm*(C), *mef*(A), *mef*(E), *msr*(A), *mre*(A), that were not tested for in the present study.

For tetracycline resistance, there are twenty-nine known tetracycline genes: 18 of the *tet* genes code for efflux pumps and 7 for ribosomal protection protein (Chopra and Roberts, 2001). The *tet*(M) gene is reported to be the most frequent tetracycline resistance gene in enterococci (Jackson *et al*., 2010; Devriese *et al*., 1992). This is partly in accord with our results where *tet*(M) was present in 33% of isolates in the pyoderma group. In both the groups, *tet*(S) (healthy [65.6%]; pyoderma [34.6]) was more commonly found. The gene, *tet*(S) encodes for ribosomal protection protein and has 79% and 72% sequence identity with *tet*(M) and *tet*(O) genes, respectively (Charpentier *et al*., 1994). The observation that few of our tetracycline resistant isolates tested negative for all the *tet* genes suggests that there are other *tet* genes that we did not screen for.

Mutations in the codon-83 and codon-87 have been associated with quinolone resistance in *E. faecalis* and *E. faecium* (Korten *et al*., 1994). In this study, amplification and sequence analysis of *gyr* A positive isolates revealed no mutation in both the codons suggesting that, the mechanism of resistance in these isolates may be due to other mutations (Korten *et al*., 1994).

Widespread dissemination of several antimicrobial resistance genes among enterococci may be attributed to horizontal transfer (Wirth, 1994). Most of the *tet* genes such as *tet*(M), *tet*(Q), *tet*(S) are commonly present on mobile genetic elements (Paulsen*et al*., 2003; Davis *et al*., 2005). The *tet* genes have been shown to transfer in enterococcal species isolated from different sources, but not from canine fecal samples. Since prevalence of resistant isolates were

high in *E. faecalis* and *E. faecium* strains, these two species were included in the intraspecies conjugal transfer of antibiotic resistant traits conjugation experiments. Some of the resistant *E. faecalis* or *E. faecium* failed to transfer their respective resistance traits, suggesting the presence of genes encoding their respective antibiotic resistant traits on non-conjugative element. In *E. faecalis,* tetracycline, erythromycin and doxycycline were transferable while tetracycline was the only antibiotic resistance trait transferred in *E. faecium*. The rate of gene transfer was relatively higher for tetracycline resistance determinants than any other antibiotics tested (Table 2.5). The conjugal gene transfer that occurred with low frequency ($\leq 10^{-13}$) for erythromycin and doxycycline resistance determinants is likely due to mutations. The only *tet* genes that were transferred were *tet*(M) and *tet*(S) which is not surprising as these two genes are known to be associated with conjugative elements (Flanngan *et al*., 1994; Roberts *et al*., 2006).

There are several virulence factors present in the enterococcal isolates that have been reported in animal infection model experiments (Jett *et al*., 1999). Here, we detected virulence traits such as haemolysins, agglutination substance, enterococcocal surface proteins, and gelatinase in our enterococcal isolates. Most of the isolates positive for the virulence determinants were either *E. faecalis* or *E. faecium*. Very few isolates were positive in the hemolysin assay with 0.5% and 2.5% prevalence in healthy and pyoderma isolates, respectively. Similar were results reported by (Gulhan *et al*., 2006) where cytolysin activity was absent in all enterococcal isolates of canine origin. None of our isolates including the hemolysin positive isolates were positive for the *cylA* gene. This may suggest the occurence of other genes in the *cyl* operon. All our isolates were tested for their casein degradation ability. The prevalence of *gelE* was higher in the *E. faecalis* isolates from pyoderma dogs than from healthy ones, as already reported by Archimbaud *et al*.,(2002). Most of the gelatinase positive isolates showed the

correlation with the expression of the *gelE* gene. We determined the occurrence of the *esp* gene in *E. faecalis* and *E. faecium* isolates, with higher incidence in the pyoderma group (52%) as compared to the healthy group (15.7%). In contrast, Harada *et al.*,(2005) reported low prevalence of *esp*-positive isolates from pet animals with most of the *esp*-positive strains from the healthy animals. The conjugative transfer in *E. faecalis* is mediated by the aggregation substance (Kreft *et al*., 1992). Although it is found mostly in *E. faecalis*, there are reports indicating the presence of *asa1* in vancomycin resistant *E. faecium* (Varaldo *et al*., 2003). In the present study, aggregation substance was found only in *E. faecalis*. It was associated more frequently with the healthy group isolates. Thus these *asa1* isolates may act as a source for horizontal transfer to other *E. faecalis* isolates in the gut of dogs which could result in a threat to public health.

We used PFGE to study the clonal structure of selected *E. faecalis* and *E. faecium* strains. The genetic diversity was defined by comparing the number of different PFGE types between both groups. Our expectation was to show a less diverse gut microbiota in pyoderma animals as compared to the healthy animals due to stress in pyoderma dogs. We found that there was no difference in diversity among *E. faecium* isolates from both groups while among *E. faecalis* isolates, pyoderma infected dogs harbored less diverse isolates as compared to healthy dogs. One of the plausible explanations for finding no difference in the diversity among the *E. faecium* isolates from both groups would be due to the variation in the number of isolates between the healthy and pyoderma group included for PFGE analysis. For *E. faecium* strains, we had only 10 isolates from the healthy group and in the pyoderma group, 15 isolates were selected for this study. Hence it is necessary to analyze large number of isolates in order to determine clonal diversity with respect to health status of animal. Cluster formation in the PFGE dendrogram depends mainly on the choice of similarity cutoff (Stenske *et al*., 2009). In the present study,

based on the similarity cutoff of 90%, (Cook *et al*., 2002) dominant genetic profiles were observed among healthy and pyoderma groups, except for *E. faecium* strains from the healthy group that showed no overlap (Fig 2.6.). The potential explanation for sharing the same PFGE pattern by the isolates from different healthy dogs is that of all the healthy dogs included in this study were owned by the people who were working at the same place. Enterococci, often reported from the hospital settings, might also exist in the community settings that are not directly linked to the hospital settings, as these are the gut commensals in humans and all warm blooded animals (Larsen *et al*., 2010). Close physical contact between pets and their owners might have provided a suitable environment for the possible transfer of these enterococcal strains to their pet animals (Guardabassi *et al*., 2004; Simjee *et al*., 2002). Another explanation for finding some clones in the same cluster might be due to the use of single restriction enzyme for digestion of DNA in the PFGE protocol used in this study (Davis *et al*., 2003). Use of additional enzymes may be required to detect mutations and other changes in the bacterial chromosome. The clones clustered in the PFGE type 'C' were originated from the same age group dogs. Among the pyoderma group isolates, an overlap was observed among dogs P1, P3, P5 (Figure 2.15.; Figure 2.17.). But none of these dogs belonged to the same age and breed, and even the time of the sample collected from these animals was different. However, this overlap infers clonal spread of these strains to some extent within the healthy and pyoderma dogs. No overlap among the *E. faecium* strains from healthy animals might be due to fewer number of isolates and also most of the isolates originated from the single animal. Thus, clonal relationships were observed within both group isolates except for *E. faecium* strains from healthy animals. Among the healthy group isolates, dog H7 harbored a most diverse population of enterococcal species (Figure 2.14; Figure 2.16). The majority of the different PFGE types identified in this study were

found in individual dogs only. The similarities of PFGE patterns within the dog suggests that there is animal to animal variation in the genetic diversity of these strains. This variation in individual animals may be attributed to factors that enhance their survival and growth in the GIT. Taken together, PFGE results show that pyoderma infection resulted in lower genotypic diversity in *E. faecalis* strains. There are studies where PFGE analysis was performed in enterococcal isolates from dogs (Jackson *et al*., 2010; Simjee *et al*., 2002; Manson *et al*., 2003), but none of these isolates were from the pyoderma infected dogs. This is the first study, where the genetic diversity of enterococcal strains in pyoderma dogs were studied.

Taken together, these data indicate that enterococci are common intestinal microbiota of dogs (De Graef *et al*., 2005). Each fecal sample showed diversity in the enterococcal strains, and this difference in the enterococcal population might be due to factors that influence the composition of intestinal microbiota such as age, sex, diet and genetic makeup of the individual animal (Tannock 1999; 2005). Data on antimicrobial resistance in dogs show that the overall resistance rates for all the enterococcal strains were low for all the antibiotics tested (range: 0-36.6%). All of our isolates were susceptible to linezolid and vancomycin antibiotics, that are commonly used for treatment of multidrug resistant gram positive bacteria such as MRSA and VRE in human healthcare. However, the proportion of resistant isolates was higher among the pyoderma group in comparison to the healthy group isolates eventhough the difference was not statistically significant. Genotyping revealed less diverse *E. faecalis* isolates from pyoderma dogs as compared to the healthy group isolates. In the pyoderma group, three dogs were never exposed to antibiotics prior to sampling (P1, P6, and P8). Antimicrobial resistance was observed among the isolates from P1 and P6 samples against tetracycline, erythromycin, ciprofloxacin and enrofloxacin. These changes might be attributed to stress on animals due to pyoderma and/or

visit to the clinic (unfamiliar environment, people) (Rostango, 2009). This may cause alteration in the intestinal pH and ultimately affect the gut microbiota (Mathew *et al*., 1996). Bacterial resistance may be indirectly affected, if these changes in the gut environment cause changes in the efflux mechanism or expression of proteins involved in antibiotic resistance. Stress-related factors might have influenced horizontal transfer of resistance genes; this might also explain the prevalence of certain resistant strains. Studies have shown that stressed animals selectively shed bacteria in their feces that are closely adherent to the intestinal mucosa (Langlois and Dawson, 1999). If such organisms harbor antibiotic resistant genes, the prevalence of resistant bacteria will be higher in the feces of animals that experience significant stress (Mathew *et al*., 2002).

Hence, our study demonstrates that antimicrobial resistance in enterococci from pyoderma dogs was higher as compared to healthy dog isolates. Pyoderma group dogs harbored less diverse population of *E. faecalis* isolates than the isolates from healthy dogs. Further studies will be necessary to assess whether this difference in the resistance prevalence and diversity was related to the host stress. Since enterococci are known to transfer resistance genes to other bacteria of same or different species, appropriate husbandry measures should be carried out to relieve animals from stress in order to reduce the prevalence of antibiotic resistant strains.

The results from this study are important from a public health point of view as most of the antibiotics employed in the present study are commonly used for treatment of infections in humans.

2.6 Conclusions

The reports on the community-acquired and nosocomial infections have identified antibiotic resistant enterococci as one of the important clinical group (Hammerum *et al.*, 2010). This study was conducted to characterize enterococci from healthy and pyoderma infected dogs. The concentration of enterococci in the fecal samples from the healthy group was $6.7 \pm 0.2 \times 10^6$ CFU/g. Considerable diversity was observed within the enteroccal population, with *E. faecalis* as the dominant species in both groups. Antimicrobial resistance in the pyoderma infected dogs was higher than the healthy dogs with highest resistance frequency observed to erythromycin (36.6%). At the species level, relatively high resistance was observed in *E. faecium* isolates. No obvious difference was observed in the virulence traits. Intra-species transfer by the conjugation assay revealed tetracycline, erythromycin and doxycycline resistance traits transferable in *E. faecalis* while in *E. faecium*, only tetracycline transferred. Genotyping of *E. faecium* strains revealed no difference in the heterogenicity while among the *E. faecalis* strains, isolates in the gut of pyoderma dogs were less diverse as compared to healthy animals.

The findings in this study suggest that although, the overall antimicrobial resistance, virulence and horizontal gene transfer remained low, pyoderma in dogs appears to be associated with higher frequency of antimicrobial resistance in enterococci. This may be due to pyoderma related stress that might have altered the resistant enterococci in canine feces. Further studies are needed to understand the mechanism by which stress induces susceptibility in the gut commensals to explain the difference in the resistance profile of pyoderma and healthy dogs.

Healthy and pyoderma infected dogs do not represent major reservoir of antibiotic resistant enterococci and serious public health risk.

2.7 Figures and Tables

Table 2.1 Information on healthy dogs

Dogs	Neuter/Spay	Age	Breed	Diet
H-1	FS	5 yr	Golden retriever/Yellow labrador mix	Maintanence diet
H-2	MN	1 yr	Terrier mix	Maintanence diet
H-3	FS	17 yr	Border Collie	Maintanence diet
H-5	FS	12 yr	Terrier mix	Maintanence diet
H-6	FS	7 yr	Pomeranian/healer mix	Maintanence diet
H-7	MN	3 yr	Labrador/ Shepherd	Maintanence diet
H-8	MN	4 yr	Yorkshire terrier+bichon	Maintanence diet
H-9	MI	6 yr	Yorkshire	Maintanence diet
H-10	MI	3 yr	Rottweiler Shepherd	Maintanence diet

Yr, Year
MI, Male intact; MN, Male neutered; FS, Female spayed

Table 2.2 Information on pyoderma dogs

Dogs	Sex	Age	Breed	History of antibiotic exposure	Antibiotics used for treatment
P-1	MI	4 months	German Shepherd	No previous exposure	N/A
P-2	MN	7 yrs	Australian Shepherd mix	9 months	cephalosporin, ampicillin, enrofloxacin
P-3	MN	5 yrs	Wheaten Terrier	1 year	metronidiazole
P-4	FS	4 yrs	Boxer	1yr and 4 months	Amoxicillin-clavulanic acid
P-5*	MN	10.5 yrs	Australian Shepherd	11 months	gentamicin (topical use), doxycycline
P-6	MN	11 yrs	German Shepherd	No previous exposure	N/A
P-7	MN	3.5 yrs	Mixed Breed	7 months	gentamicin (topical use), no systemic antibiotics
P-8	FS	4.5 yrs	Dalmatian	No previous exposure	N/A
P-9*	FS	14 yrs	Australian Shepherd	7 months	cephalosporin, gentamicin (tropical use)

N/A, Not applicable
MI, Male intact; MN, Male neutered; FS, Female spayed
* History of chronic pyoderma

Table 2.3 Primer sets for PCR used in this study

Gene	Sequence(5’-3’)	Product size(bp)	Reference
Species specific genes			
E. faecalis	F-TCAAGTACAGTTAGTCTTTATTAG R-CGATTCAAAGCTAACTGAATCAGT	941	Kariyama *et al*.,(2000)
E. faecium	F-TTGAGGCAGACCAGATTGACG R-TATGACAGCGACTCCGATTCC	658	Kariyama *et al*.,(2000)
E. casseliflavus	F-CGGGGAAGATGGCAGTAT R-CGCAGGGACGGTGATTTT	484	Malen *et al*.,(1995)
E. gallinarum	F-GGTATCAAGGAAACCTC R-CTTCCGCCATCATAGCT	822	Malen *et al*.,(1995)
16S rRNA	F-GGATTAGATACCCTGGTAGTCC R-TCGTTGCGGGACTTAACCCAAC	320	Kariyama *et al*.,(2000)
sodA	F-CCITAYICITAYGAYGCIYTIGARCC R-ARRTARTAIGCRTGYTCCCAIACRTC	438	Poyart *et al*.,(2000)
Antibiotic resistance genes			
tet(A)	F-GCTACATCCTGCTTGCCTTC R-CATAGATCGCCGTGAAGAGG	210	Ng *et al*.,(2001)
tet(C)	F-CTTGAGAGCCTTCAACCCAG R-ATGGTCGTCATCTACCTGCC	418	Ng *et al*.,(2001)
tet(Q)	F-TTATACTTCCTCCGGCATCG R-ATCGGTTCGAGAATGTCCAC	904	Ng *et al*.,(2001)
tet(M)	F-GTGGACAAAGGTACAACGAG R-CGGTAAAGTTCGTCACACAC	406	Doherty *et al*.,(2000)
tet(S)	F- CATAGACAAGCCGTTGACC R-ATGTTTTTGGAACGCCAGAG	667	Ng *et al*.,(2001)
tet(K)	F-TCGATAGGAAACAGCAAGTA R-CAGCAGATCCTACTCCTT	169	Ng *et al*., (2001)
tet(O)	F-AACTTAGGCATTCTGGCTCAC R-TCCCACTGTTCCATATCGTCA	515	Ng *et al*., (2001)
tet(W)	F-GAGAGCCTGCTATATGCCAGC R-GGCCGTATCCACAATGTTAAC	168	Aminov *et al*.,(2001)
erm(B)	F-GAAAAGGTACTCAACCAAATA R-AGTAACGGTACTTAAATTGTTTAC	639	Sutcliffe *et al*.,(1996)
gyr(A)	F-CGGGATGAACGAATTGGGTGTGA R-AATTTTACTCATACGTGCTTCGG	141	Korten *et al*.,(1994)
Virulence genes			
gelE	F-TATGACAATGCTTTTTGGGAT R-AGATGCACCCGAAATAATATA	213	Vankerckhoven *et al*.,(2004)
asa1	F-GCACGCTATTACGAACTATGA R-TAAGAAAGAACATCACCACGA	375	Vankerckhoven *et al*.,(2004)
cylA	F-ACTCGGGGATTGATAGGC R-GCTGCTAAAGCTGCGCTT	688	Vankerckhoven *et al*.,(2004)
esp	F-AGATTTCATCTTTGATTCTTGG R-AATTGATTCTTTAGCATCTGG	510	Vankerckhoven *et al*.,(2004)

F, forward; R, reverse
I = A, T, G or C; Y = C or T; R = A or G

Table 2.4 Concentration of enterococci in the feces of healthy and pyoderma dogs

	Samples	Mean±SEM (CFU per gram)	Number of isolates (n) #
Healthy	H-1	$8.8 \pm 1.6 \times 10^5$	20
	H-2	$2.6 \pm 0.2 \times 10^5$	20
	H-3	$4.7 \pm 0.1 \times 10^7$	20
	H-5	$2.6 \pm 0.3 \times 10^6$	20
	H-6	$4.5 \pm 0.6 \times 10^5$	20
	H-7	$4.7 \pm 2.7 \times 10^5$	20
	H-8	N/A	20
	H-9	$5.3 \pm 0.8 \times 10^5$	20
	H-10	$1.5 \pm 0.1 \times 10^6$	20
Mean±SEM/Total		$6.7 \pm 0.2 \times 10^6$	180
Pyoderma	P-1	N/A	20
	P-2	17.3×10^5	20
	P-3	N/A	20
	P-4	N/A	20
	P-5	N/A	20
	P-6	7.0×10^5	20
	P-7	N/A	20
	P-8	N/A	20
	P-9	N/A	20
Mean/Total		1.2×10^6	180

N/A, Not applicable (rectal swabs)
#, selected for further analysis randomly

Table 2.5 Intra-species transfer of antibiotic resistance traits in *E. faecalis* and *E. faecium* isolates

Wild isolates	Total isolates	Enterococcus Recipient	Broth mating/Transfer rate			Filter mating/Transfer rate		
			n (%)	T/D Mean±SEM	T/R Mean±SEM	n (%)	T/D Mean±SEM	T/R Mean±SEM
Tetracycline								
E. faecalis	30	OG1SSp	7(23.3)	$3.8 \pm 2.1x10^{-4}$	$10.3 \pm 6.6x10^{-8}$	8 (26.6)	$4.5 \pm 1.9x10^{-5}$	$4.4 \pm 1.7x10^{-6}$
E. faecium	35	TX5034	14(40.0)	$1.3 \pm 0.6x10^{-2}$	$3.0 \pm 1.0x10^{-7}$	19(54.2)	$2.0 \pm 1.7x10^{-3}$	$2.0 \pm 1.2x10^{-5}$
Erythromycin								
E. faecalis	6	OG1SSp	1(20)	1.0×10^{-8}	0.3×10^{-11}	1(20)	7.4×10^{-10}	4.6×10^{-13}
E. faecium	10	*E. faecium*(45-26)	0	N/A	N/A	0	N/A	N/A
Doxycycline								
E. faecalis	4	OG1SSp	1(25)	$2.0 x10^{-9}$	$1.4 x10^{-12}$	1(25)	$3.4 x10^{-11}$	$2.6 x10^{-12}$
E. faecium	3	TX5034	0	N/A	N/A	0	N/A	N/A
Gentamicin								
E. faecalis	10	OG1SSp	0	N/A	N/A	0	N/A	N/A
E. faecium	5	*E. faecium*(38-42)	0	N/A	N/A	0	N/A	N/A
Streptomycin								
E. faecalis	8	*E. faecalis* (41-31)	0	N/A	N/A	0	N/A	N/A
E. faecium	5	ATCC-51559	0	N/A	N/A	0	N/A	N/A
Enrofloxacin								
E. faecalis	3	JH2-2	0	N/A	N/A	0	N/A	N/A
E. faecium	8	ATCC-51559	0	N/A	N/A	0	N/A	N/A
Ampicillin								
E. faecalis	1	*E. faecalis* (41-31)	0	N/A	N/A	0	N/A	N/A
E. faecium	5	ATCC- 19454	0	N/A	N/A	0	N/A	N/A

T/D, Number of transconjugants per donor; T/R, Number of transconjugants per recipient
N/A, Not applicable

Table 2.6 Intra-species transfer of *tet* genes from *E. faecalis* and *E. faecium*

			Broth mating/Transfer rate			Filter mating/Transfer rate		
Wild isolates	**Enterococcus Recipient**	***tet* genes**	**n (%)**	**T/D Mean±SEM**	**T/R Mean±SEM**	**n (%)**	**T/D Mean±SEM**	**T/R Mean±SEM**
E. faecalis	OG1SSp	*tet*M (n=21)	7 (33.3)	$3.8 \pm 2.1 \times 10^{-4}$	$10.3 \pm 6.6 \times 10^{-8}$	8 (38.0)	$4.5 \pm 1.9 \times 10^{-5}$	$4.4 \pm 1.7 \times 10^{-6}$
		*tet*A (n=5)	0	N/A	N/A	0	N/A	N/A
		*tet*S (n=21)	0	N/A	N/A	0	N/A	N/A
E. faecium	TX5034	*tet*M (n=5)	0	N/A	N/A	3 (60.0)	$3.0 \pm 2.9 \times 10^{-5}$	$3.0 \pm 2.9 \times 10^{-6}$
		*tet*O (n=12)	0	N/A	N/A	0	N/A	N/A
		*tet*S (n=18)	14 (77.8)	$1.3 \pm 0.6 \times 10^{-2}$	$3.0 \pm 1.0 \times 10^{-7}$	16 (88.9)	$2.3 \pm 0.2 \times 10^{-3}$	$2.3 \pm 1.4 \times 10^{-5}$

T/D, Number of transconjugants per donor; T/R, Number of transconjugants per recipient
N/A, Not applicable

Figure 2.1 Diversity of enterococci from canine fecal samples

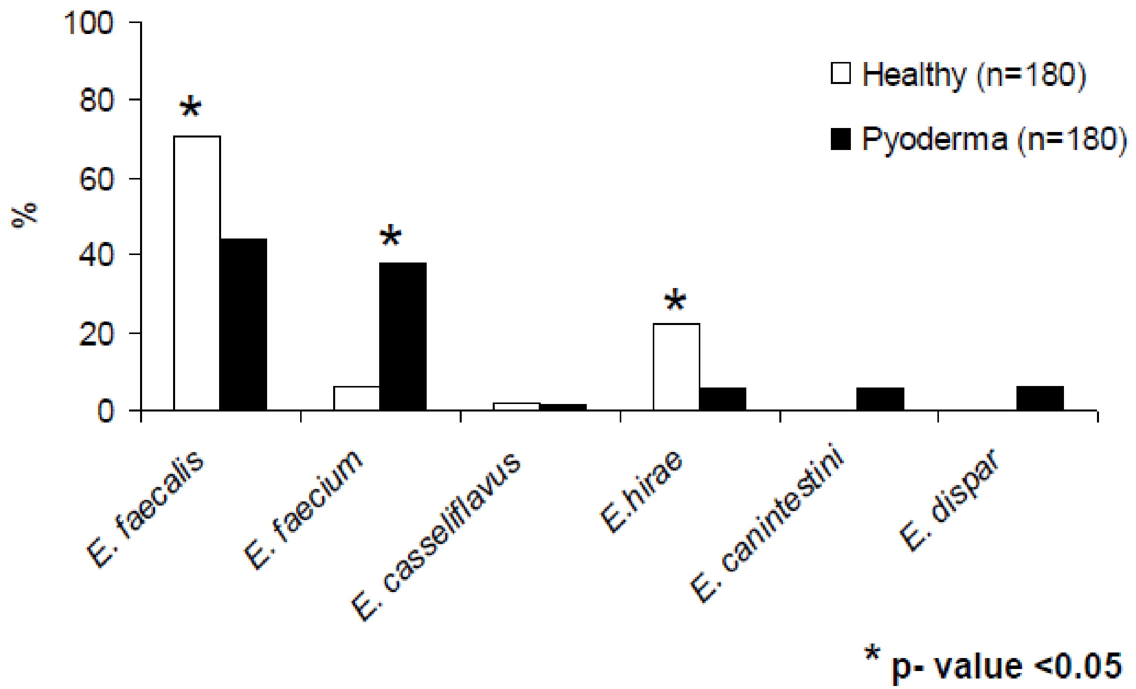

Figure 2.2 Diversity of enterococci in individual healthy dogs

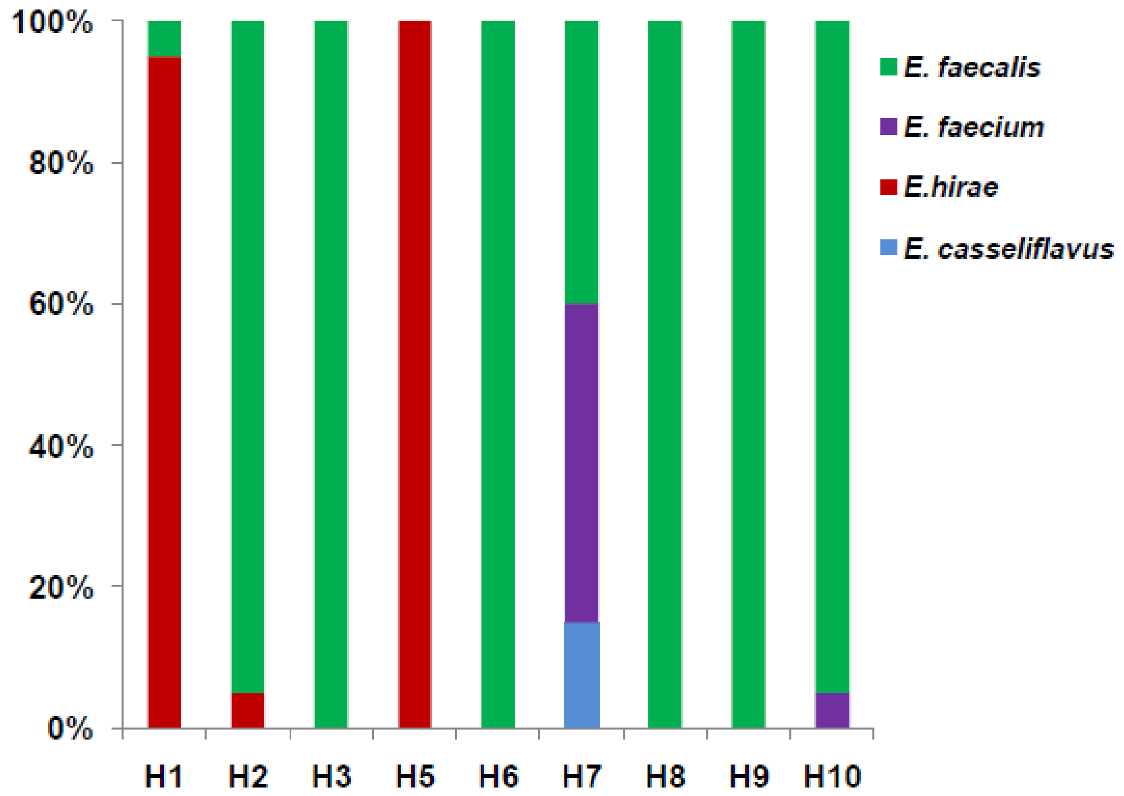

Figure 2.3 Diversity of enterococci in individual dogs with pyoderma

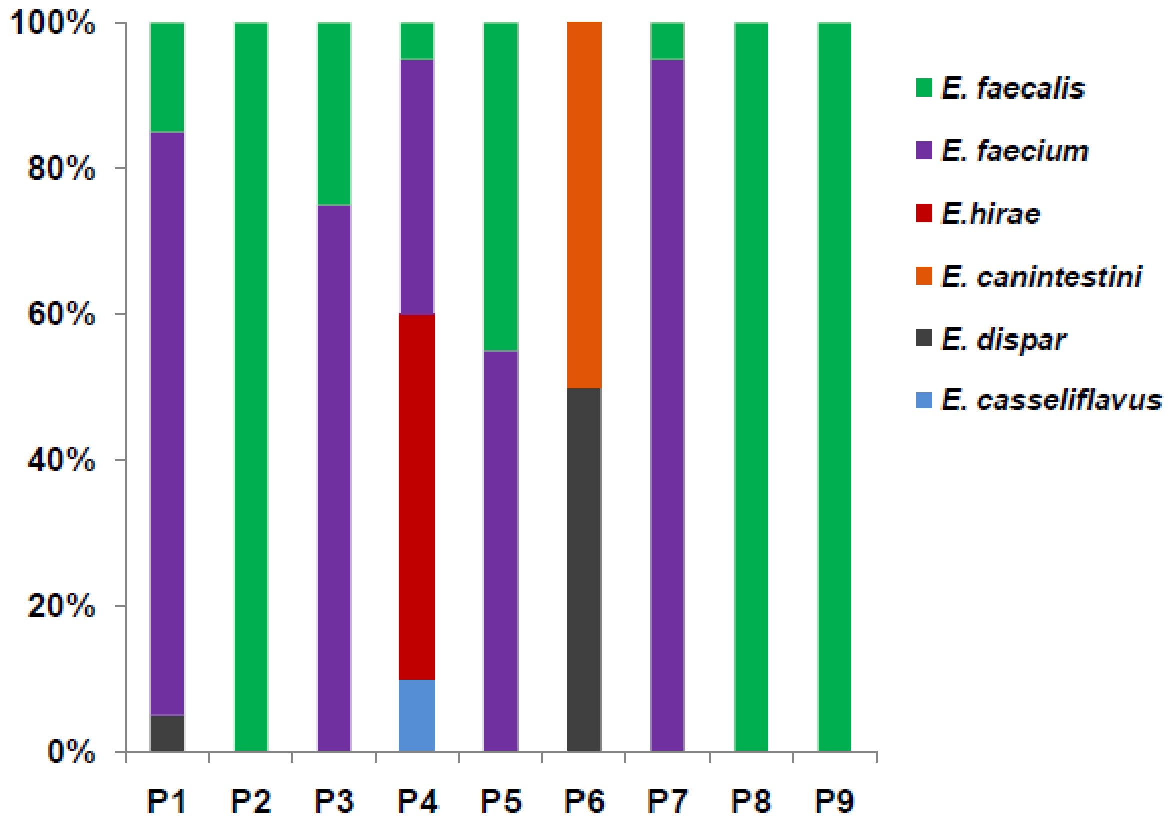

Figure 2.4 Antibiotic resistance profile (%) of enterococci from canine fecal samples

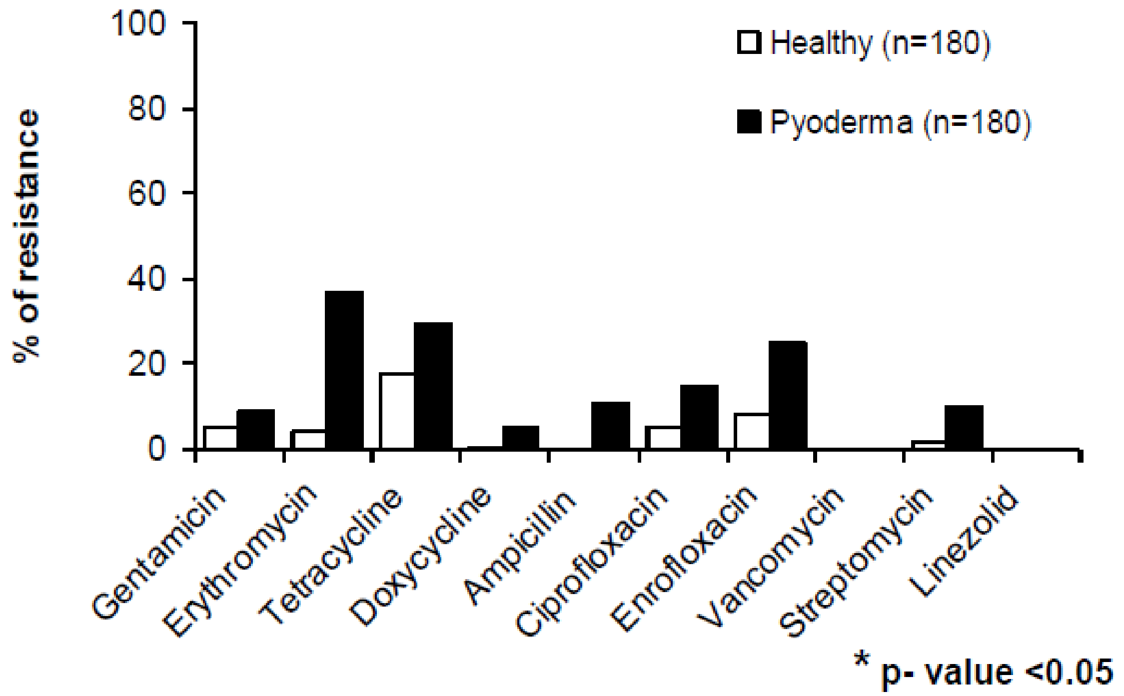

Figure 2.5 Antibiotic resistance profile (%) of *E. faecalis* from canine fecal samples

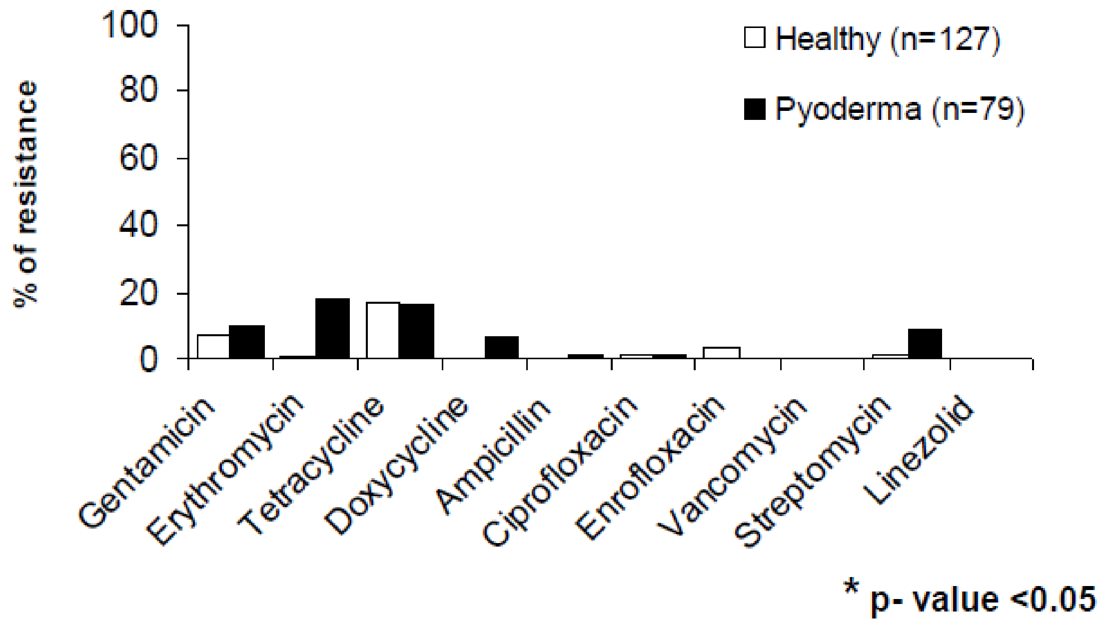

Figure 2.6 Antibiotic resistance profile (%) of *E. faecium* from canine fecal samples

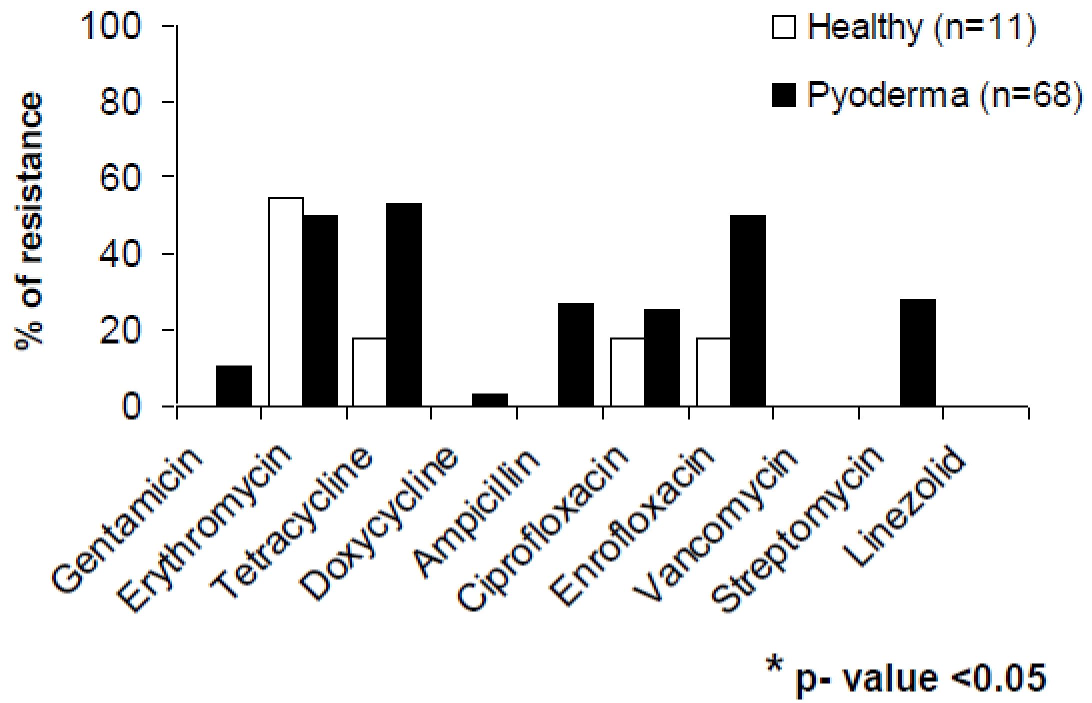

Figure 2.7 Antibiotic resistance profile (%) of *E. hirae* from canine fecal samples

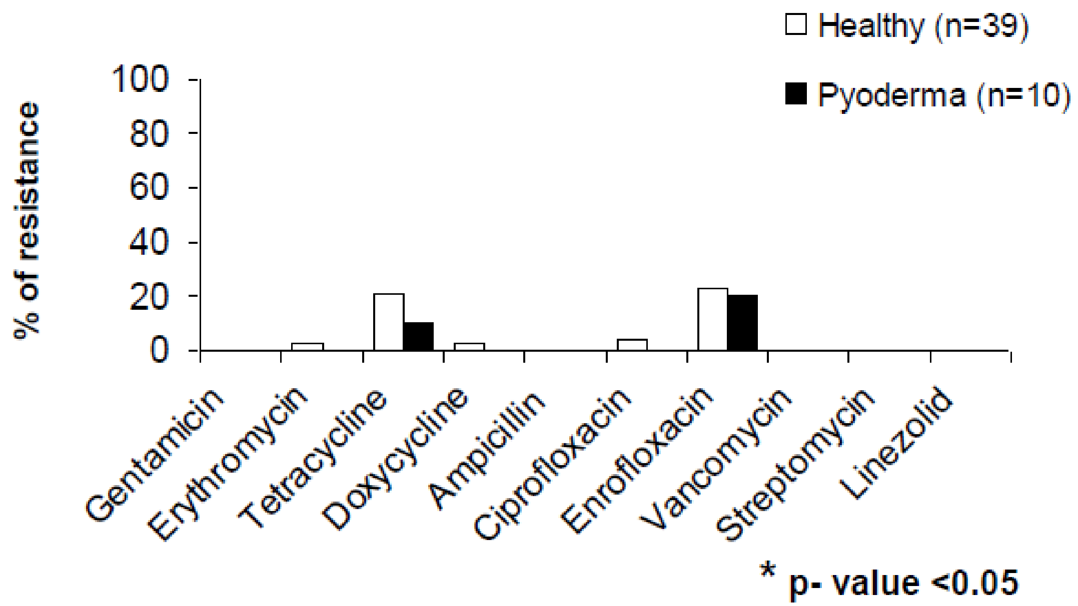

Figure 2.8 Antibiotic resistance profile (%) of *E. dispar* from canine fecal samples

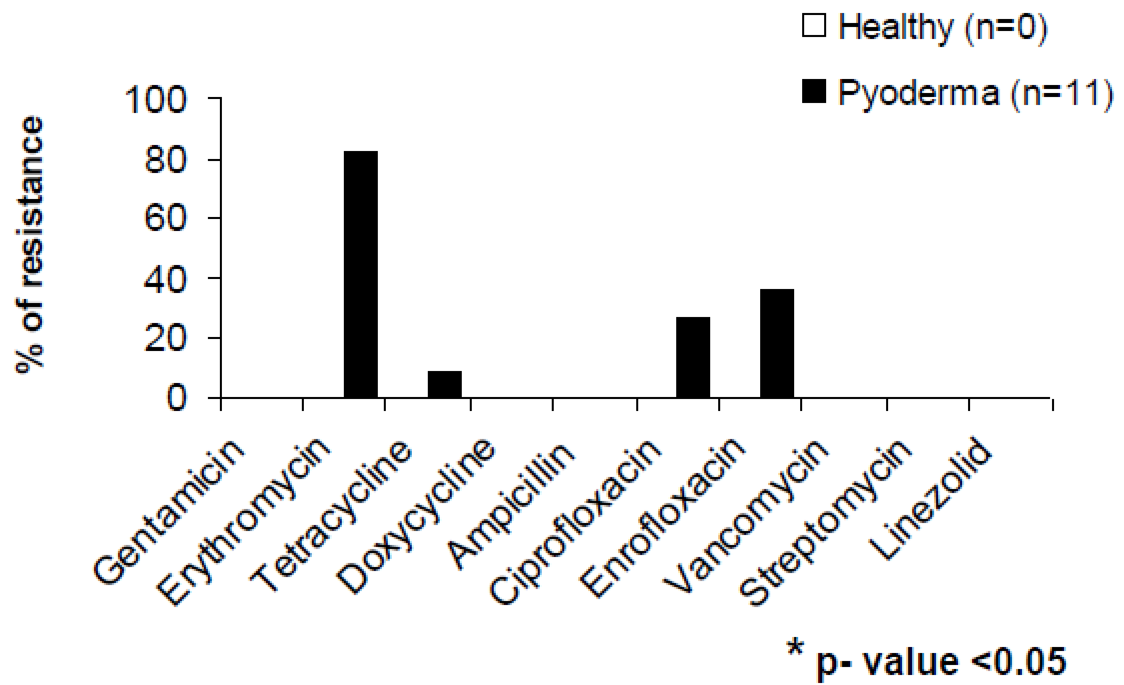

Figure 2.9 Antibiotic resistance profile (%) of *E. canintestini* from canine fecal samples

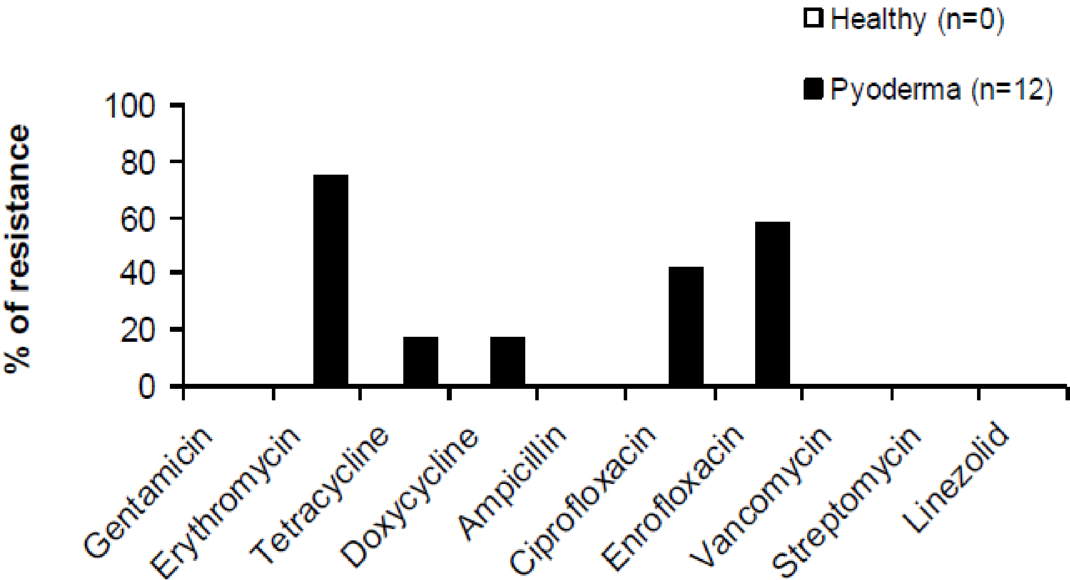

Figure 2.10 Antibiotic resistance profile (%) of *E. casseliflavus* from canine fecal samples

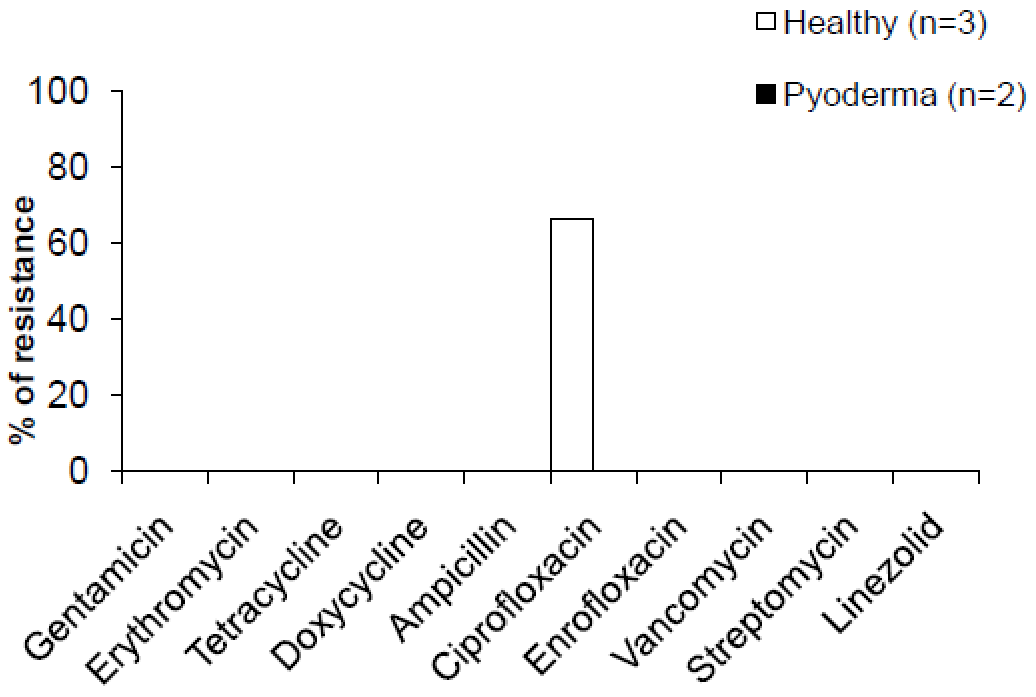

Figure 2.11 Prevalence and diversity of *tet* genes from the isolates phenotypically resistant to tetracycline

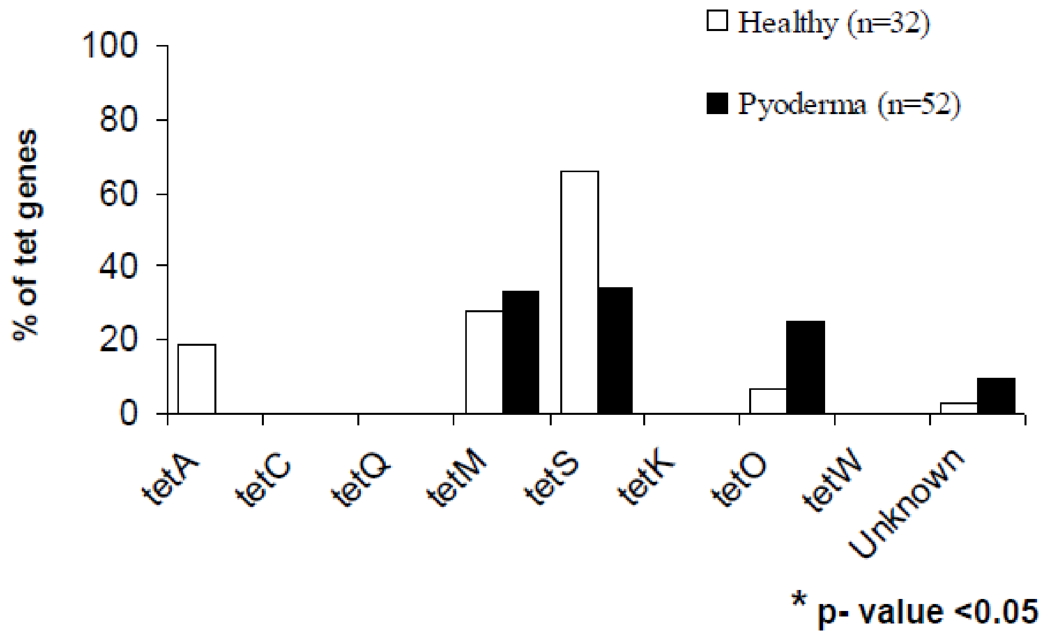

Figure 2.12 Profile and the prevalence of the *esp* and *gelE* gene in *E. faecalis* and *E. faecium* and *asa1* gene in *E. faecalis*

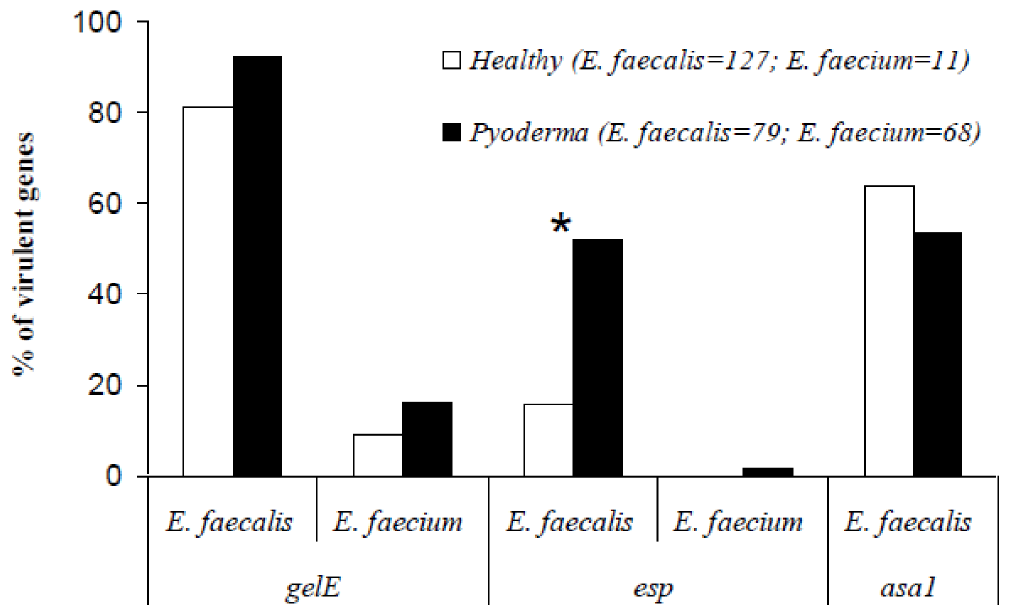

Figure 2.13 Correlation of *gelE* and gelatinase phenotype in *E. faecalis*

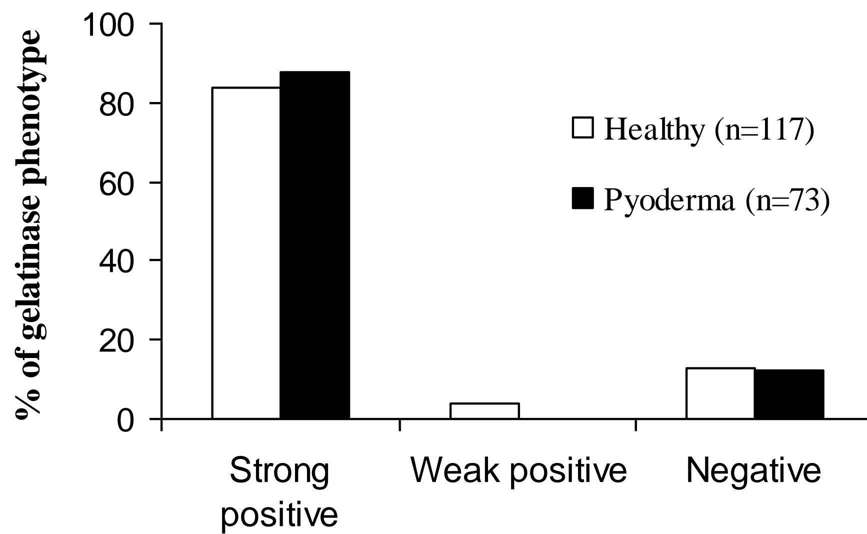

Figure 2.14 Clonal diversity of *E. faecalis* from healthy dogs

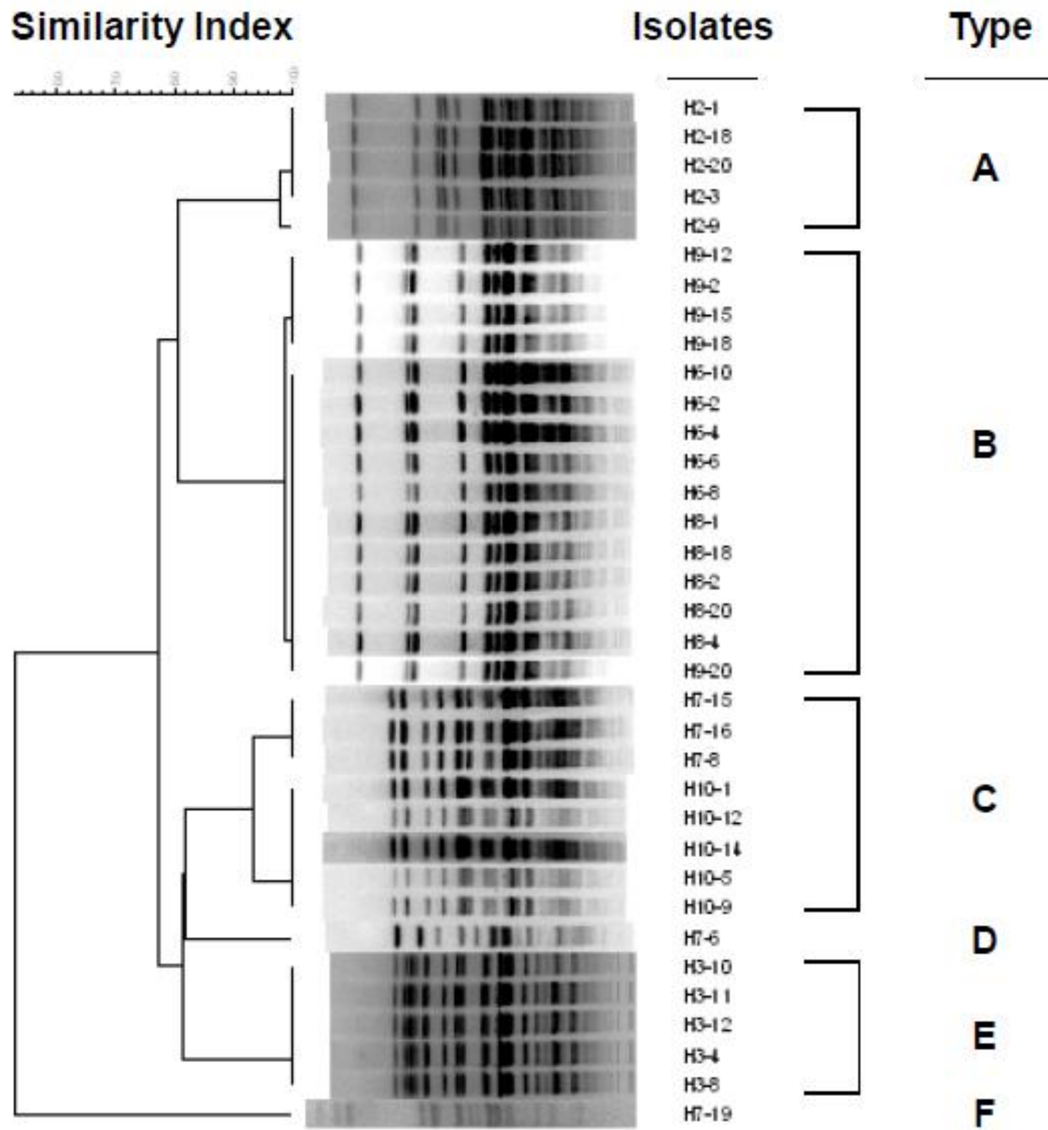

Figure 2.15 Clonal diversity of *E. faecalis* from pyoderma dogs

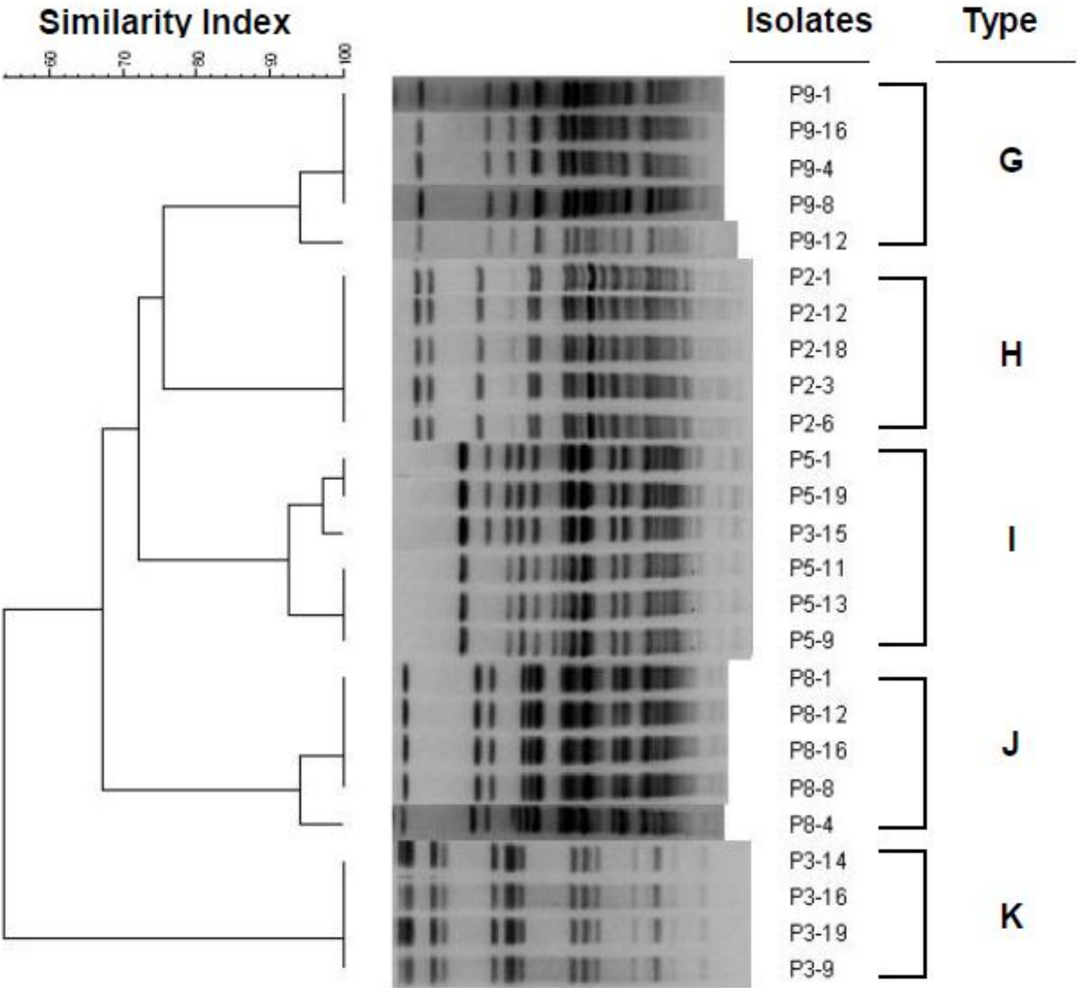

Figure 2.16 Clonal diversity of *E. faecium* from healthy dogs

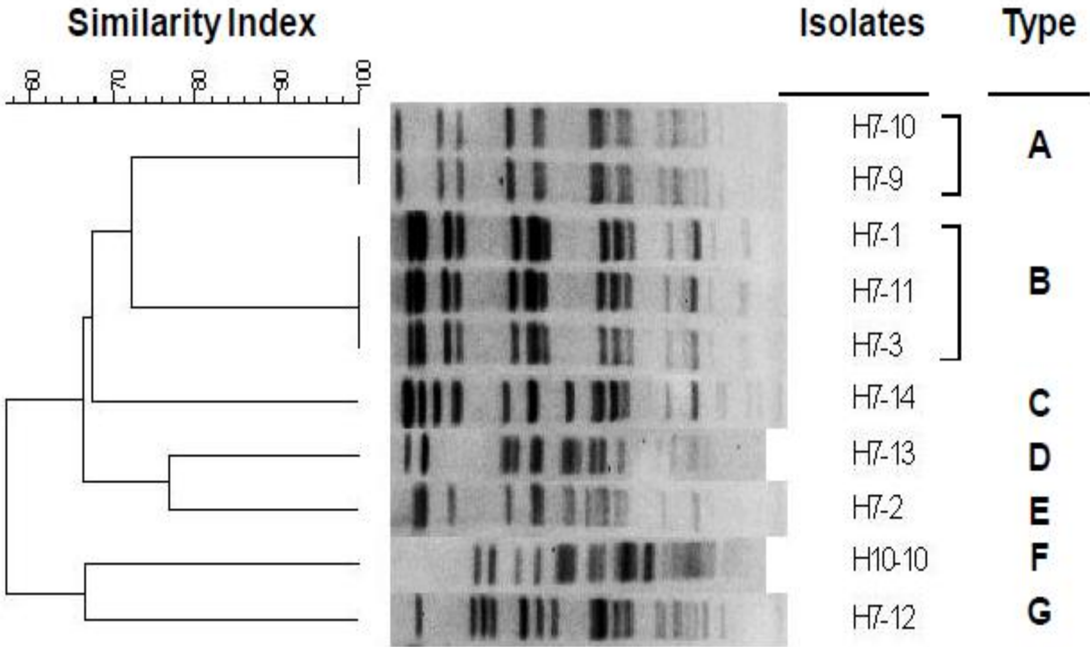

Figure 2.17 Clonal diversity of *E. faecium* from pyoderma dogs

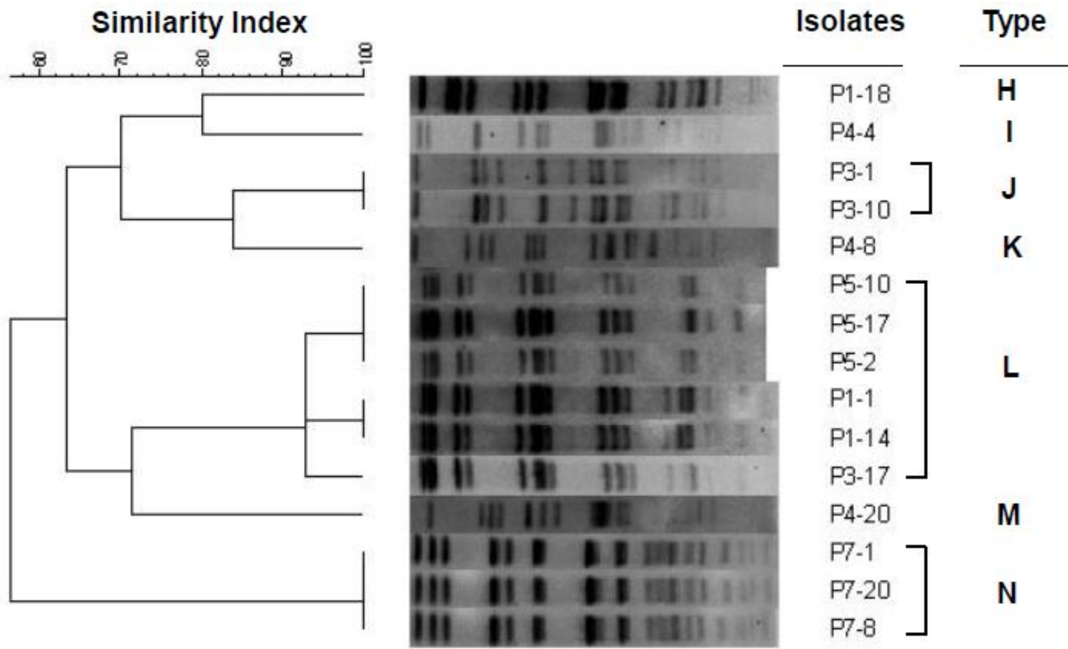

Table 2.7 Alignment of sequence of *gyrA* gene on selected quionolone resistant isolates

Isolates	Tyr	His	Pro	His	Gly	Asp	Ser (Codon 83)	Ala	Ile	Tyr	Glu (Codon 87)	Ser	MET	Val	Arg	MET	Ala	Glu
ANV-4_*E.faecalis* AS	Tyr	His	Pro	His	Gly	Asp	Ser	Ala	Ile	Tyr	Glu	Ser	MET	Val	Arg	MET	Ala	Glu
ACHL01000060 *E. faecium* AS	Tyr	His	Pro	His	Gly	Asp	Ser	Ala	Ile	Tyr	Glu	Ser	MET	Val	Arg	MET	Ala	Glu
ANV-3_*E.faecalis* AR	Tyr	His	Pro	His	Gly	Asp	Ser	Ala	Ile	Tyr	Glu	Ser	MET	Val	Arg	MET	Ala	Glu
AF060881 *E.faecium* AR	Tyr	His	Pro	His	Gly	Asp	Arg	Ala	Ile	Tyr	Glu	Ser	MET	Val	Arg	MET	Ala	Glu
AF060882 *E. faecium* AR	Tyr	His	Pro	His	Gly	Asp	Ser	Ala	Ile	Tyr	Lys	Ser	MET	Val	Arg	MET	Ala	Glu
D1-4 *E.faecalis**	Tyr	His	Pro	His	Gly	Asp	Ser	Ala	Ile	Tyr	Glu	Ser	MET	Val	Arg	MET	Ala	Glu
H7-3 *E. faecium**	Tyr	His	Pro	His	Gly	Asp	Ser	Ala	Ile	Tyr	Glu	Ser	MET	Val	Arg	MET	Ala	Glu
H7-8 *E. faecium**	Tyr	His	Pro	His	Gly	Asp	Ser	Ala	Ile	Tyr	Glu	Ser	MET	Val	Arg	MET	Ala	Glu
H7-9 *E. faecium**	Tyr	His	Pro	His	Gly	Asp	Ser	Ala	Ile	Tyr	Glu	Ser	MET	Val	Arg	MET	Ala	Glu

Isolates	Pro	Phe	Ser	Tyr	Arg	Tyr	MET	Leu	Val	Asp	Gly	His	Gly	Asn	Phe	Gly	Ser	Val
ANV-4_*E.faecalis* AS	Pro	Phe	Ser	Tyr	Arg	Ala	MET	Leu	Val	Asp	Gly	His	Gly	Asn	Phe	Gly	Ser	Val
ACHL01000060 *E. faecium* AS	Pro	Phe	Ser	Tyr	Arg	Tyr	MET	Leu	Val	Asp	Gly	His	Gly	Asn	Phe	Gly	Ser	Val
ANV-3_*E.faecalis* AR	Pro	Phe	Ser	Tyr	Arg	Ala	MET	Leu	Val	Asp	Gly	His	Gly	Asn	Phe	Gly	Ser	Val
AF060881 *E.faecium* AR	Pro	Phe	Ser	Tyr	Arg	Tyr	MET	Leu	Val	Asp	Gly	His	Gly	Asn	Phe	Gly	Ser	Val
AF060882 *E.faecium* AR	Pro	Phe	Ser	Tyr	Arg	Tyr	MET	Leu	Val	Asp	Gly	His	Gly	Asn	Phe	Gly	Ser	Val
D1-4 *E.faecalis**	Pro	Phe	Ser	Tyr	Arg	Ala	MET	Leu	Val	Asp	Gly	His	Gly	Asn	Phe	Gly	Ser	Val
H7-3 *E. faecium**	Pro	Phe	Ser	Tyr	Arg	Tyr	MET	Leu	Val	Asp	Gly	His	Gly	Asn	Phe	Gly	Ser	Val
H7-8 *E. faecium**	Pro	Phe	Ser	Tyr	Arg	Tyr	MET	Leu	Val	Asp	Gly	His	Gly	Asn	Phe	Gly	Ser	Val
H7-9 *E. faecium**	Pro	Phe	Ser	Tyr	Arg	Tyr	MET	Leu	Val	Asp	Gly	His	Gly	Asn	Phe	Gly	Ser	Val

Isolates	Asp	Gly	Asp	Gly	Ala	Ala	Ala	MET	Arg	Tyr	Thr	Glu	Ala	Arg	MET	Ser
ANV-4_*E.faecalis* AS	Asp	Gly	Asp	Gly	Ala	Ala	Ala	MET	Arg	Tyr	Thr	Glu	Ala	Arg	MET	Ser
ACHL01000060 *E. faecium* AS	Asp	Gly	Asp	Gly	Ala	Ala	Ala	MET	Arg	Tyr	Thr	Glu	Ala	Arg	MET	Ser
ANV-3_*E.faecalis* AR	Asp	Gly	Asp	Gly	Ala	Ala	Ala	MET	Arg	Tyr	Thr	Glu	Ala	Arg	MET	Ser
AF060881 *E.faecium* AR	Asp	Gly	Asp	Gly	Ala	Ala	Ala	MET	Arg	Tyr	Thr	Glu	Ala	Arg	MET	Ser
AF060882 *E.faecium* AR	Asp	Gly	Asp	Gly	Ala	Ala	Ala	MET	Arg	Tyr	Thr	Glu	Ala	Arg	MET	Ser
D1-4 *E.faecalis**	Asp	Gly	Asp	Gly	Ala	Ala	Ala	MET	Arg	Tyr	Thr	Glu	Ala	Arg	MET	Ser
H7-3 *E. faecium**	Asp	Gly	Asp	Gly	Ala	Ala	Ala	MET	Arg	Tyr	Thr	Glu	Ala	Arg	MET	Ser
H7-8 *E. faecium**	Asp	Gly	Asp	Gly	Ala	Ala	Ala	MET	Arg	Tyr	Thr	Glu	Ala	Arg	MET	Ser
H7-9 *E. faecium**	Asp	Gly	Asp	Gly	Ala	Ala	Ala	MET	Arg	Tyr	Thr	Glu	Ala	Arg	MET	Ser

*, Isolates from this study
AS, antibiotic susceptible (negative control); AR, antibiotic resistant (positive control)

References

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Chapter 3

Gut microbial diversity of healthy dogs

3.1 Abstract

The gastrointestinal microbiota of mammals play an important role in protecting the host from exogenous pathogenic microorganisms, and an imbalance among these microbes can lead to the development of disease. Our knowledge about these complex microbial communities is mostly based on the studies done on human gut commensals. As in humans, disruptions of gut microbiome can have an impact on the health of the dogs, but portion of the budget is allocated to small animal veterinary practices surprisingly, only few studies have been reported. In this study, we surveyed the gut microbial community from ten healthy dogs. Bacterial diversity was determined by the bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP) of about 600bp of the 16S rRNA gene. We characterized a total of 76,758 sequences obtained from ten fecal samples and determined the bacterial richness and diversity (3% and 5% divergence). Bacterial sequences were assigned to twelve phyla and 178 genera. *Firmicutes* represented the most dominant phylum. Five of 12 most common phyla were shared among all the microbial communities. The composition of the microbial community differed among the dogs in both the abundance and richness. Based on 3% dissimilarity, the number of operational taxonomical units (OTUs) (analyzed by MOTHUR) was 183 and the Shannon diversity index was estimated as 3.09 (mean). Our results indicate that the H-4 sample is the sample with great diversity at the species level and the H-9 sample at the phylum level, while the H-5 sample has the most diverse taxa. Twelve different genera were consistently identified in all the fecal samples. Among them *Lactobacillus*, *Ruminococcus*, *Turicibacter*, *Clostridium*, and *Fusobacterium* represented >2% of

the sequences and showed wide diversity in the bacterial species. Highest bacterial diversity was observed among the *Clostridium* genus (53 spp.) and lowest among the species belonging to the genus, *Turicibacter* (one spp). The dominant bacterial genuses identified in ten samples were *Turicibacter*, *Megamonas*, *Lactobacilli*, *Escherichia*, *Clostridium*, and *Fusobacterium*. In conclusion, this study provides data to support the hypothesis that the gut microbiota of healthy dogs is diverse and also provides baseline information about the canine gut microbial community in healthy animals.

Key words: bTEFAP, healthy dogs, fecal microbiota

3.2 Introduction

Commensal bacteria are considered to play an important role in the mammalian body. These are composed of an enormous number of microorganisms, forming a complex community of microbiota residing at various sites of the body such as the skin, the gastrointestinal, genitourinary and respiratory tracts (Chiller *et al.*, 2001; Hull *et al.*, 2007; Neish 2009; Verstraelen, 2008). The most densely colonized region in a vertebrate animal is the GIT. The cell densities in the colon are estimated to be 10^{12} cells/ml (Whitman *et al.*, 1988). The composition of this gut microbial community is likely to be influenced by host-microbe co-evolution coupled with selection pressure over time (Lee *et al.*, 2006). The interactions among this consortium of gut microbiota are critical to the health of the host animal. They play an important role in immune functions, protection from pathogens, nutrient acquisition, and the structure and functional development of gastrointestinal tract (GIT) (Sekirov *et al.*, 2010). Alterations in these GIT communities will affect their protective and immunomodulatory functions and, hence, maintaining their balance is necessary for the maintenance of health (Backhead *et al.*, 2005; Palming *et al.*, 2006).

Little is known about the bacterial composition in the GIT of healthy dogs. Similar to other mammals, dogs also likely harbor diverse microbiota in their GIT. The microbial content in the stomach of the dog is 10^4 to 10^5 CFU/gm (colony forming units per gram) (Benno *et al.*, 1992). Based on the culture dependant studies, in the stomach, aerobic and anaerobic bacteria are equally distributed with Gram positive bacteria being more dominant than Gram negative bacteria (Benno *et al.*, 1992). Along the small intestine, the number of bacteria progressively

increases along the duodenum, jejunum and ileum. In the duodenum and jejunum, the microbial population accounts for 10^5 to 10^6 CFU/ g of content and in the ileum is 10^7 CFU/g of content (Benno *et al*., 1992; Johnston, 1999). The numbers of aerobic, facultative anaerobes and anaerobes in the jejunum are equal (Mentula *et al*., 2005). Some of the common bacteria reported to be present in the small intestine of dogs include *Eubacterium*, *Bacteroides*, *Clostridium*, *Fusobacterium*, *Bifidobacterium*, and *Lactobacillus* among the anaerobes and *Streptococcus*, *Staphylococcus*, *Pasturella*, *Escherichia*, and *Enterobacter* among the aerobic and facultative anaerobes (Benno *et al*., 1992; Davis et al., 1977; Johnston, 1999). Along the GIT, the largest number of microbes is harbored by the large intestine, the caecum, and colon and ranges from 10^9 to 10^{10} CFU/gm of content (Greene, 1998). Gram negative aerobic bacteria and spore/non spore forming anerobic bacteria dominate in the large intestine of dogs (Greene, 1998). The predominant bacteria present in this part of intestine include *Eubacterium*, *Bacteroides*, *Clostridium*, *Peptococcus*, *Bifidobacterium*, *Lactobacillus* and *Streptococcus* (Benno *et al*., 1992; Davis et al., 1977; Greene, 1998).

Most of the studies available on dog GIT microbiota provide information on the effect of age, diet or antibiotics on the gut microbial structure (Simpson *et al*., 2002; Middelbos *et al*., 2009; Suchodolski *et al*., 2010). In 2002, Simpson *et al*., studied the gut microbial diversity in dogs belonging to different age groups and breeds that were fed different fiber diets. This study included 18 dogs belonging to two different age groups (young and old) and 3 breeds (German shepherds, English setters, and Miniature schnauzers). They were fed on high or low fiber diet. Using the culture method Simpson *et al*.,(2002) identified *Bacteroides*, *Fusobacteria*, *Lactobacilli* and *Streptococci* as the major bacterial groups while *Enterococci*, *Clostridia*, *Bifidobacteria* and *Eubacteria* represented the minor groups from the dog fecal samples. The

inclusion of 10% fiber in the diet of the animals decreased the number of *Bacteroides* in older dogs compared to the number of *Bacteroides* in younger dogs. Similarly, the number of *Clostridia* increased in German shepherds and *Fusobacteria* in English setters. Suchodolski *et al.*,(2009) evaluated the shift in the jejunal microbial community of healthy dogs (with jejunal fistula) fed with the antibiotic, tylosin, for 14 days. Feeding tylosin changed the bacterial composition of jejunal microbiota. Some of the bacterial taxa such as *Fusobacteria*, *Bacteriodales* and *Moraxella* decreased while *Enterococcus*-like organisms, *Pasturella* spp and *Dietzia* spp increased. Screening of the samples on 14th day after the cessation of antibiotic treatment revealed that some of the bacterial taxa had completely vanished. Middelbos *et al.*,(2010) demostrated that changes in the gut microbiota of healthy dogs could be induced by dietary fiber. *Fusobacterium*, *Firmicutes* and *Bacteriodetes* were the most dominant phyla. When the animals were fed with the control diet, *Fusobacterium*, *Firmicutes*, *Bacteriodetes*, *Proteobacteria*, and *Actinobacteria* were present while feeding these animals a fiber diet showed the presence of *Fusobacterium*, *Firmicutes*, *Bacteriodetes*, and *Proteobacteria* phyla. The effect of dietary fiber varied among individual dogs with respect to the phylogenetic diversity of their gut flora. However, not much information is available about microbial diversity in clinically healthy dogs.

Several techniques are available,(culture dependent and culture independent) to study the microbial diversity of GIT microbiota (Sekirov *et al.*, 2010). Most studies have used culture-based techniques to determine the microbial diversity in the GIT microbiota of dogs (Greetham *et al.*, 2002; Mentula *et al.*, 2005; Benno *et al.*, 1992; Davis *et al.*, 1977; Greene, 1998; Johnston, 1999). Greetham *et al.*,(2002) studied the gut microbiota of Labrador Retrievers using cultural and genotypic approaches. Different culture medium were used for determining the number of

total aerobes and anaerobes, coliforms, *Bifidobacteria*, *Lactobacilli*, Gram positive bacteria, *Clostridia* and *Bacteroides*. Among all the bacterial species identified, *Bacteroides* were the dominant species in all of the samples followed by Gram positive cocci, *Clostridia*, aerobes, and lactic acid bacteria, respectively. The culture grown on these media were also identified by the 16S rRNA sequencing. Sequence analysis data revealed the presence of new bacterial diversity that had not been identified with the culture method. Mentula *et al*.,(2005) compared the microbial communities of the small intestine and in the feces of Beagel dogs using a culture technique. The fecal samples were dominated by anerobes while in the small intestine, both aerobic and anerobic bacteria were present in about equal numbers. In the small intestine, *Staphylococcus*, Gram negative rods, and yeast were more prevalent while in the fecal samples, Gram positive rods, other than *Clostridium*, *Streptococci*, *Bacteroides* and coliforms, were the dominant bacteria. However, it is estimated that >80% of all gut microbiota cannot be cultivated in the laboratory (Sekirov *et al*., 2010) and hence the full species diversity was likely not fully assessed (Ward *et al*., 1990; Greetham *et al*., 2002). Several molecular methods for phylogenetic analysis are available and are commonly used to evaluate the microbial composition in fecal samples (Simpson *et al*., 2002; Middelbos *et al*., 2009). The development of such types of studies began when the domains of *Bacteriae*, *Archae* and *Eucarya* were proposed, based on the small subunit ribosomal DNA sequences (Woese *et al*., 1990). The conserved regions within these ribosomal DNA genes can be used to survey the uncultivable microbial diversity in the samples (Hugenholtz *et al*., 1998; Pace *et al*., 1985; Pace 1997). Several molecular techniques, such as DNA microarray, fluorescent in situ hybridization, and qPCR are available, but they cannot identify novel sequences (Bae *et al*., 2005; Sekirov *et al*., 2010). Identification based on

polymerase chain reaction amplification, cloning and sequencing of the single target gene can be time consuming (Randazzo *et al*., 2002).

A metagenomic approach that included cloning and sequencing of 16S rRNA has been used to study the microbial community of the GIT of dogs by Suchodolski *et al*.,(2008). They identified 4 phyla: *Firmicutes*, *Fusobacteria*, *Bacteroides*, and *Proteobacteria* from the GIT. *Lactobacillales* were present in all parts of intestine. *Enterobacteriales* were more abundant in small intestine while *Fusobacteriales* and *Bacteroidales* were more abundant in ileum and colon and *Clostridium* was abundant in duodenum, jejunum, ileum and colon. A similar technique has been used to compare the gut microbial communities of healthy dogs to dogs with inflammatory bowel disease (Xenoulis *et al*., 2008). This technique is more expensive compared to other sequencing techniques such as 454 pyrosequencing (Sekirov et al., 2010).

The metagenomic approach coupled with parallel DNA sequencing based on pyrosequencing can be used to study the bacterial diversity by comparing the sequence of the target gene from the sample to the available database. This method is more sensitive as it can detect less abundant species as well. Such an approach has been used to explain high bacterial diversity in the GIT microbiota of swine and cattle (Dowd *et al*., 2008a, 2008b).

In this study, we used bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). Pyrosequencing is a DNA-sequencing method based on the “sequence by synthesis” method (Armougom *et al*., 2009). During the enzymatic synthesis of the complementary DNA strand from the single-stranded DNA template, the nucleotide incorporation produced by DNA polymerase (DNA synthesizing enzyme) is accompanied by the release of inorganic pyrophosphate (PPi). An enzyme, ATP sulfurylase, subsequently converts PPI to ATP. This ATP oxidizes luciferase to luciferin and the signal light is generated. The amount of light is detected

by a charge coupled device (CCD) camera and is displayed as peak in a pyogram. Unincorporated dNTPs and excess ATPs are degraded by a nucleotide degrading enzyme, Apyrase. Since the added nucleotide is known, the nucleotide sequence of the target DNA can be determined based on the signal peaks of the pyogram (Figure 3.1) (Armougom *et al*., 2009).

bTEFAP is an expansion of pyrosequencing technique. The target DNA is amplified on the DNA capture beads by oil emulsion PCR procedure. After amplification, DNA that is attached to the bead is denatured and then sequenced using a normal pyrosequencing method. In this method tagged fusion primers are incorporated for the sample identity. This enables sequencing of dozens of samples at a time, thus allowing a high throughput (Figure 3.2).

The results retrieved from our analysis of fecal microbiota of healthy dogs are based on having employed bTEFAP technique using 16S rRNS as the target gene. We had hypothesized that the GIT tract of healthy animals would have a highly diverse community.

3.3 Materials and methods

3.3.1 Collection of fecal samples

Ten clinically healthy household dogs were included in this study. All dogs were fed with commercial diet and none of them were exposed to antibiotics at least for an year prior to the sample collection. Freshly laid fecal samples were collected from these healthy dogs and stored in falcon tubes on wet ice and then kept frozen at -80°C until analysis as freezing has no effect on the bacterial diversity (Wu *et al*., 2010)

3.3.2 Extraction of DNA

Upon thawing, DNA extraction was performed from 1g of canine fecal sample taken from the center to minimize environmental contamination effect using QIAamp stool DNA mini

kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The extracted DNA (1µl) was quantified using Nanodrop ND-1000 spectrophotometer (Nanodrop technologies, Wilmington DE).

3.3.3 bTEFAP sequencing PCR

3.3.3.1 Universal primer primary PCR

DNA samples were diluted to 100ng/µl. Initially, a primary PCR was performed for amplification of 600 base pairs (bp) of 16S rRNA using 16S universal universal Eubacterial primers, 530F and 1100R. The forward primer sequence was (5'–GTG CCA GCM GCN GCG G-3') and reverse primer was (5' –GGG TTN CGN TCG TTG-3'). The PCR reaction mixture consisted of 50 µl HotStarTaq Plus Master Mix Kit and 100ng of DNA template. Amplification conditions were as follows: initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30 sec, followed by annealing at 60°C for 40 sec, elongation at 72°C for 1 min and final extension step of 5 min at 72°C.

3.3.3.2 Linker-Tag-Universal Primer secondary PCR

The primary amplicons were incorporated with specific sample tag and FLX linker sequence for the identification of individual sample sequence from multiple samples in a single PicoTiterPlate (Roche, Nutley, New Jersey). For this purpose, secondary PCR was performed. Primers were designed with different tag sequences for FLX amplicon sequencing. The forward fusion primer consisted of sequence adaptor A, sample specific tag and eubacterial primer 530F. The reverse fusion primer consisted of sequence adaptor B, and eubacterial primer 1100R. The PCR conditions were same and 1µl of original PCR reaction was used as a template.

3.3.4 AmBead purification

After secondary amplification, the composite sample was prepared by mixing together equal volume of all the PCR products from different samples. The pooled amplicons were cleaned using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA) according to manufacturer's instructions.

3.3.5 bTEFAP FLX massively parallel pyrosequencing

The purified amplicon containing 9.6 E+06 double-stranded DNA molecules/µl with average size of 625bp were used for further processing. These samples were amplified after combining with 9.6 million DNA capture beads using emulsion PCR (Hori *et al.*, 2007). Recovery and enrichment of amplified DNA capture beads was followed by denaturation of DNA that is immobilized on the beads by PCR by using NaOH. The samples were then used for FLX pyrosequencing using Genome Sequencer FLX system (Roche) according to manufacturer's instructions.

3.3.6 bTEFAP sequence data analysis

The sequencing data were processed within Microsoft®. NET (Microsoft Corp, Seattle, WA) development environment. Raw reads obtained from the FLX sequencing run output files, were sorted into individual samples files based on the 100% homology to assigned tag sequence and >150bp read length. All of the sequences were assembled using CAP3 (Huang and Madan *et al.*, 1999) and sequences were blasted against RDP-II 16S database and GeneBank (http://ncbi.nlm.nih.gov). BLASTn best hits for the reads with sequence identity >98% and tentative consensus sequence length of 260bp were evaluated to the genus and species level. The genus clustering and their relative predicted proportion in the given sample was determined by a

post processing algorithm (% = [#sequences from an organism / total number of sequences from the sample] x 100%).

3.3.7 Phylogenetic assignment, alignment and clustering of 16S rRNA gene fragments

Based on the best BLAST hit, each sequence was linked with its appropriate taxonomic identification. The taxonomic assignment was followed by sequence alignment and distance matrix formation using MUSCLE and DNAdist programmes, respectively. Based on their sequence dissimilarity at 1%, 3% and 5%, they were clustered into operational taxonomic units (OTU).

3.3.8 Biodiversity

At each dissimilarity level, the rarefaction curves were generated and an estimation of species richness was performed by using ACE (abundance based coverage estimator), Chao1 and Shannon diversity indexes to predict the approximate frequency of species in our 16S rRNA library from the fecal samples of ten healthy dogs.

3.4 Results

3.4.1 Characteristics of pyrosequencing data

An application of bTEFAP pyrosequencing technique was used for the analysis of fecal microbiota from dogs. Sequences were generated from the middle region (600 bp) of 16S rDNA genes in the bacterial DNA, and the libraries were constructed from ten fecal samples from healthy dogs. A total of 76,758 sequences were obtained from the pyrosequencing run for all samples. Each unique sequence read was searched against the Ribosomal Database Project (RDP) using the BLASTN tool. After the best match hits that met the required criteria (materials

and methods), 56,320 sequences remained for analysis (Table 3.1). The number of sequences varied among the animals from 4622 to 6102 with the mean of 5632.

3.4.2 Abundance of microorganisms in gut microbiota of healthy dogs

Aligned sequences were grouped into operational taxonomic units (OTU) at the 3% and 5% divergence levels (Table 3.2). At the species level, the number of OTUs (mean±SEM)in the ten libraries ranged from $1.83 \pm 0.1 x 10^2$, while at genus level, a range of $1.39 \pm 0.08 x 10^2$ OTUs was observed. A rarefaction curve analysis based on the number of sequences in each sample shows that the curve at species, genus and phylum level in all the samples had reached the plateau, which indicates that these libraries were completely sampled (Figure 3.3).

3.4.3 Biodiversity

Based on the species richness estimators, ACE, at 3% dissimilarity, the OTUs ranged from $2.50 \pm 0.1 x 10^2$and at 5% dissimilarity, from $1.90 \pm 0.09 x 10^2$; and for Chao1, at 3% dissimilarity, the OTUs ranged from $2.5 \pm 0.1 x 10^2$and at 5% dissimilarity, $1.90 \pm 0.1 x 10^2$. Based on the species diversity index, Shannon diversity, at 3% dissimilarity, the OTUs ranged from 1.95-3.73 and at 5% dissimilarity, from 1.81-3.23 (Table 3.2).

Among the ten samples tested, H4 had the highest species richness (ACE-305.45, Chao1-307.54) and at 5% dissimilarity, H9 had the highest species richness (ACE-237.20, Chao1-237.20) (Table 3.2). But the H5 sample was the most diverse sample at 3% and 5% dissimilarity with values of 3.73 and 3.23 respectively for Shannon diversity index (Table 3.2). This shows that microbial community from H5 had high evenness.

3.4.4 Bacterial composition in the gut microbiota of healthy dogs

Based on the phylogenetic classification of the sequences inherited from RDP-II database, about 178 different bacterial genera belonging to 12 different phyla were identified (Table 3.3).

3.4.5 Phyla level

Each fecal sample was represented by ≥ 6 different phyla out of which 5 phyla were present in the gut of all the dogs (Figure 3.4). Of the total bacterial sequences, *Firmicutes* represented the dominant phylum in our library. The other most dominant phyla detected were *Fusobacteria*, *Proteobacteria*, *Bacteriodetes*, *Spirochaetes*, *Actinobacteria*, *Chloroflexi* with sequences ranging from (mean) 1-5%. The rest of the sequences were distributed among the phyla (mean %) *Lentisphaerae* (0.06%), *Fibrobacteres* (0.03%), *Verrucomicrobia* (0.01%), *Tenericutes* (0.01%) and *Synergistetes* (0.01%) and comprised less than 1% of the intestinal bacterial population at the genus level (Figure 3.4).

In the most dominant phylum, *Firmicutes*, the percentage of sequences ranged between 95.9-36.9% with a mean of 77.1%. The majority of the sequences (range) belonging to the genus *Turicibacter* (77.2-0.3%), *Lactobacillus* (65.5-0.01%), *Megamonas* (38.13 and 0.07%), *Ruminococcus* (28.7-1.05%) and *Clostridium* (21.6-1.06%) were identified. All these species were ubiquitous in all of the 10 samples, with the exception of the *Megamonas* species, which was present in 9 out of 10 samples.

Sequences belonging to the phylum *Fusobacteria* ranged between 22.7-0.1% with a mean of 5.3%.The dominant genus in this phylum was *Fusobacterium* (22.4-0.1%).

In the phylum *Proteobacteria*, (25.9-0.4%) with a mean of 5.1%, the more prevalent genera were *Escherichia* (22.3-0.01%) and *Moraxella* (5.7-0.02%), which were present in 8 out of 10 samples.

Species belonging to the phylum *Bacteriodetes* were also identified and ranged between 12.6-0.02% with a mean of 3.3%. *Bacteroides* species were detected in 9 fecal samples in this study.

The other phylum that was encountered in the feces was *Spirochaetes,* which ranged from 15.7-0.01% with a mean of 1.8%.

3.4.6 Genus level

Out of 178 genera present in our library, only twelve common bacterial genera (mean sequence %) were found in all of the libraries: *Leuconostoc* (0.16), *Weissella* (0.23), *Lactobacillus* (26.75), *Streptococcus* (1.15), *Lactococcus* (0.13), *Ruminococcus* (5.84), *Turicibacter* (13.11), *Clostridium* (9.54), *Roseburia* (1.85), *Dorea* (1.28), *Eubacterium* (1.30), and *Fusobacterium* (6.89). Among these, *Lactobacillus*, *Ruminococcus*, *Turicibacter*, *Clostridium*, and *Fusobacterium* represented >2% of the sequences. A wide diversity in the bacterial species was observed among these genera except for *Turicibacter*, from which only one bacterial species, *Turicibacter sanguinis* was dominating in all the samples (Figure 3.6; 3.7; 3.8; 3.9; 3.10). The highest diversity was observed among the *Clostridium* genus, which was comprised of 53 different bacterial species (Figure 3.10).

Figure 3.5 shows the bacterial diversity at the genus level in individual dogs with ≥2% (sequences) cut off. The bacterial composition in each dog was different. The dominant sequence in each fecal sample was as follows: H-1, -8 *Turicibacter*; H-2 *Megamonas*; H-3, -6, -7, and -10 *Lactobacillus*; H-4 *Escherichia*; H-5 *Clostridium*; H-9 *Fusobacterium.*

3.5 Discussion

The gut commensals play important role in terms of nutrional, developmental, defensive and physiological processes in the host body (Mackie *et al*., 1999). In the U.S., the number of pet animals is increasing (Municipality of anchorage animal care and control services, statrtegic plan, 2006). The U.S. ranks first among the worlds top ten countries and has the largest pet dog population (http://www.mapsofworld.com/world-top-ten/countries-with-most-pet-dog-population.html). Based on market research statistics, from 2007, the pet population accounts for more than 72 million. The average expenditure cost per household per pet is estimated to be $366 in U.S. (http://www.avma.org/reference/marketstats/sourcebook.asp). In comparison to the studies done on other animals such as cattle and pigs, the data available on the canine gut microbial community are very limited.

Most of the information available with respect to the canine gut microbial community is based on cultivation approach (Benno *et al*., 1992; Davis et al., 1977; Greene, 1998; Greetham *et al*., 2002; Mentula *et al*., 2005). The data available from this technique are considered to be inadequate, as only 1-20% of the total gut microbiota is cultivable in the laboratory conditions (Sekirov *et al*., 2010); and thus, microbial diversity is greatly underestimated. The assessment of microbial diversity by using molecular techniques has facilitated the study of this diverse microbial community harbored by the host. DNA-based techniques have been recently used to study the gut microbiota in different animals including cattle, pigs, monkeys and dogs (Durso *et al*., 2010; Dowd *et al*., 2008a; McKenna et al., 2008; Suchodolski *et al*., 2009; Dowd *et al*., 2008a). Those studies that were done on dogs focused mainly on determining the effect of diet and antibiotics on the fecal microbial community of dogs (Middelbos *et al*., 2010; Suchodolski *et al*., 2009).

Our analysis of 16S rDNA sequences from the fecal microbiota of healthy dogs revealed a wide diversity of microorganisms, indicating that the digestive tract has a complex community of microbes. A total 12 phyla with the most prevalent being *Firmicutes* and five minor phyla (mean ≤ 1% sequences), were encountered in the fecal samples. *Lactobacillus, Ruminococcus, Fusobacterium, Turicibacter* and *Clostridium* represented some of the dominant bacterial genera in this study. *Firmicutes* is also reported to be the dominant phylum in the cattle, macaque and human gut microbiome (Durso, *et al*., 2010; McKenna *et al*., 2008; Eckburg *et al*., 2005). Our study shows great dog-to-dog variation in the composition of fecal microbiota of individual dogs.

Most of the genera belonging to the phylum *Firmicutes* (86%) and *Actinobacteria* 94% were Gram positive bacteria (86%) while in the remaining phyla identified in this study, Gram negative bacteria formed the major group. In the phylum, *Proteobacteria,* 91% of bacterial genera were Gram negative bacteria. while *Chloroflexi* consisted of 50% Gram negative bacteria. In the phyla, *Bacteroidetes, Tenericutes*, *Synergistetes*, *Fusobacteria*, *Spirochaetes*, *Lentisphaerae*, *Fibrobacteres*, and *Verrucomicrobia*, all the bacterial genera were identified as Gram negative. In the present study Gram positive bacteria dominated the dog fecal sample and this matches with the previously published data (Mentula *et al*., 2005). The main bacterial genera present in healthy dogs include *Lactobacillus*, *Clostridium*, *Streptococci* and *Bacteroides* (Maskell and Johnson, 1993; Mentula *et al*., 2005) and were also identified in our samples.

The fact that the rarefaction curves reached a stable value suggests complete estimation of species richness in all ten samples. Hence, most bacterial diversity has been included in our sampling size.

We used the nonparametric estimators ACE and Chao1 to estimate richness in the ten libraries. Based on our statistical analysis of the OTUs, a difference in the relative abundance

(ACE, Chao1) and diversity (Shannon diversity) of bacterial taxa in all the samples was observed, which may be because the fecal samples from dogs belonging to different age groups, and breeds fed different diets were compared. Based on Chao1, the estimated species richness values (mean) at 3% sequence dissimilarity was 254.51. This richness estimate is similar to that observed in humans (264) (Eckburg *et al.*, 2005) and is much lower than that reported in food animals such as cattle (637) (Durso *et al.*, 2010).

In this study, most of our samples did not show great difference in their diversity index (3% dissimilarity) (Table 3.2). The average estimate of the diversity index for all of the ten animals was 3.09, a value lower than that observed in cattle (4.85) (Durso *et al.*, 2010) at 3% sequence dissimilarity. Although the diversity index is widely accepted for determining the biodiversity, and it takes into account both species richness and evenness, two communities with same index value cannot be considered to have same bacterial diversity as there may be differences with respect to high or low evenness or richness, respectively (Kennedy, 1999).

In the present study, *Lactobacilli*, *Clostridium*, *Turicibacter*, *Megamonas*, *Fusobacterium*, and *Ruminococcus* were among the dominant bacterial genera that were distributed among the dogs. For bovine feces, *Prevotella* is reported as the most common bacterial genus (Durso *et al.*, 2010), and in dairy cattle *Clostridrium* is most commonly found (Dowd et al., 2008b). Among macaques and humans, the *Clostridrium* spp. are reported to be the dominant bacteria in their digestive tract (Durso *et al.*, 2010; Eckburg *et al.*, 2005).

Several studies have examined the gut microbial community in dogs (Suchodolski *et al.*, 2009; Middelbos *et al.*, 2010; Greetham *et al.*, 2002; Mentula *et al.*,2005; Simpson *et al.*, 2002). Suchodolski *et al.*, (2009) studied the effect of antibiotic tylosin on the microbial diversity in the canine jejunum using bTEFAP analysis. They identified 10 different bacterial phyla, with

Proteobacteria being the most dominant phylum. Inter-individual responses were observed for specific taxa in each of the dogs. During tylosin administration, the proportion of *Enterococcus*-like organisms, *Pasteurella*, and *Dietzia* increased. Bacteria belonging to the family *Spirochaetes*, *Streptomycetaceae*, and *Prevotellaceae* were not recovered 2 weeks after antibiotic treatment. On the other hand, according to the data gathered by Middelbos *et al*., (2010), when they looked at the effect of fiber diet on the gut community, they found that *Fusobacteria, Firmicutes*, and *Bacteroidetes* were the dominant phyla in the gut communities of healthy dogs, which agree with the data in our study. Two phyla *Lentisphaerae*(<1%) and *Fibrobacteres*(<1%) were identified in our study that were not previously reported in healthy dogs.

The number of OTUs (5%) and richness (5%) estimates in the present study was lower than that observed in the study done by Suchodolski *et al*., (2009) and higher than the number obtained by Middelbos *et al*.(2010). In the pyrosequencing studies conducted by Suchodolski *et al*.,(2009) and Middelbos *et al*.,(2010) the mean values for the following indices (5%) were as follows: OTU – 143, ACE – 191, Chao1 – 197; OTU – 135, ACE – 186, and Chao1 – 177, respectively. Based on the Shannon diversity index, a less diverse population was observed in our samples compared to what observed by Suchodolski *et al*., (2009) study. These differences in the richness and diversity likely reflects differences in the age, breed (Simpson *et al*., 2002) and diet of the individual dogs (Middelbos *et al*., 2010) and differences in the sampling site of intestine (Mentula *et al*., 2005). It must also be noted that in both of the above mentioned studies, Suchodolski *et al*., (2009) and Middelbos *et al*.(2010), the sample sizes were small (≤ 6 samples).

Thus, in the present study, bTEFAP analysis was used to study the microbial community present in the feces of healthy dogs. Additionally, using the bar-coded primers allowed the

bTEFAP technique to sequence ten samples simultaneously. Despite these advantages, the pyrosequencing technique, like any other molecular techniques, also has limitations. Because of the sequence conservation of the target gene, 16SrRNA, bTEFAP may not detect bacterial microbiota with divergent target sequences. The smaller length of the reads produced during pyrosequencing may allow for lower unique discrimination. Moreover, all of these sequence studies carried out in dogs, including the present study, rely on the PCR step and this may also affect the number of bacteria observed due to PCR's bias with respect to the variation in the primer binding site of the target gene (vonWintzingerode *et al*., 1997). DNA extraction bias due to incomplete extraction can also affect abundance and composition of the diverse bacterial community (Martin-Laurent *et al*., 2001).

The results from this study suggest that there is difference in the composition of gut microbial community among individual healthy dogs. This difference may be attributed to age, diet and genetic make-up of the individual animals. It has also been noted that healthy dogs harbor more diverse bacterial population in their GIT as compared to dogs under selective pressure (Suchodolski *et al*., 2009).

3.6 Conclusion

Gut microbiota, the most abundant commensals in a vertebrate animal, are important to the health of the host animal. The main objective of this research was to study the diverse habitat of the microbial community in the canine gut. Until recently, assessing microbial diversity completely was hindered due to the limitations of the traditional cultivation methods. Most of the microbes present in the GIT do not grow in laboratory culture media. Development of molecular techniques such as pyrosequencing allowed the analysis of diverse microbial community in the mammalian gut without culturing limitations.

Using bTEFAP technique in this study, we determined the diversity in the fecal microbiota of the healthy dogs. A total of ten apparently healthy dogs were included in this study. The total number of sequences per sample ranged from 4622 to 12925. After quality trimming, a total of 56,320 sequences were used for further analysis. The sequences from ten libraries were grouped into OTUs based on 3% and 5% sequence dissimilarities (corresponding to species and genus level, respectively). The number of OTUs ranged from 118 to 224 and 88 to 166 at 3% and 5% sequence dissimilarity, respectively. At the species level, the mean values for ACE, Chao1 and the Shannon diversity index were estimated as 253, 254, and 3.09, respectively. A total of 12 phyla and 178 genera were identified among the ten fecal samples.

Our results indicate that the GIT of dogs is composed of a diverse population of bacteria. The rarefaction analysis revealed that there was sufficient sampling, indicating that this data likely represent the entire gut microbiota. Our study found two phyla *Lentisphaerae* (<1%) and *Fibrobacteres* (<1%) that have not been previously reported in healthy dogs.

Overall, the data revealed high variability in the composition of gastrointestinal communities of healthy dogs. This study serves as baseline information for future studies that aim to determine the gut microbial community structure in any pathological conditions, or for examining the impact of various factors on them or other related issues.

3.7 Figures and Tables

Figure 3.1 Principle of pyrosequencing technology (Armougom and Rauolt, 2009)

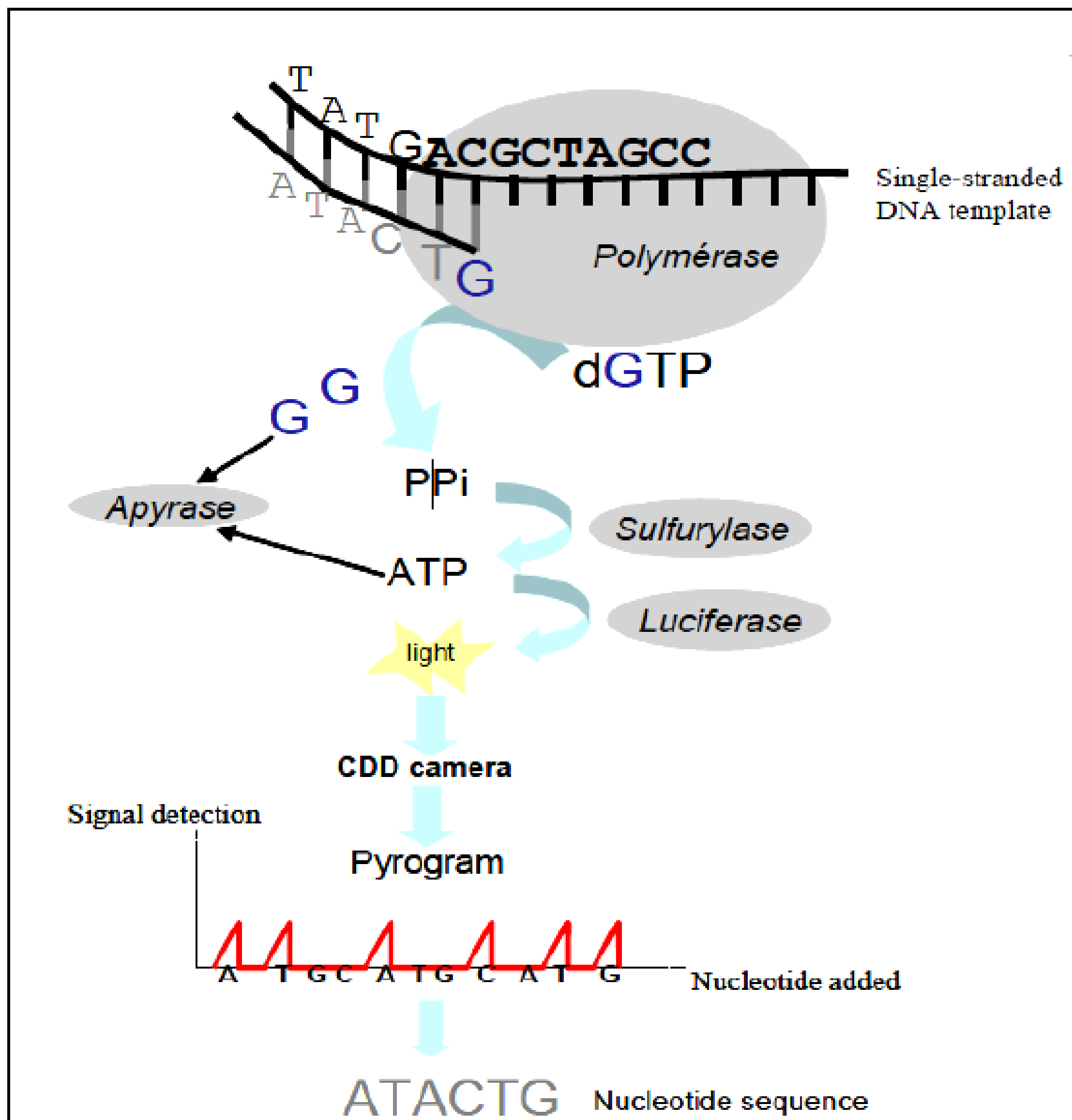


Figure 3.2 Graphical depiction of bTEFAP technique (Dowd *et al*., 2008)

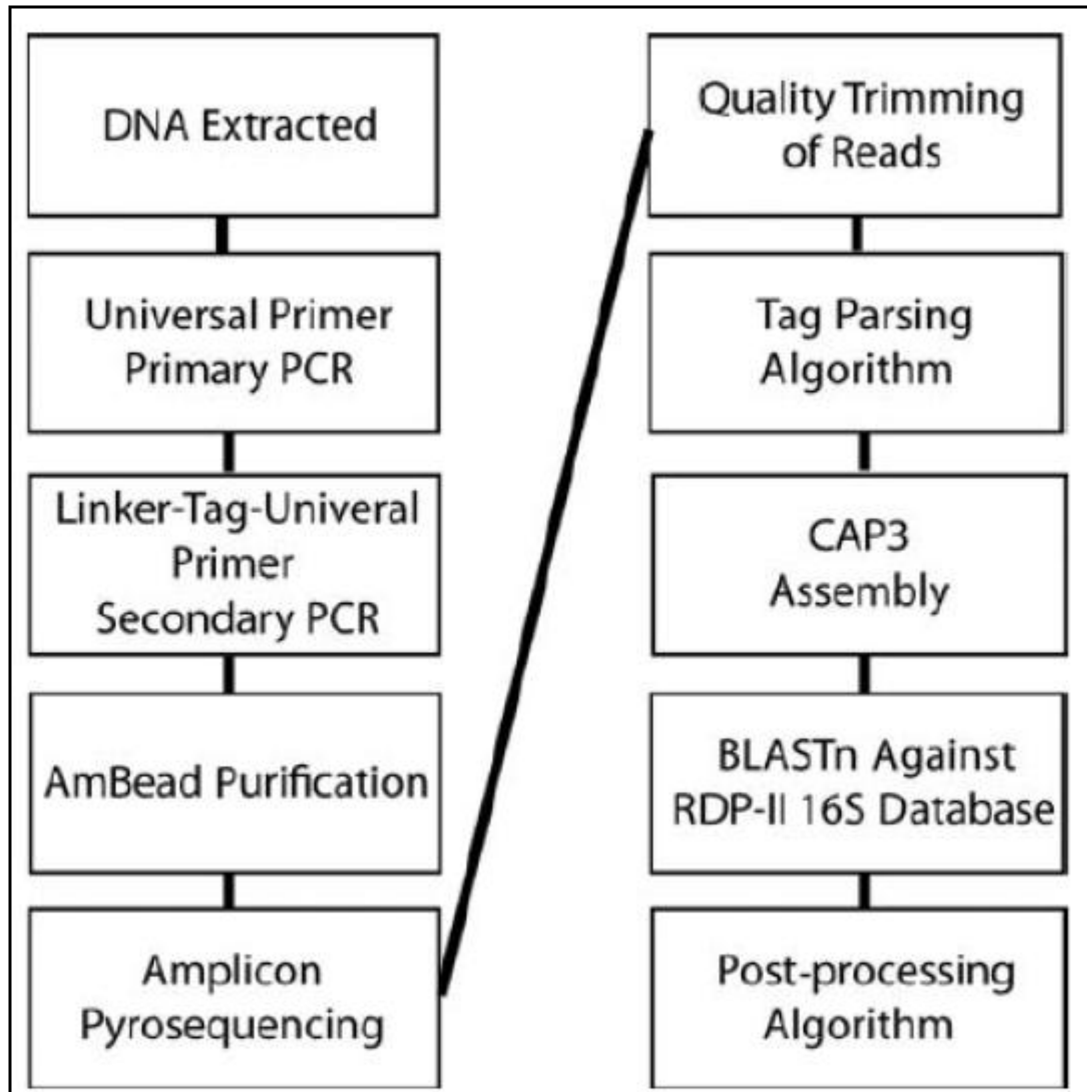


Table 3.1 Number of reads and trimmed sequences per sample of canine feces microbiota

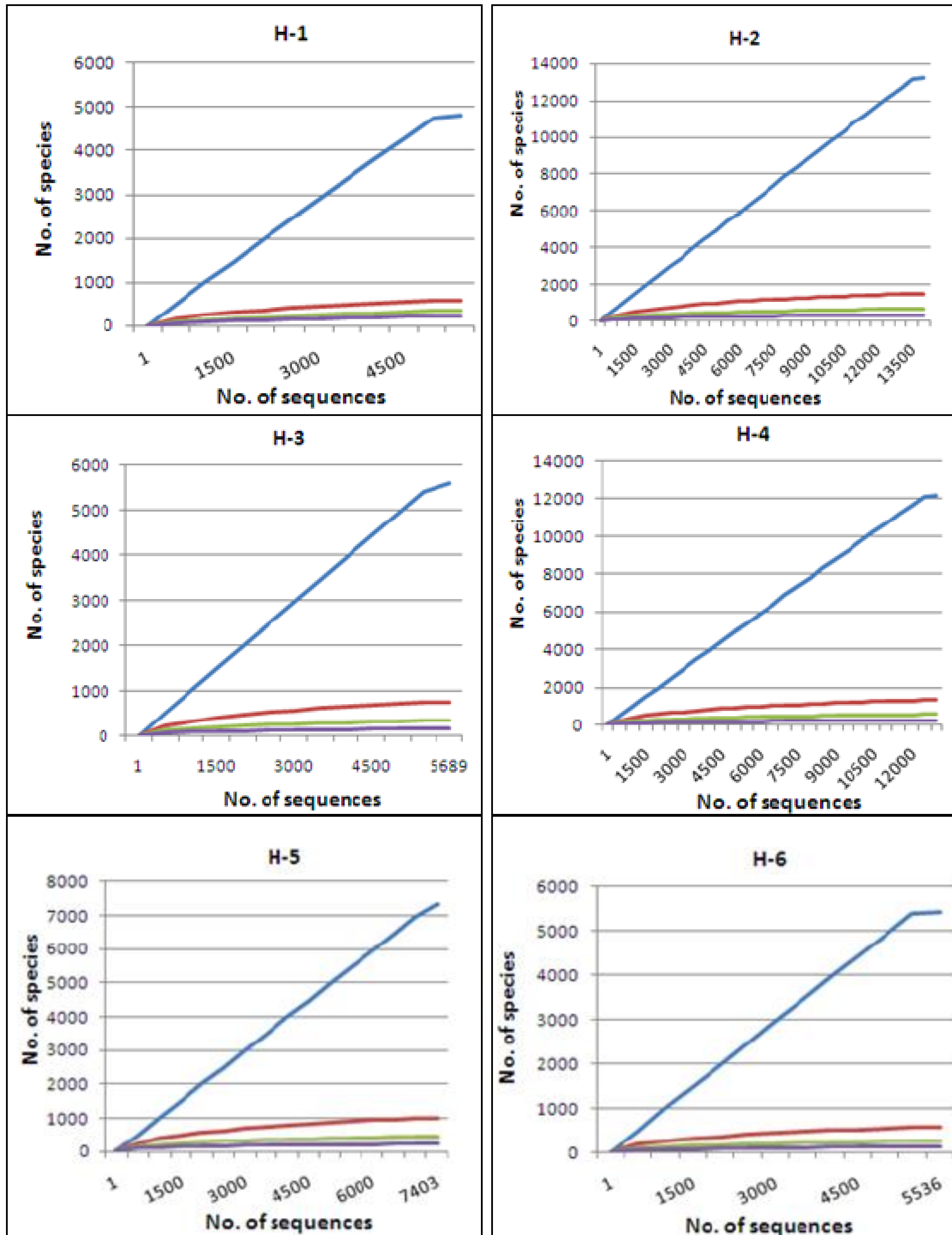
Sample #	# of sequences	
	Total sequences	**Trimmed sequences**
H-1	4622	4622
H-2	11886	6101
H-3	5218	5218
H-4	11363	6102
H-5	6634	6101
H-6	5191	5191
H-7	4915	4915
H-8	8138	6102
H-9	12925	6102
H-10	5866	5866

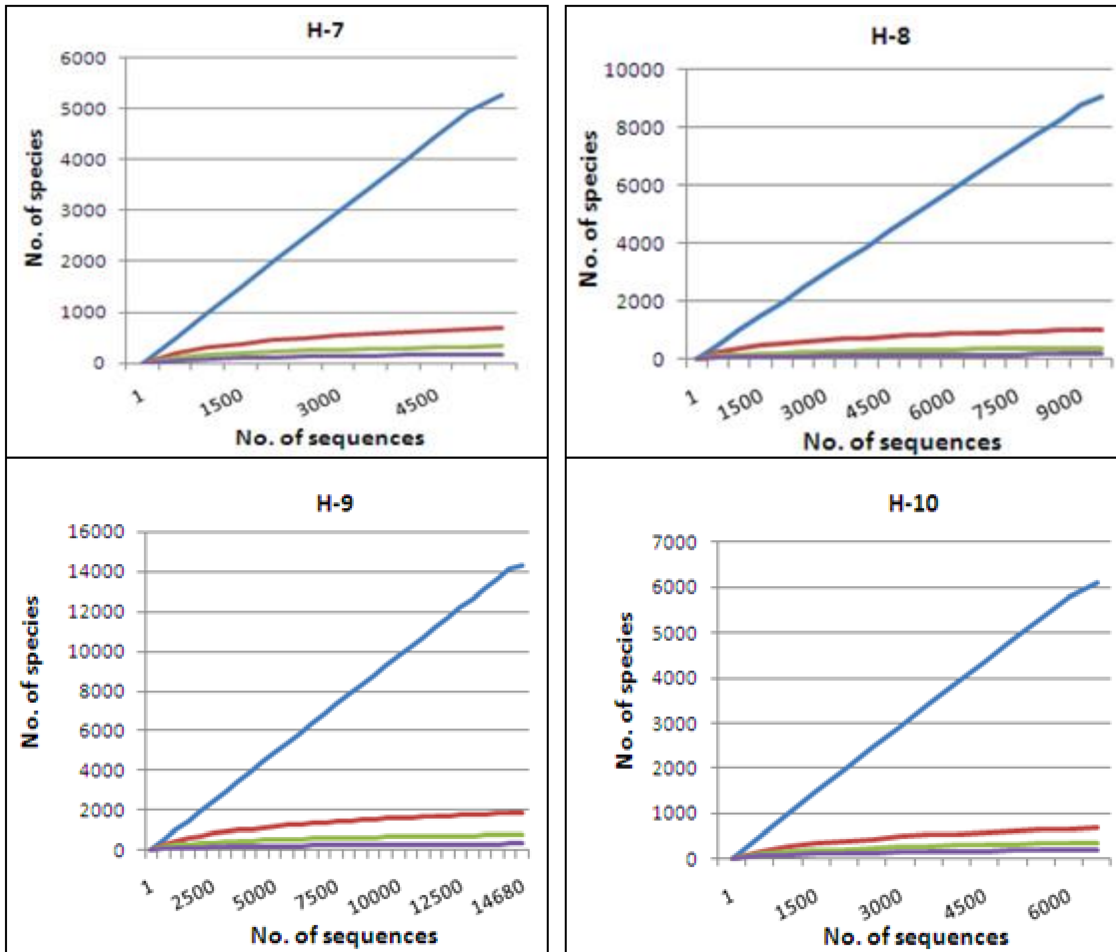
Table 3.2 Species richness and diversity indices from fecal microbiota of healthy dogs

Sample #	No. of OTUs	Species richness		Species diversity
		ACE	Chao1	Shannon index
3% DNA sequence dissimilarity				
H-1	146	243.57	234.64	1.95
H-2	218	284.48	278.00	3.17
H-3	176	210.17	209.71	3.17
H-4	195	305.45	307.54	3.50
H-5	221	289.57	284.67	3.73
H-6	160	239.16	247.00	3.23
H-7	173	216.63	218.55	3.02
H-8	118	182.44	193.00	2.81
H-9	224	304.77	271.54	3.35
H-10	204	258.70	300.47	3.00
5% DNA sequence dissimilarity				
H-1	119	196.89	183.11	1.81
H-2	166	211.25	226.11	2.91
H-3	133	153.03	146.79	2.87
H-4	141	199.93	201.06	2.90
H-5	166	213.74	198.25	3.23
H-6	122	181.23	183.88	2.62
H-7	134	174.04	180.50	2.42
H-8	88	136.09	129.33	2.45
H-9	161	237.20	228.05	3.03
H-10	162	200.90	226.40	2.54

OTU, Operational taxonomic unit, ACE, Abundance –based coverge estimator; Chao1, Richness estimator

Figure 3.3 Rarefaction analysis of dog fecal samples




H-7
6000
5000
4000
3000
2000
1000
0
No. of species
1
1500
3000
4500
No. of sequences
H-8
10000
8000
6000
4000
2000
0
No. of species
1
1500
3000
4500
6000
7500
9000
No. of sequences
H-9
16000
14000
12000
10000
8000
6000
4000
2000
0
No. of species
1
2500
5000
7500
10000
12500
14680
No. of sequences
H-10
7000
6000
5000
4000
3000
2000
1000
0
No. of species
1
1500
3000
4500
6000
No. of sequences

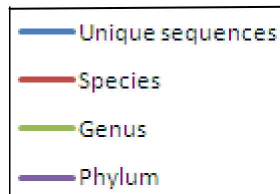

Unique sequences
Species
Genus
Phylum

Figure 3.4 The number of bacterial phyla detected in the canine fecal samples

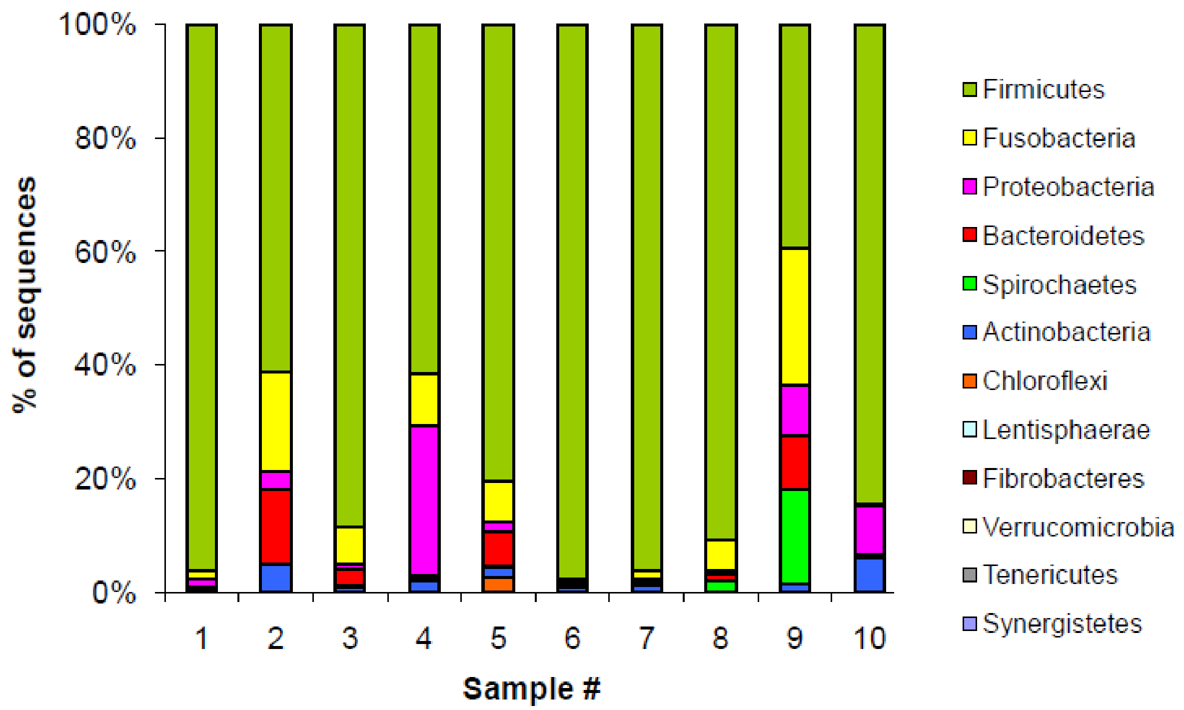
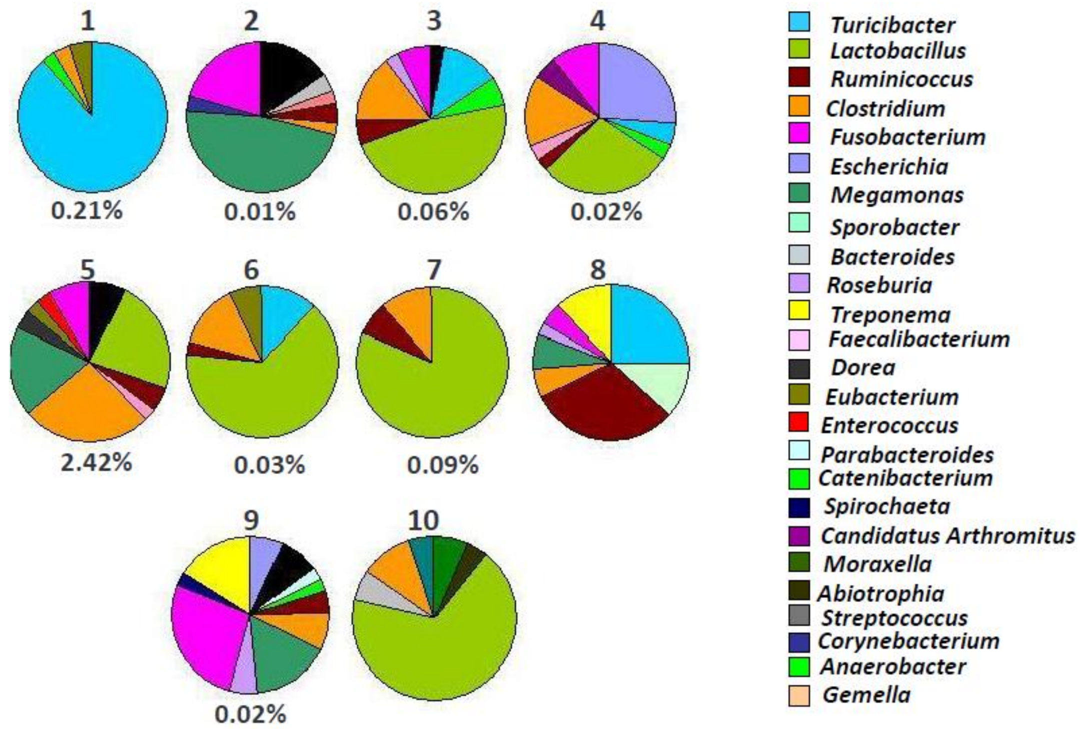


Figure 3.5 Bacterial diversity on the genus level in the dog fecal samples



*Numbers below each pie diagram indicates the percentage of sequences corresponding to *Enterococcus* genus in each sample

Figure 3.6 Structure of *Lactobacillus* spp. diversity in the fecal samples

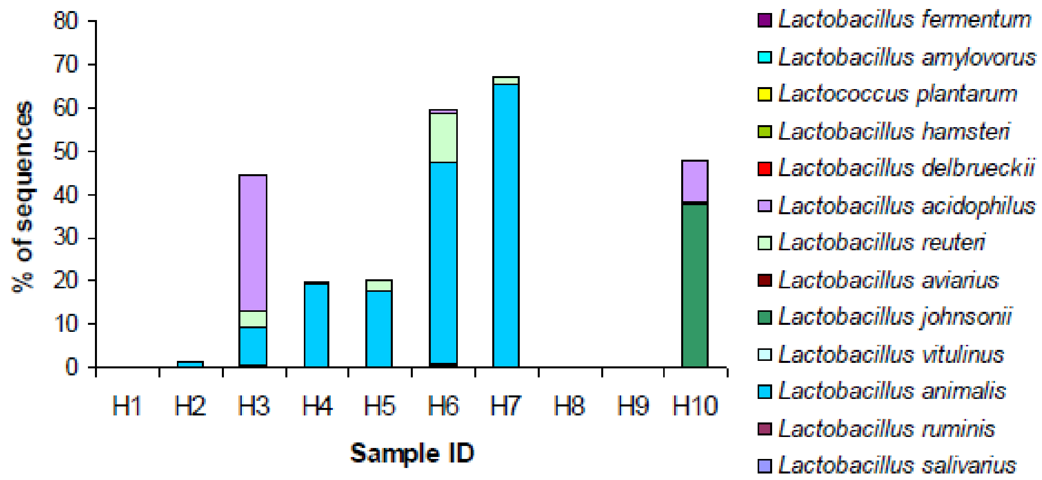


Figure 3.7 Structure of *Ruminococcus* spp. diversity in the fecal samples

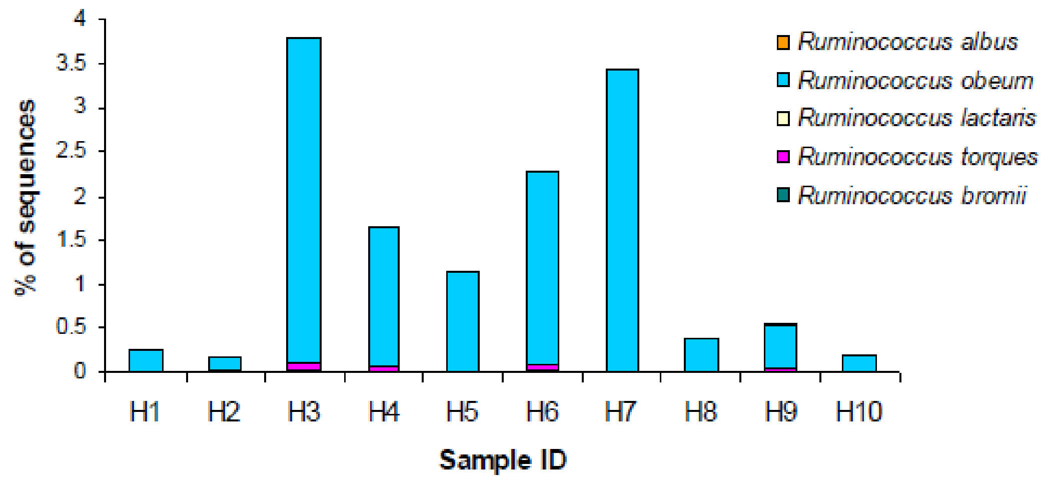


Figure 3.8 Structure of *Fusobacterium* spp. diversity in the fecal samples

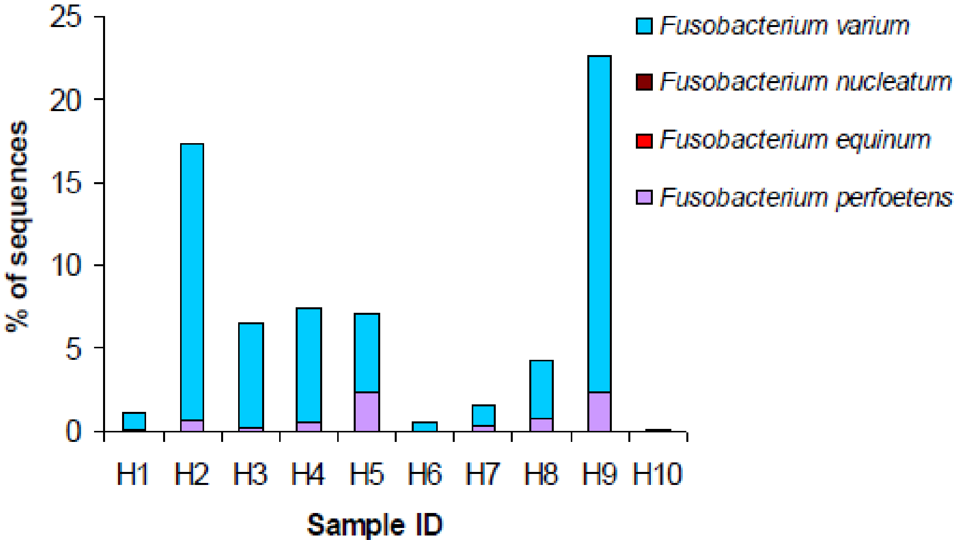


Figure 3.9 Structure of *Turicibacter* spp. diversity in the fecal samples

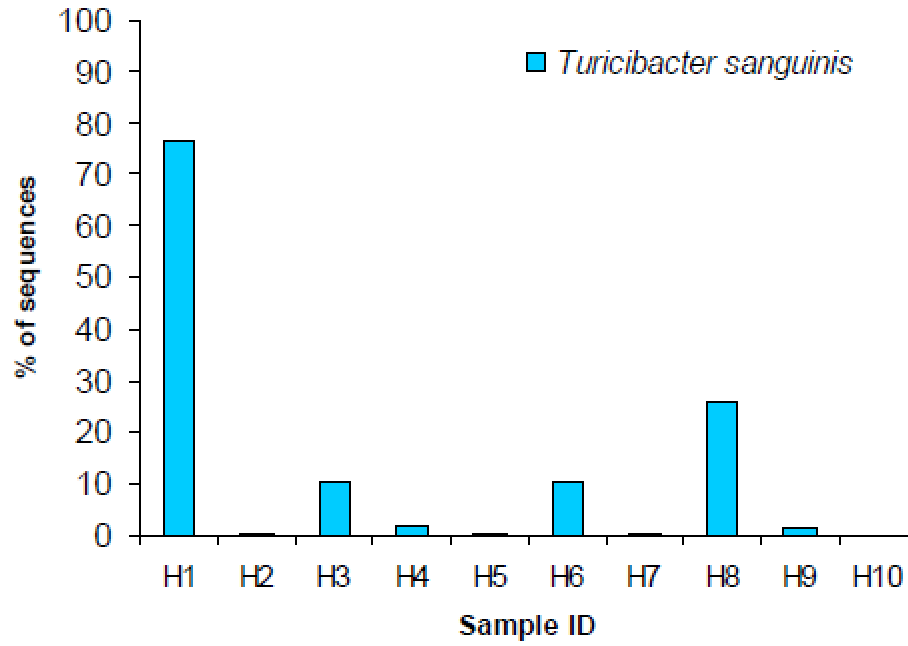


Figure 3.10 Structure of *Clostridium* spp. diversity in the fecal samples

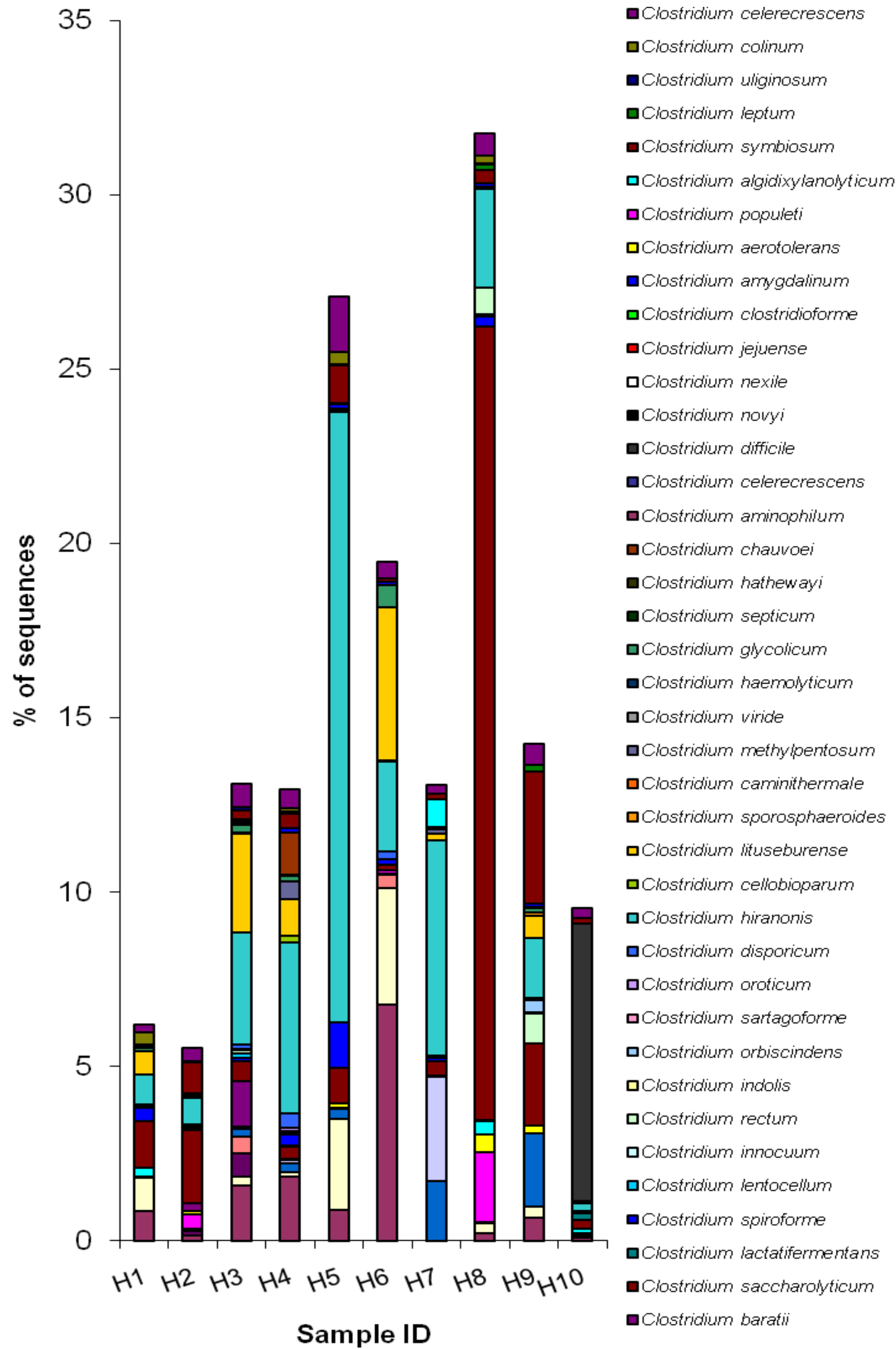


Table 3.3 Phylogenetic classification of sequences obtained from the dog fecal samples

			Healthy dog #	H- 1	H- 2	H- 3	H- 4	H- 5	H- 6	H- 7	H- 8	H- 9	H- 10
Phylum	**Order**	**Family**	**Genus**										
Proteobacteria			Total sequences (%)	1.5	2.94	0.98	25.95	1.62	0.59	0.49	0.44	8.43	8.36
	Enterobacteriales	Enterobacteriaceae	*Morganella*	0.15									
			Citrobacter		0.02	0.03		0.01	0.03			0.04	0.01
			Shigella	0.09		0.01	4.49	0.03				0.64	0.36
			Enterobacter				0.01					0.01	
			Klebsiella	0.01				0.03					
			Pantoea					0.01				0.01	0.01
			Escherichia	0.33		0.33	21.27	0.36		0.01	0.01	5.68	1.14
			Cronobacter									0.01	
	Pseudomonadales	Moraxellaceae	*Moraxella*	0.64	0.27	0.03		0.06	0.02		0.03	0.75	5.70
			Acinetobacter	0.01				0.02	0.06			0.01	0.04
		Pseudomonadaceae	*Pseudomonas*			0.08							
	Burkholderiales	Oxalobacteraceae	*Massilia*							0.02			
		Alcaligenaceae	*Sutterella*		1.17	0.33	0.15	0.27	0.01	0.22	0.03	0.16	0.31
			Achromobacter	0.03				0.03	0.03			0.01	
		Comamonadaceae	*Curvibacter*										0.01
			Brachymonas										0.01

			Acidovorax	0.07							0.01	0.01	
		Burkholderiaceae	*Lautropia*	0.07									
			Burkholderia	0.03	0.05			0.01	0.39	0.01		0.07	0.01
		Burkholderiales Genera incertae sedis	*Leptothrix*	0.01	0.01						0.01		
	Legionellales	Coxiellaceae	*Rickettsiella*									0.02	
	Pasteurellales	Pasteurellaceae	*Actinobacillus*					0.02				0.03	0.18
			Pasteurella									0.81	
			Haemophilus							0.02	0.04	0.02	0.10
			Psychrobacter		0.02								0.02
			Bibersteinia									0.03	0.15
			Volucribacter										0.06
	Neisseriales	Neisseriaceae	*Vogesella*			0.01							
			Microvirgula			0.01	0.02						
			Alysiella										0.01
			Aquitalea					0.02					
			Neisseria				0.01					0.09	0.09
	Aeromonadales	Succinivibrionaceae	*Succinivibrio*	0.01	0.54	0.01		0.29	0.05		0.01	0.01	
			Anaerobiospirillum	0.01	0.75			0.42				0.01	
			Ruminobacter			0.01							
		Aeromonadaceae	*Aeromonas*									0.01	
	Rhodobacterales	Rhodobacteraceae	*Paracoccus*		0.04								

			Rubellimicrobium			0.12							
	Campylobacterales	Campylobacteraceae	*Campylobacter*		0.02								
		Helicobacteraceae	*Helicobacter*		0.04	0.01		0.04			0.29	0.01	
	Rhizobiales	Rhizobiaceae	*Sinorhizobium*							0.01		0.01	
			Amorphomonas							0.12			
		Hyphomicrobiaceae	*Devosia*		0.01					0.01	0.01		
	Xanthomonadales	Xanthomonadaceae	*Xanthomonas*									0.01	
			Rhizobium	0.03									
			Stenotrophomonas							0.07		0.01	
			Lysobacter										0.15
		Sinobacteraceae	*Steroidobacter*		0.01								
	Vibrionales	Vibrionaceae	*Vibrio*					0.01					
	Caulobacterales	Caulobacteraceae	*Brevundimonas*	0.01									
Bacteriodetes			Total sequences (%)	0.14	12.60	2.6	0.66	5.97	0.02	0.54	1.09	8.85	0.45
	Sphingobacteriales	Sphingobacteriaceae	*Sphingobacterium*								0.01		
	Bacteroidales	Bacteroidaceae	*Bacteroides*	0.03	12.54	2.5	0.62	5.91		0.40	1.05	6.78	0.30
		Porphyromonadaceae	*Porphyromonas*	0.03									
			Tannerella	0.01	0.01	0.03	0.01	0.01		0.03		0.01	
			Parabacteroides	0.01		0.03			0.01	0.05	0.01	2.05	0.04
		Prevotellaceae	*Hallella*				0.01	0.01	0.01	0.03	0.01		0.01
		Rikenellaceae	*Alistipes*		0.01								

			Rikenella	0.05	0.03	0.03	0.01	0.04		0.03	0.01	0.01	0.01
	Sphingobacteriales	Sphingobacteriaceae	*Pedobacter*	0.01									
	Flavobacteriales	Flavobacteriaceae	*Chryseobacterium*		0.01	0.01	0.01						
			Bergeyella										0.09
Firmicutes			Total sequences (%)	95.9	57.9	85.1	60.16	78.66	97.37	92.59	84.62	36.99	82.17
	Erysipelotrichales	Erysipelotrichaceae	*Allobaculum*		0.23	0.96	0.48	0.63	0.20	0.70			0.01
			Coprobacillus	0.48		0.13	0.43	1.46	0.05	0.07			0.03
			Bulleidia	0.09	0.08	0.09	0.06	0.03	0.29	0.09			
			Turicibacter	77.21	0.47	10.95	3.94	1.28	10.93	1.26	23.44	1.44	0.20
			Catenibacterium	2.52		5.64	2.81		0.10	0.40		2.18	
			Erysipelothrix	0.01	0.01								
	Lactobacillales	Carnobacteriaceae	*Alloiococcus*		0.01							0.02	0.02
			Anaerostipes	0.15		0.01	0.01						
			Atopostipes		0.01								
			Granulicatella		0.15			0.06				0.01	0.04
			Carnobacterium		0.11			0.11					
		Aerococcaceae	*Facklamia*		0.21			0.31				0.01	0.23
			Aerococcus		0.91			0.08				0.01	0.23
			Abiotrophia	0.01				0.42				0.25	4.34
		Leuconostocaceae	*Leuconostoc*	0.29	0.05	0.20	0.01	0.11	0.36	0.18	0.13	0.09	0.20
			Weissella	0.21	0.05	0.43	0.04	0.28	0.36	0.14	0.17	0.24	0.42

		Lactobacillaceae	*Lactobacillus*	0.03	1.14	41.41	23.3	19.15	58.84	65.53	0.01	0.05	58.04
			Paralactobacillus										
		Carnobacteriaceae	*Trichococcus*		0.04			0.09				0.01	
		Streptococcaceae	*Streptococcus*	0.05	3.09	0.40	0.66	0.65	0.29	0.88	0.09	0.34	5.13
			Lactococcus	0.13	0.01	0.16	0.01	0.34	0.29	0.25	0.03	0.02	0.09
	Bacillales	Bacillaceae	*Bacillus*		0.02								
			Paucisalibacillus		0.01								
		unclassified Bacillales	*Aerosphaera*		0.13			0.01					
		Bacillales Family XI. Incertae Sedis	*Gemella*		2.25			0.41				0.19	0.78
		Staphylococcaceae	*Salinicoccus*		0.46			0.08					0.01
			Macrococcus		0.01			0.13					0.01
			Staphylococcus		1.10	0.03		0.02	0.05	1.15		0.12	0.33
			Jeotgalicoccus	0.03	0.17			0.41				0.05	0.15
		Planococcaceae	*Sporosarcina*	0.01									
	Clostridiales	Ruminococcaceae	*Sporobacter*	0.11	0.01	0.58	0.20	0.53	0.44	0.7	10.58		
			Ruminococcus	1.98	3.36	4.64	2.35	4.10	2.68	5.7	28.70	3.92	1.05
			Acetivibrio	0.01								0.01	0.01
			Fastidiosipila										0.01
			Acetanaerobacterium				0.86						
			Anaerofilum		0.34		0.04			0.34		0.01	
			Anaerotruncus				0.23	0.31	0.01	0.03	0.07	0.18	

			Faecalibacterium			0.17	2.66	2.11	0.27	0.81	0.10	1.01	0.70
			Papillibacter	0.01	0.01	0.08	0.21	0.23	0.03	0.01		0.10	0.03
		Peptostreptococcaceae	*Filifactor*		0.08								0.03
			Peptococcus		0.01		0.03				0.01	0.01	
			Peptostreptococcus		0.10			0.03				0.20	
		Clostridiaceae	*Ethanoligenens*				0.06					0.01	0.10
			Clostridium	3.03	2.06	13.19	12.55	21.57	12.69	9.26	5.80	6.35	8.97
			Candidatus Arthromitus	1.30	0.27		3.99	0.21				0.07	
			Megamonas	0.97	38.13	0.83	0.15	14.95	0.15	0.07	6.70	13.24	
			Anaerobacter	4.39	0.67		0.01	0.18	0.01		0.03	0.36	0.07
			Sarcina				0.02						
		Clostridiales Family XI. Incertae Sedis	*Peptoniphilus*								0.01		
			Anaerovorax		0.06	0.16	0.08	0.01	0.10	0.28	0.09		0.01
			Finegoldia			0.01							
			Soehngenia		0.04							0.04	0.45
		unclassified Clostridiales	*Frigovirgula*					0.04				0.01	0.01
		Peptococcaceae	*Desulfotomaculum*	0.02			0.01						
		Lachnospiraceae	*Roseburia*	0.31	1.20	2.5	1.62	1.74	1.43	1.89	2.34	4.96	0.56
			Oribacterium				0.01					0.01	
			Lachnobacterium					0.01					
			Coprococcus		0.01					1.92	0.01	0.01	

			Butyrivibrio		0.01	0.01	0.01		0.03	0.05	0.01	0.01	
			Lachnospira	0.07		0.24	0.35	0.27	0.03	0.16			
			Dorea	1.10	0.75	1.22	1.74	3.26	1.28	0.39	1.94	0.65	0.51
			Pseudobutyrivibrio	0.05	0.01		0.01	0.01			0.01		
			Oribacterium				0.01					0.01	
		Clostridiales Family XI. Incertae Sedis	*Helcococcus*		0.17							0.04	0.15
			Tissierella		0.01								
		Veillonellaceae	*Succiniclasticum*			0.01	0.01		0.05	0.18	0.03		0.01
			Dialister			0.03							
			Veillonella	0.11	0.05	0.06		0.02	0.05		0.05		0.06
			Mitsuokella								0.01		
			Acidaminococcus		0.19	0.01	0.05	0.59		0.19			
		Eubacteriaceae	*Eubacterium*	1.22	0.09	0.95	1.15	2.43	6.36	0.05	0.03	0.74	0.01
	Thermolithobacterales	Thermolithobacteraceae	*Thermolithobacter*							0.01		0.01	
Chloroflexi			Total sequences (%)	0.21	0.02	0.06	0.02	2.42	0.03	0.09		0.10	
	Anaerolinaeles	Anaerolinaceae	*Levilinea*		0.01								
			Enterococcus	0.21	0.01	0.06	0.02	2.42	0.03	0.09		0.10	
Actinobacteria				0.08	4.40	0.78	1.81	1.84	0.82	0.92	0.01	1.13	5.97
	Actinomycetales	Propionibacteriaceae	*Luteococcus*		0.01								
			Propionibacterium		0.12				0.46	0.12			
		Actinomycetaceae	*Actinomyces*		0.45		0.01	0.04			0.01	0.02	0.20

			Arcanobacterium		0.01							0.01	0.80
		Micrococcaceae	*Micrococcus*		0.01								
			Rothia							0.07			0.01
			Cellulosimicrobium		0.01								
			Arthrobacter							0.07			
		Nocardiaceae	*Rhodococcus*		0.02								
			Friedmanniella		0.02			0.01					
		Dermabacteraceae	*Dermabacter*	0.01	0.13							0.01	0.09
			Brachybacterium		0.02			0.03					
		Promicromonosporaceae	*Cellulosimicrobium*		0.01								
		Microbacteriaceae	*Crocebacterium*				0.01						
			Pseudoclavibacter		0.06								
			Leucobacter					0.01					0.07
		Micrococcaceae	*Kocuria*		0.08			0.09				0.01	
		Micromonosporineae	*Micromonospora*		0.01								
			Polymorphospora		0.01								
			Salinispora		0.04							0.01	
			Actinoplanes		0.01							0.01	
		Cellulomonadaceae	*Cellulomonas*			0.01							
		Corynebacteriaceae	*Corynebacterium*	0.05	2.73	0.01		1.14				0.20	4.20
		Propionibacteriaceae	*Brooklawnia*		0.01								

		Dietziaceae	*Dietzia*	0.01	0.01			0.01					
		Intrasporangiaceae	*Ornithinimicrobium*	0.01									
		Streptosporangiaceae	*Astrosporangium*		0.02								
		Gordoniaceae	*Gordonia*		0.05								
	Coriobacteriales	Coriobacteriaceae	*Collinsella*		0.47	0.75	0.27	0.47	0.12	0.64		0.75	1.29
			Eggerthella		0.01		0.23			0.01			0.01
			Asaccharobacter							0.01			
			Slackia		0.02	0.01	1.29	0.01	0.24				
		Brevibacteriaceae	*Brevibacterium*		0.06			0.03				0.01	0.20
Tenericutes			Total sequences (%)			0.01							
	Anaeroplasmatales	Anaeroplasmataceae	*Anaeroplasma*			0.01							
Synergistetes			Total sequences (%)									0.01	
	Synergistales	Synergistaceae	*Aminomonas*									0.01	
Fusobacteria			Total sequences (%)	1.16	16.69	6.37	8.97	6.94	0.50	1.53	5.1	22.72	0.11
	Fusobacteriales	Fusobacteriaceae	*Fusobacterium*	1.16	16.69	6.37	8.97	6.92	0.50	1.53	4.29	22.42	0.11
			Leptotrichia					0.02				0.01	
			Cetobacterium			0.03					0.18	0.29	
Spirochaetes			Total sequences (%)	0.22	0.01	0.27	0.30	0.20	0.22	0.03	1.77	15.70	0.03
	Spirochaetales	Spirochaetaceae	*Spirochaeta*	0.03			0.07	0.06		0.03	1.77	2.37	0.03
			Treponema	0.19	0.01	0.27	0.23	0.14	0.22		11.22	13.33	0.189
Lentisphaerae			Total sequences (%)						0.06				

	Victivallaceae		*Victivallis*						0.06				
Fibrobacteres			Total sequences (%)	0.21	0.01			0.01	0.03	0.01	0.01	0.01	
	Fibrobacterales	Fibrobacteraceae	*Fibrobacter*	0.21	0.01			0.01	0.03	0.01	0.01	0.01	
Verrucomicrobia			Total sequences (%)				0.01						
	Opitutales	Opitutaceae	*Opitutus*				0.01						

Grey boxes indicate the sequences greater than 2%

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Chapter 4

Analysis of antibiotic and metal resistance genes in diverse bacteria of gastrointestinal tract of healthy dogs

4.1 Abstract

The problem of antimicrobial resistance is becoming a major concern in terms of treatment failures, economic losses and the inability to use antimicrobial agents. During the past two decades, the increase in the pet animal population and their close contact with humans has raised concerns regarding the transmission of resistant bacteria from pet animals to humans. Lack of surveillance programs makes evaluating the epidemiology of antimicrobial resistance in companion animals difficult. The present study determined the diversity of resistance genes in the fecal microbiota of healthy dogs. Using a spotted DNA microarray, we determined the presence of antibiotic and metal resistance genes in the fecal microbiota of 8 healthy dogs. Our microarray data revealed that the overall prevalence of antibiotic and metal resistance was low. Our results showed that among the antimicrobial agents, several tetracycline, erythromycin and aminoglycoside resistance genes were detected while among the metal resistance genes tested, four copper resistance genes were found. However, most of these originated from one of eight dogs that had history of antibiotic treatment for chronic skin disease one year prior to our sampling.

Key words: microarray, healthy dogs, antibiotic and metal resistance genes

4.2 Introduction

The occurrence of resistance to antimicrobial agents by pathogens and commensals is causing treatment failures in human and veterinary medicine. The cost associated with treating resistant bacterial infections in the U.S. is estimated to be $4 billion annually (McGowan *et al.*, 2001). The interaction of the antibiotics, microorganism, and host plays a role in the prevalence and persistence of antimicrobial resistance (Dowling, 1996). The resistant bacteria are maintained in the environment due to continuous selective pressure both by the routine use of antibiotics and by environmental conditions in which the co-selection of resistance genes and compensatory mutations occur due to the lack of fitness costs (Bjorkman *et al.*, 2000). Thus, the development of multidrug resistant bacteria in turn directly reduces the clinical efficacy of the antimicrobial agents. This has led to the inability to use of some antimicrobial agents because their therapy has stopped being effective. The impact of antimicrobial resistance on clinical outcome varies depending on the level of resistance (at different hospitals and geographical areas) (Hidron *et al.*, 2008), site of infection, and availability of novel or alternative therapeutic options (Rice 2009). Once they develop resistance to antimicrobial agents, the ability of bacteria to transmit their resistance genes to other bacteria or acquire new resistant determinants into their pre-existing conjugative element is alarming.

Evidence suggests that commensal bacteria represent a reservoir of antimicrobial resistance genes (Salyers *et al.*, 2004). The intestinal tract is considered the largest reservoir of commensal bacteria within the mammalian body (Berends *et al.*, 2001). Furthermore, the gut is considered to be an ideal environment for the resistance gene transfer among bacteria (Lester *et al.*, 2004; Scott 2002). The GIT of mammals is composed of a diverse population of microbes (>

10^{14}) (Andremont, 2003) which colonize specific regions of the intestine. These enteric commensals can acquire or donate resistance among themselves as well as to other bacteria that enter the intestine through the fecal-oral route (Salyers *et al*., 2004). This resistant microbiota when excreted with feces may eventually be the potential source for resistance transfer to other mammals including humans. It has been suggested that investigating the antimicrobial resistance in commensals of healthy animals will be valuable in understanding their contribution to the increased antimicrobial resistance scenario, as they might be harboring resistance genes and under optimal conditions can transfer these genes to pathogenic bacteria (Guillemot *et al*., 2001).

Due to the impact of the resistant bacteria on human and animal health, concerns have been expressed regarding the role of companion animals as a reservoir of resistant bacteria. The issue of antimicrobial resistance is becoming an emerging problem in these animals as well (Weese *et al*., 2008). Close contact and habitat sharing between humans and companion animals might serve as a source of resistant bacteria in humans (Clarke, 2006). Despite the increasing concern over this issue, only few studies have been carried out to evaluate the antimicrobial resistance in dogs in U.S. and most of the investigations were focused on *Staphylococcus intermedius* and *Escherichia coli* (Cohn *et al*., 2003; Rachal *et al*., 2009; Keefe *et al*., 2010). Till date no study has examined the diversity of resistance genes in the gut of the healthy dogs.

With the recent advances in molecular techniques, several new approaches are available for the analysis of diverse microbial communities. A DNA microarray is a parallel detection system, which makes detecting thousands of genes simultaneously possible. Due to the availability of a large number of sequence data for antimicrobial resistance genes, this technique is well suited for identification of these genes (Call *et al*., 2003). A DNA microarray consists of thousands of gene specific spots containing conserved regions of the targeted genes. These gene

specific probes are immobilized by covalent attachment to solid surfaces such as glass slides. When the fluorescently labeled target DNA is added to the chip, binding and hybridization of the target DNA to the specific probe occurs. These hybridized targets are detected by the fluorescence of the reporter molecule (fluorescent dye having been added to the target DNA) emitted by laser excitation. The fluorescence intensity is then measured with a scanner.

In this study, we used a spotted DNA microarray to detect 227 antimicrobial resistance genes encoding 30 different antimicrobial agents and 99 metal resistance genes from healthy dog fecal samples using one sequence specific probe for each gene. Studying antimicrobial resistance in commensal bacteria provides information about different resistance mechanisms present in the bacteria that exist at the same site. The specific aims of this study were: (1) to identify antibiotic resistance genes and (2) to identify metal resistance genes.

4.3 Materials and methods

4.3.1 Collection of fecal samples

Eight healthy dogs were included in this study. Fresh fecal samples were collected and used for analysis.

4.3.2 Template DNA extraction

Genomic DNA was extracted from 500 mg fecal sample using ZR soil Microbe DNA Kit™ and its manufacturers recommended methods. The extracted DNA was purified using Geneclean Turbo kit (MP Biomedicals). The purified DNA (1µl) was quantified using Nanodrop ND-1000 spectrophotometer (Nanodrop technologies, Wilmington DE). The samples with DNA concentration 1.7-2.0 ng/µl were used for microarray analysis. This DNA was analyzed in the

laboratory at Department of Pathobiology, College of Veterinary Medicine, Kansas State University (Peterson *et al*., 2010).

4.3.3 Preparation of labeled DNA

4.3.3.1 Labeling

Labeling of DNA was performed in amber tube, in a dark room using BioPrime Plus Array CGH Genomice Labeling System (Invitrogen Co., Carlsbad, CA) according to manufacturer's directions. DNA was labeled with Alexa Flour 555 via random priming using 400U/µl Klenow fragment. Briefly, the reaction mixture consisted of 20µl Alexa Fluor 555, 1.5 µg template DNA and adjusting the volume to 44 µl with sterile water. The labeling reaction mixture was incubated at 95°C for 10 minutes in heating block, and chilled on ice for 5 minutes. The reaction mixture was vortexed, and kept on the ice. This mixture was then subjected to extension with Klenow fragment by adding 10x nucleotide mix 555 (5µl), and Exo-Klenow fragment (1µl). After mixing and centrifugation, the reactions were carried out for 2 hours in a sealed film canister.

4.3.3.2 Purification of labeled DNA

Labeled DNA fragments were purified using Qiagen PCR purification column in the dark. This mixture present in the spin column was centrifuged for 1min at 10K x g and the flow-through was discarded.

4.3.3.3 Washing and elution

Washing two times with washing buffer was performed in order to separate labeled DNA from the free dye. For this purpose 650µl washing buffer (available in the kit) with ethanol (100%) was added to the column. This was further followed by spinning and discard of flow-

through. Then the columns were spinned with the caps opened for 3 min at 12K x g for drying the membrane. The washing step was followed by elution step where the column was transferred to a new amber tube and 15µl the elution buffer (10mM Tris HCl, pH 8.5) was added, incubated at room temperature for 1 min and centrifugation at 11K x g for 2 min. The amount of incorporated dye was quantified by using Nanodrop ND-100 spectrophotometer with microarray feature in order to determine the overall labeling efficiency.

4.3.4 Microarray hybridization

4.3.4.1 Slide preparation

The microarray slides were printed by Genetix QArray2 System slide printer (Genetix, Hampshire, UK) crosslinked in a UV Stratlinker 2400. Each slide was printed with two identical fields. The array contained 489 oligonucleotide (70-mer) probes that consisted of 227antimicrobial resistance genes conferring resistance to 30 antibiotics and 99 metal resistance genes. The slides were prehybridized by incubating at 42°C for one hour with shaking in the blocking solution (0.1% BSA; 5X SSC; 1% SDS) to block the nonspecific binding of the probe. The slides were spun dried by centrifugation at approximately 2200 x g. Finally, the elevated coverslips were applied over the oligo fields.

4.3.4.2 Hybridization

The labeled DNA was dried with a SpeedVac and the volume was adjusted to 14µl with water. Labeled DNA was mixed with equal volume of 2X hybridization mix (2%SDS; 30X SSC; 50% formamide) and appropriate 25mer (1µl). The mixture was incubated at 80°C for 5 min, chilled on ice, spinned and applied to the slide under the coverslip. Hybridization was performed overnight in a chamber at 42°C.

4.3.4.3 Post hybridization washing

Hybridized slides were transferred to the container containing wash 1 solution and were slightly agitated until the coverslips were floated off. Then the slides were washed for 10 min in each wash buffers: 10X SSC + 0.2% Sarkosyl; 10X SSC; 0.2X SSC at room temperature in the dark. After the last wash, the slides were immersed quickly in distilled water and spun dried.

4.3.5 Data analysis

A GenePix slide reader (Molecular Devices, Sunnyvale, CA) scanner with laser wavelength of 532 nm for Cy3 and 635 nm for Cy5 generated TIF images of the slide for analysis. The images were visualized and the software linked it to GenePix Array list (GAL) file created by the slide printer. This GAL file provides information about the layout and each feature within the block of microarray. The median pixel intensities were measured from each probe and the data were stored in the GenePix report (GPR) file. Finally, the average signal intensities for each probe were determined and used for further analysis.

The data were analyzed by 3 methods: TIGR MultiExperiment viewer programe (TIGR, Rockville, MD), relative pathogen signal ratio (RPS) and Frye analysis.

TIGR MultiExperiment viewer programe (TMeV) (TIGR, Rockville, MD) with one color setting is used for the analysis purpose. Briefly, the GPR file was uploaded and basic analysis were done by visualization of fluorescence intensity of the identified spots in order to get a quick overview of the sample.

For RPS and Frye analysis, the GPR file data are uploaded in the Wildcat Fetch 1.0 analysis programe. The output file obtained is the histogram with X-axis showing the genes and RPS or Frye ratios on the Y-axis. Based on the histogram, a threshold is determined and positive

or negative determinations are made. Based on the threshold value, the RPS and Frye analysis is performed.

In the RPS analysis, the output file contains two sets of data for each gene i.e RPS data and RPS after PCV (Print control value) data. The RPS analysis data are obtained by dividing the average signal intensity measured for each probe by intensity of positive control probes (EUB and Frye 3).

$$\text{RPS data} = \frac{\text{Average signal intensity of each probe}}{\text{Signal intensity of positive controls}}$$

For RPS after PCV data, the average signal intensity measured for each probe is divided by PCV (Peterson *et al*., 2009).

$$\text{PCV} = \frac{\text{Mean of the median 25 mer signal of a particular spot}}{\text{Mean of the median 25 mer signal of all spots on the chip}}$$

$$\text{RPS after PCV} = \frac{\text{Average signal intensity of each probe}}{\text{PCV}}$$

Generally, when RPS values were ≥ 0.25, the spot was scored positive.

Further analysis was performed by Frye analysis, where also the output file consisted of two sets of data for each gene i.e Frye data and Frye after PCV data. For Frye data, average signal intensity of each probe were divided by median value of hybridization intensity of all 70-mer. When Frye ratio was ≥ twice the median value of hybridization intensity of all 70-mer, the hybridization was considered positive.

$$\text{Frye ratio} = \frac{\text{Average signal intensity of each probe}}{\text{Median value of hybridization intensity of all 70-mer}}$$

Frye after PCV were calculated by dividing Frye ratio by mean of the median 25 mer signal of all spots on the chip

$$\text{Frye after PCV} = \frac{\text{Frye ratio}}{\text{PCV}}$$

Generally, when Frye values were ≥ twice the median value of hybridization intensity of all 70-mer, the hybridization was considered positive.

4.4 Results

Of 8 dogs included in this study, none were associated with antibiotic exposure for at least 1 year prior to sample collection. All dogs were considered healthy by the owners during the time of fecal collection. To detect antibiotic resistance genes and metal resistance genes in diverse gut microbiota, we used a 70-mer oligonucleotide probe consisting of conserved regions (300-400bp) of the genes for screening canine fecal samples.

4.4.1 Composition of antibiotic resistance genes in diverse fecal microbiota

A microarray hybridization of genomic DNA extracted from the intestinal microbiota revealed the presence of several antimicrobial resistance (AMR) genes in the fecal samples. An overview of the result from the microarray array analysis is shown in Table 4.1. The microarray showed positive hybridization to probes corresponding to 10 different antimicrobial groups, e.g., tetracyclines, aminoglycosides, fluroquinilones, macrolides, streptogramins, lincosamide, beta-lactams, glycopeptides, chloramphenicol and sulpha group drugs. Of 227 probes designed for different antibiotic resistance genes, 33 different resistance genes were detected in our samples.

Most of the fecal samples (6 of 8) positive for antimicrobial resistance genes belonged to only two or three different groups of antibiotics except for the two samples (H-4 and H-10) in which the antimicrobial resistance genes belonged to ≥5 antibiotic groups.

Sample H-1 and H-2 showed resistance to tetracycline antibiotic only. Resistance to aminoglycosides was observed in all the fecal samples, with the exception of sample, H-1 and H-2. Macrolide resistance was detected in H-4 and H-7, and H-4 harbored most of the resistance genes. Dog H-10 was the only dog that showed positivity for fluroquinolone resistance gene,

parC. Beta-lactam and glycopeptides resistance determinants were observed only in H-4 and H-10.

Dog H-4 harbored most of the resistance determinants as compared to other dogs included in the study. Resistance to chloramphenicol, lincomycin, streptogramin, and sulphanilamide antibiotics were observed only in this sample. This dog harbored diverse resistance genes to macrolide, lincosamide, streptogramin, beta-lactam, tetracycline, aminoglycoside and glycopeptides.

Each canine sample showed positivity for ≥2 antimicrobial resistance genes. Diverse resistance genes belonging to the same category were observed for the following antibiotics: macrolides, tetracycline, and aminoglycosides. Tetracycline was the only antibiotic against which resistance was detected in all samples with *tet*(O) as the most prevalent genotype. All tetracycline resistant samples harbored at least two *tet* genes. The *tet* genes *tet*(O), *tet*(C), and *tet*(Y) were detected in ≥ 50% of the samples. The *aadE* gene coding for putative aminoglycoside 6-adenyltransferase was the most prevalent aminoglycoside resistance determinant in our samples.

4.4.2 Composition of metal resistance genes in diverse fecal microbiota

Genes showing resistance to metals such as arsenic, cadmium, copper, cobalt/nickel, transferable copper, copper/zinc/cadmium and copper/silver (Table 4.2.) were detected by the corresponding oligonucleotide probes in the microarray. For the samples carrying metal resistance genes, most of them had one or two resistance genes, except the sample from dog H-4 possessed 11 resistance genes conferring resistance to cadmium, copper, copper/zinc/cadmium, cobalt and transferrable copper.

Four of the fecal samples tested (H-3, H-5, H-7, and H-8) were negative for all of the metal resistance genes tested. Among the samples that were tested positive, H-1 and H-10 harbored resistance determinant to only one metal, arsenic and cadmium, respectively. Copper and copper/zinc/cadmium resistance genes were present in sample H-2. Diverse resistance genes were detected for copper, and all of them were harbored by dog H-4. The gene, *czcA2 copABCD* conferring resistance to copper/zinc/cadmium was most prevalent (25%) among all the metal resistance genes identified in our samples.

Table 4.3 shows the list of antimicrobial and metal resistance genes that were absent in our analyzed samples.

4.5 Discussion

The problem of antimicrobial resistance in bacteria is a global concern. The proliferation of these resistant bacteria can be driven by several factors. Studies have shown that genes conferring resistance to metals can play an important role in the dissemination of antimicrobial resistance genes (Summers, 2002). Multiple resistance genes encoding for metals and antibiotics are commonly localized together on the same mobile genetic elements such as plasmids and transposons conferring co-resistance (Summers, 2002). Sometimes cross resistance can also be observed in bacteria, in which a single enzyme can function as an efflux pump for both metals and antibiotics (Hayashi *et al.*, 2000). In both cases, co-resistance and cross resistance, selection of one gene can result in the co-selection of other resistance genes in bacteria (Wright, 2007).

The prevalence of antimicrobial resistance in commensal bacteria in various ecosystems suggests that commensal bacteria play an important role in the dissemination of antimicrobial resistance genes (Salyers, 2006). It is often proposed that food animals are responsible for the resistance development in humans through the transfer of resistant bacteria via food

(Guardabassi *et al.*, 2004). But antimicrobial resistance in the commensal bacteria associated with pet animals with respect to the antibiotic resistance pool and its role in the horizontal gene transfer is not well known. In small animal veterinary practice, the antimicrobial agents used for therapy also include the ones that are liscensed for use in human medicine. Consequently, development of resistance in the commensals of these animals can pose a risk for the zoonotic transmission to humans. The transfer of variety of resistant bacteria and resistance genes from pets to humans has been reported (Manian, 2003). Hence, our overall objective was to determine the diversity of antibiotic and metal resistance genes in the healthy canine gut microbiota.

It is a well known fact that there are several mechanisms by which bacteria can develop resistance to a particular antibiotic (Roe and Pillai, 2003). These resistance mechanisms are conferred by the alteration in the genes encoding the protein that is the antibiotic target site. Hence, several antimicrobial resistance genes are distributed among the bacteria conferring resistance to a single antibiotic group (Chopra and Roberts, 2001). Thus, the heterogenecity among the antimicrobial resistance genes makes the use of molecular techniques such as polymerase chain reaction (PCR) and southern blotting to detect resistance genes in a single isolate cumbersome and time consuming. Moreover, false-negative results can be obtained if any variants of the target genes are present (Cockerill III FR, 1999).

Microarrays have been previously used for the detection of viruses and resistance determinants in bacteria. Microarray technology can be used for screening large amounts of genetic information simultaneously in a single assay. This technique can be used to detect the variety of genes harbored by any bacteria from the extracted DNA. In the present study, we used microarray analysis for a microarray containing 489 oligomers representing 227 antimicrobial and 99 metal resistance genes (Peterson *et al.*, 2010) to determine the overall diversity of

antibiotic and metal resistance genes in the feces of healthy dogs. A microarray has an advantage over traditional PCR in that the identification of the target gene is based on its internal sequences and not on the length of the PCR product (Liu, 2009). Additionally, the microarray used in this study consisted of oligonucleotide probes (Peterson *et al*., 2010), which had the advantage of greater specificity than PCR probes (Kane *et al*., 2000). Several studies have been carried out in which DNA microarray has been used to detect the resistance genes in bacteria (Call *et al*., 2003; Frye *et al*., 2006; Monecke *et al*., 2003; Van Hoek *et al*., 2005). Call *et al*., (2003) developed a DNA microarray for the detection of diverse tetracycline resistance genes. A DNA microarray for the detection of genes conferring resistance to macrolide, lincosamide and streptogramin antibiotics was developed by Cassone *et al*., (2006). The assay we used was based on the direct detection of the genes using a single fluorescence dye.

Based on the microarray results of the eight samples tested, all samples were identified as having resistance to at least one antibiotic. The overall prevalence of resistance genes was low in the healthy animals. Diversity among resistance genes was observed for the antibiotics: tetracycline, macrolide and aminoglycosides. Most of the resistance genes for antibiotics and metals were harbored by the sample H-4. According to the information obtained from the owner, this dog had a history of skin problems and had been on antibiotic therapy (gentamicin, neomycin, polymyxin B sulphate, cephalexin) several times two years before the sample was collected. This may be a possible explanation for the presence of most of the resistance genes we found in the fecal microbiota of dog H-4. It also indicates long lasting effects of antibiotic treatment on the gut microbiota.

The high prevalence of tetracycline resistance observed in our samples is not surprising, since widespread use of this antibiotic has contributed to high rates of resistance (Roberts, 1996).

The PCR analysis for tetracycline resistance genes from the fecal enterococcal isolates (results from Chapter 2) correlated with the microarray profile for tetracycline resistance genes. Two of our samples were positive for glycopeptides resistance genes, *vanD* and *vanH*. However, *vanD* gene is located on the chromosome and is not self transferrable (Depardieu *et al*., 2003). Multiple resistance genes in combination are required for vancomycin resistance, and *vanH* is one gene that encodes for an enzyme that is essential for resistance (Murray, 1998).

However, further verification of all of the positive resistance genes needs to be done with a PCR assay to confirm the microarray results. Moreover, one of the drawbacks of this approach is the detection limit. Without enrichment of the samples, the detection limit of the microarray assay used in this study is 10^9 CFU/g of the fecal material (Peterson *et al*., 2009). In the present study the samples were analyzed without enrichment. Hence, the presence of resistance genes in low abundances probably has not been detected. To our knowledge this is the first reported instance of antibiotic and metal resistance in healthy dog fecal samples tested with DNA microarray.

4.6 Conclusion

Antimicrobial use is often considered as the reason for the emergence and dissemination of resistant bacteria, thus posing a potential public health hazard. Indeed, the natural inhabitants of the GIT of healthy animals are known to harbor resistance genes (van den Bogaard, and Stobberingh, 1999; Witte, 2000). The gut commensals can acquire or donate the resistant determinants among themselves or among the bacteria that enter the intestine through the fecal-oral route (Salyers *et al*., 2004). Food animals are often considered as the source for the resistant bacteria and transfer of these bacteria to humans is considered to be via food chain or direct contact (Kruse, 1999). In contrast to the food animals, the role of companion animals as a reservoir of antimicrobial resistance genes has not been explored. Close physical contact and common habitat sharing among the pets and their owners can increase owners at the risk of acquisition of resistant genes harbored by resistant bacteria from pet animals. There are reports indicating that resistant *Staphylococcus* spp can be transferred from small animal pets to humans via contact (Clarke, 2006).

The present study was undertaken to assess the overall diversity of antibiotic and metal resistance genes in the fecal microbiota of healthy dogs using microarray analysis. Genes conferring resistance to chloramphenicol, macrolide, lincomycin, streptogramins, fluoroquinolones, beta-lactams, sulpha drugs, tetracycline, aminoglycosides, and glycopeptides were detected and most of them originated from the dog sample H-4. Among the metal resistance genes tested, the fecal samples were positive for the following metals: cobalt/nickel, copper, copper/silver, arsenic, cadmium, and copper/zinc/cadmium. However, most of them originated

from the dog H-4. Thus, the overall prevalence of antibiotic resistance genes harbored by the healthy dogs was low except for one dog that was positive for most of the resistance genes.

To our knowledge, this is the first report to study the diverse antimicrobial and metal resistance genes in the gut of healthy dogs using microarray. The results obtained in this study provide the baseline information on the diversity of antimicrobial resistance genes in the fecal microbiota of healthy dogs. The data show that healthy dogs do not represent an important reservoir of resistance genes and consequently public health risks.

4.7 Figures and Tables

Figure 4.1 Principle of DNA microarray

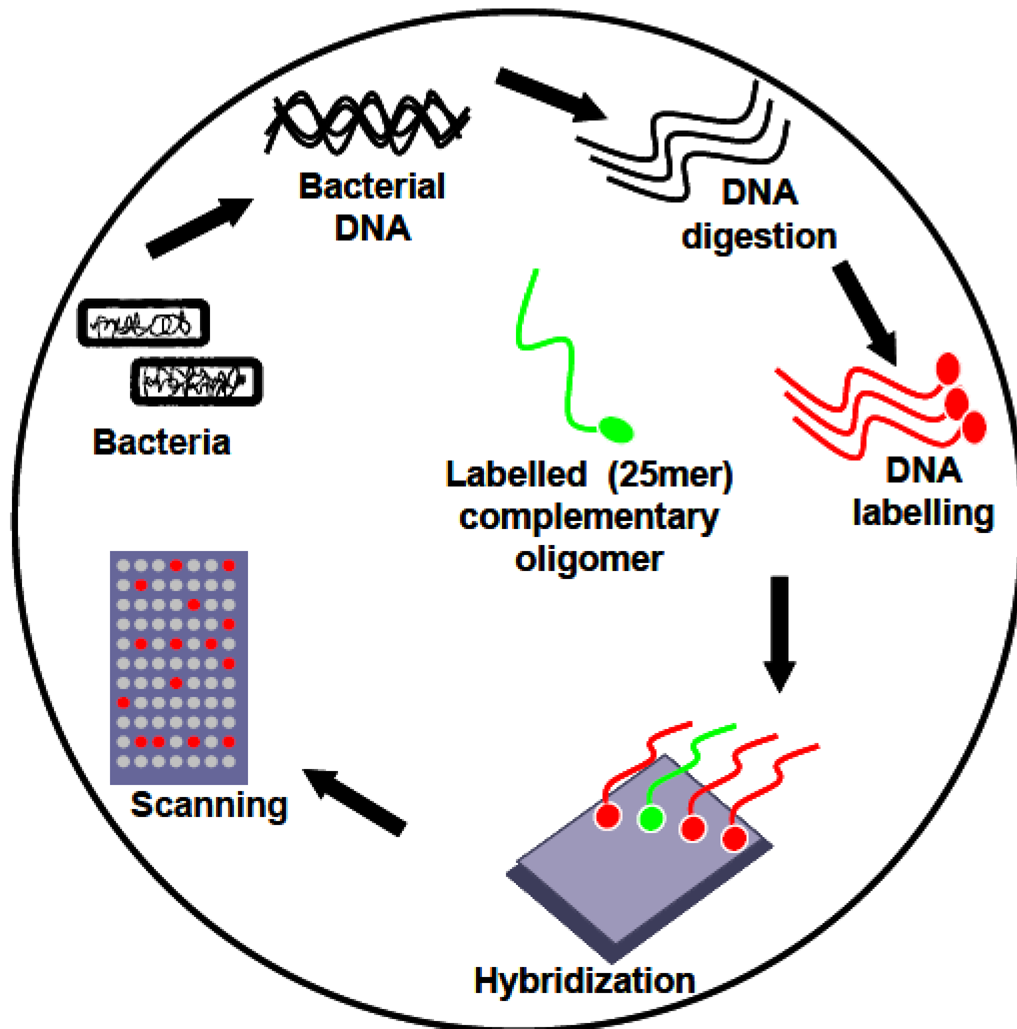

Table 4.1 Microarray analysis for detection of antibiotic resistance and metal resistance genes

Samples #	H-1	H-2	H-3	H-4	H-5	H-7	H-8	H-10
Chloramphenicol								
catQ				■				
Macrolide								
ereB				■				
erm(TM)2				■				
ermX						■		
mef(A/E)				■				
Lincomycin								
linA				■				
carA				■				
Streptogramins								
sat(G) vat(E-8)				■				
vat(B)				■				
Fluroquinilones								
parC								■
Beta-lactams								
bla2				■				■
blaZ				■				■
ccrB								■
penA				■				
Trimethoprim								
dfrA1 2				■			■	
Sulphanilamide								
sulII				■				
Tetracycline								
tetC		■			■	■		■
tetD							■	
tetM			■					■
tetO	■	■	■	■	■	■	■	
tet37			■					
tetY					■	■	■	■
tet31				■				
tetR								■
tet38				■				
tetA	■							

Aminoglycosides								
aadE				■	■	■	■	
*aac(6)-Ib**				■				
aac(6)-Im				■				■
ant(4)-Ia			■					
aphA-3							■	
Glycopeptides								
vanD				■				■
vanH				■				

Filled block (■), positive; blank block (□), negative

Table 4.2 Microarray analysis for detection of metal resistance genes

Samples #	H-1	H-2	H-3	H-4	H-5	H-7	H-8	H-10
<u>METALS</u>								
Arsenic								
arsD	■							
Cadmiun								
cadD3								■
cadD4				■				
Copper								
cusA		■		■				
cusB				■				
cusF				■				
cusS				■				
Copper/Zinc/Cadmium								
czcA2 copABCD		■		■				
Cobalt/Nickel								
cnrB				■				
Transferable copper								
tcrB				■				

Filled block (■), positive; blank block (□), negative

Table 4.3 Microarray analysis: Samples negative for the following resistance genes

Antibiotic groups	Resistance genes
Chloramphenicol	cat(TC), cat(B), cat(DP1), cat(S), cat-4, cat(P), flo ,cat-86, catp(XX), cfr, cat(DPS), cat(LM), cat(TC), cat(B)
Macrolide	erm(TR), ere(A), ere(A2), ere(B), erm(A), erm(B), erm(C), erm(D), erm(F), erm(G), ermQ, ermT, erm(TR)2, ermY, erm(BCT), ole(B), ole(C), srm(B), tlc(C), mef(A), mef(B), msr(A), mph(K), mph(B), mph(A), mph(BM)
Lincosamide	lmr(A), lin(A)2, lnu(A), lnu(B), lin(B)
Streptogramins	vga(A), vat(C), sat(A), sat(G), sat4, vgb(A), vgb(B), vat(A), vat(D), vat(E), vga(B)
Beta-lactam	ccr(B), pbp4 , mec(A), mec(A-2),
Rifamycin	arr-3
Streptothricin	sat(4)
Tetracycline	tet(B), tet(E), tet(G), tet(H), tet(J), tet(K), tet(L), tet(AP), tet(U), tet(V), otr(C), tcr, tet(BP), tet(Q), tet(S), tet(T), tet(32), tet(36), otr(A), tet(X), tet(Z), tet(W), tet(30), tet(33), tet(35), tet(34), tet(39)
Glycopeptides	ble, van(A), van(B2), van(D), van(E), van(G), van(H), van(R), van(X), van(Y)
Sulpha drugs	sulII, dfr(A), dfr(D)

Microarray analysis: Samples negative for the following resistance genes (cont.)

Metals	**Resistance genes**
Quaternary ammonium	qac
Aluminium	BnALMT2, BnALMT1, yba(X)
Arsenic	ars(G), ars(B), ars(C), ars(H), ars(R)
Cadmium	cad(A), cad(D2), cad(D), col(R) col(S)
Chromate	cys(A)
Cobalt/Nickel	cnr(A), cnr(B), cnr(C), cnr(H), cnr(T), cnr(X), cnr(Y), ncr(A),ncr(B), yoh(M)
Copper	cop(B), cop(C), cop(D), cop(P), cue(O), cue(R), pco(B), pco(C), pco(D), pco(R), pco(S)
Copper/Silver	cus(C)

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