Lactobacilli in the gastrointestinal tract of dog and wolf

– Isolation, identification and characterization of *L. reuteri*

Anton Pallin

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Abstract

Since the first wolf was taken as a companion animal a lot have happened with the biology of these animals. Is it possible that changes in the gut microbiota of the domestic dog are involved in the increasing numbers of animals with gastrointestinal disorders? Probiotics have been proven to promote the immune system of dogs by increasing concentrations of neutrophils and monocytes, decreasing the fragility of erythrocytes and increasing the serum IgG concentrations. Probiotics have also been shown to improve the fecal consistency, fecal dry matter and defecation frequency of dogs. The aim of this study was to find out if the bacterial counts in the gut of dogs differed from the gut of wolves, with the focus on lactobacilli. The presence of *Lactobacillus reuteri* and characterization of such strains in wolves and dogs was also analyzed to find a possible probiotic candidate. Fecal specimens from five dogs and intestine content from four wolves were collected and lactobacilli were analyzed and isolated by cultivation on the selective substrate Rogosa. Bacterial enumerations were noted and colonies with morphological differences were collected and isolated before the characterization was done using reuterin assay, rep-PCR and gel electrophoresis. A representative for each rep-PCR type was identified using 16S rRNA gene sequencing. Bacteria identified as *L. reuteri* was characterized further using methods for testing bile tolerance, detection of the genes *ureC* and *pduC*, mucus binding capacity and antibiotic susceptibility. A handful of isolates were also characterized by determining the gene sequence of a cell surface protein specific for *L. reuteri*. The content of lactobacilli in the fecal samples of dogs ranged from 10^6 to 10^8 cfu g^{-1} feces and in intestine of wolves from 10^3 to 10^5 cfu g^{-1} gut content. Nevertheless, not all of the bacteria grown on the Rogosa agar were lactobacilli, species of *Streptococcus* were also isolated. Even though the total counts of bacteria were higher in dog, the number of different species of isolated lactobacilli was higher in the wolf. In the samples from dog, two species of *Lactobacillus* were isolated; *L. animalis* and *L. reuteri* while in the wolf samples another five species were isolated. A total of 32 *L. reuteri* were isolated from the subjects and these could be divided into 10-12 different strains according to the characterization methods. Among the 13 isolated *L. reuteri* analyzed by the different characterization methods, four were reuterin positive and two were urease positive. Some of the strains bound well to intestinal mucus while others did not bind at all. The bile tolerance also differed between the strains but most of them, except for one strain, survived a concentration of 5% bile. The results from the antibiotic susceptibility test showed a close relation between the strains, with resistance against streptomycin and tetracycline. Some of these strains were isolated from both dog and wolf whereas other strains could only be found in dog or wolf. This was established using the different characterization methods as well as analyzing the gene sequence of a cell surface protein referred to as "protein x". A new method for isolating *L. reuteri* were also tested, called immunomagnetic isolation. The results from this method were both positive and negative, this since the beads bound to some strains of *L. reuteri* but not all. The Dynabeads also bound to lactobacilli in a dog sample but not to any bacteria in a wolf sample.

Sammanfattning

Ända sedan den första vargen domesticerades har en hel del skett när det gäller dessa djurs biologi. Är det möjligt att förändringar i tarmens mikrobiota hos hundar är orsaken bakom den ökande frekvensen av djur med mag- och tarmproblem? Probiotika har bevisats ha positiv effekt på immunförsvaret genom att öka koncentrationen av neutrofiler och monocyter, öka erytrocyters motståndskraft och genom att öka serumkoncentrationen av IgG. Probiotika har även visat sig förbättra konsistensen på hundars träck, torrsubstansen i träcken och antalet tarmtömningar. Syftet med detta arbete var att ta reda på om antalet laktobaciller i tarmen hos hund skiljer sig från antalet hos varg. Förekomsten av *Lactobacillus reuteri* och karaktäriseringen av sådana stammar i varg och hund analyserades också i ett försök att hitta en probiotisk kandidat. Träckprover från fem hundar och prover med tarminnehåll från fyra vargar samlades in och odlades på medium (Rogosa agar) specifikt anpassat för tillväxt av arter av *Lactobacillus*. Beräkningar av bakterieantalet antecknades och kolonier med morfologiska skillnader plockades och isolerades innan karaktäriseringen genomfördes med hjälp av reuterin analys, rep-PCR och gelelektrofores. En representant från varje rep-PCR-typ identifierades med hjälp av 16S rRNA gensekvensering. Bakterier identifierade som *L. reuteri* karaktäriserades ytterligare genom användning av metoder för att undersöka galltolerans, detektionen av generna *ureC* och *pduC*, bindning till tarmslem samt känslighet mot antibiotika. Ett fåtal isolat karaktäriserades även genom att bestämma gensekvensen för ett cellyteprotein som är specifikt för *L. reuteri*. Bakterieinnehållet i träckproven från hund varierande från 10^6 till 10^8 cfu g^{-1} träck och i tarmproven från varg varierade innehållet från 10^3 till 10^5 cfu g^{-1} tarminnehåll. Dock var inte alla bakterier som växt på Rogosa agarplattorna laktobaciller, arter av *Streptococcus* and *Pediococcus* kunde även påvisas. Även om det totala antalet bakterier var större i hund än i varg, kunde en större diversitet av bakterier ses i varg. I prover från hund isolerades två olika arter av *Lactobacillus*, *L. animalis* och *L. reuteri* medan det i varg isolerades ytterligare fem arter. Totalt isolerades det 32 *L. reuteri* från alla prover och dessa isolat kunde sedan bli uppdelade i 10-12 olika stammar med hjälp av de olika karaktäriseringsmetoderna. Av de 13 isolerade L. reuteri som analyserats av de olika karaktäriseringsmetoderna, var fyra reuterin-positiva och två ureas-positiva. Några av stammarna band bra till tarmslem (mucus) medan andra inte band alls. Galltoleransen varierade även mellan de olika stammarna men de flesta, förutom en stam, överlevde en koncentration på 5 % galla. Resultaten från testet för känslighet mot olika antibiotika visade på liknande resultat för samtliga stammar, där resistens mot streptomycin och tetracyklin kunde ses. Några av stammarna isolerades från både hund och varg medan andra bara isolerades från antingen hund eller varg. Detta framgick efter karaktäriseringen med hjälp av de olika metoderna samt genom analys av gensekvensen för ett cellyteprotein kallat "protein x". En ny metod som kallas "immunomagnetic isolation" som skulle användas för att isolera *L. reuteri* testades också. Resultaten blev både positiva och negativa eftersom kulorna band till några stammar av *L. reuteri* men inte till alla. Kulorna, eller "Dynabeadsen", band också till laktobaciller i prov från hund men inte till några bakterier i prov från varg.

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1. Introduction

The wild wolf is a highly topical subject in Sweden and has been so for many years. Some consider them to be a nuisance and regard them as pests while other see them as a natural part of the Scandinavian ecosystem and want to increase the number of wolves. One of the reasons why some people hate wolves may be due to the fact that the wolves occasionally kill and eat cattle and companion animals. Even though many dog owners have a negative attitude towards wild wolves because of this, knowledge of the biology and microbiota of the wolf might actually be valuable in understanding some of the ailments that afflict the dog. With the domestication and breeding of the dog numerous of less favorable traits have occurred, the modern lifestyle have also had its effect on the health of the dog just as it has affected humans. Different kinds of stress connected to the modern lifestyle have led to the development of different gastrointestinal defects and diseases in both humans and dogs. With the promising results of a number of different probiotic strains on the gastrointestinal health of humans, the focus has shifted slightly to study the effect of probiotics on our companion animals as well.

Since wild wolves do not have gut problems in the same extent as the domestic dogs it would be interesting to study the reason for this difference. The diet might be one factor that influences the microbial health and hence the health of the host, another possible reason for the better gastrointestinal health of the wolf might be an altogether different microbiota compared to the one in domestic dogs. Does the wild wolf have a different quantity of certain bacteria in the gut or other species/strains of bacteria that might contribute to a healthier environment in the gut? Have the modern lifestyle and breeding of the dogs disrupted the natural balance in the gastrointestinal tract? These are some of the questions that would be interesting to figure out, especially since a human equivalence of wild versus "domestic" is not possible to study. Dogs are also very useful as a tool within scientific research since they live in the same environment as humans and are exposed to the same potential allergens. This makes them better than e.g. mice that live in a strict and controlled laboratory environment when used in studies. Diseases that frequently afflict humans such as cancer and diseases in the heart are also very frequent in dogs. Since the genetic variation of the pure breed dogs today is very low, fewer individuals (dogs) are needed for each study than it would be if humans were used in the study. This makes it easier to find the genes that are responsible for different diseases. (Björnerfeldt, 2007) Answers to these questions and the finding of the genes responsible for certain diseases might aid the development of a new probiotic strain for dogs and hopefully solve some of the gastrointestinal problems that afflict more and more animals.

1.1 Domestication of dogs

The dog family (*Canidae*) consists of a group of carnivores that can be divided into 38 species. They can either be solitary hunters like the fox or social as the wolf, jackal and the coyote. This group of animals is well spread across the world and can be found everywhere except Antarctica and a few oceanic islands. Discussion whether or not the domestic dog (*Canis lupus familiaris*) originates from the wolf (*Canis lupus*) or the golden jackal (*Canis aureus*) has been discussed since before Darwin's time. Today it is certain that the wolf is the ancestor of the dog. This fact has been established through studies of behavior, vocalizations, morphology and molecular biology. (Clutton-Brock, 1995) Analyzes of the mitochondrial DNA from dogs of different breeds and wolves from different place around the world has also been performed. (Vilà et al., 1997) These results support the hypothesis that wolves are the ancestor of dogs by comparing these results with result from coyotes and jackals.

Remains of wolf bones have been found in caves and other sites dated back to as early as 400 000 years ago. This suggests that man might have hunted the wolf for their furs and meat but it could also mean that they kept pups to be tamed and used as companion animals. The wolves that

became less submissive would most likely have been killed or driven away, but the ones that were kept might very well be the precursors of the domestic dog. The earliest findings of a domesticated dog was discovered in a grave in Germany and was dated at 14 000 years BP (before present). Such findings suggest that the dog was the first animal to become domesticated by humans. Since the use of long distance projectile weapons was widespread at this time, it is possible that the dogs were used to help track the wounded animals or even bring them down. This should have contributed to a higher success rate for the hunters. (Clutton-Brock, 1995)

The biological changes that happened during the time of domestication due to inbreeding and breeding for certain traits meant that the dog became less and less like their wild forbears. Changes as coat colors, carriage of the ears and tail, proportion of the limbs and overall size, were all attributes suited for a place in the human society and less for the wild. A life among humans has also given the dog less acute sight and hearing, and that the dog retained juvenile characteristics and behavior into adult life. The earliest changes to the animals were however a reduction in size, this was due to the fact that they were given a different diet and less food and this acted as a strong natural selection. Small animals would have had better chances of surviving on a small amount of food and this meant that they could continue spreading their genes. (Clutton-Brock, 1995)

1.2 Gastrointestinal health of dogs

The lifestyle, diet and breeding of the dogs have led to a number of visual differences compared to their forbears, the wolf, but also a number of negative health effects. Among these health effects are a number of different gut problems.

The domestic dog can be afflicted with a number of gastrointestinal diseases, which can be associated with nonspecific alternations in the intestinal microbiota. One of these is a common disorder called small intestinal dysbiosis, which also can be referred to as small intestinal bacterial overgrowth or antibiotic-responsive diarrhea. Previous studies suggested that the disease was due to an increased bacterial count in the duodenum and the patients responded well to antibiotics unfortunately the diarrhea often came back after termination of the therapy. More recent studies have on the other hand found no correlation between an increased bacteria count in the duodenum and the disease. (Suchodolski, 2011)

Recently there have also been studies that suggest that inflammatory bowel disease (IBD) in dogs has to do with the commensal intestinal microbiota. The presently proposed reason behind the disease is that there is an abnormal interaction between commensal intestinal microbiota and the intestinal immune system in these individuals. Animal studies have shown that IBD only develops if bacteria are present. The hypothesis is that intestinal inflammation causes a dysbiosis toward gram negative bacteria. This in turn lead to reduced number of commensal bacterial groups, which reduces the effect the intestinal microbiome has on the immune response. The microbiome can no longer suppress unusual immune responses and the result will be an alarm of intestinal inflammation. Other recent studies have also revealed some of these changes in the microbiome in dogs and cats with IBD compared to healthy animals. It appears that for example the counts of *Enterobacteriaceae* were significantly higher in the afflicted animals. Higher concentrations of *Proteobacteria* have also been found in the duodenum of IBD dogs. Alongside these increasing counts of bacteria there have also been revealed that the proportion of *Bacteroidales* and *Clostridiales* were reduced in IBD dogs. (Suchodolski, 2011) Even though the results from these studies suggest that the reason for the inflammation might be due to the concentration of certain bacteria in the gut it is hard to determine the caus. Is it the bacteria that causes the inflammation or is it the inflammation that make it possible for these bacteria to grow better?

1.3 Diet of wild wolves

The diet contributes greatly to the bacterial composition of the intestinal tract. Different types of bacteria that are provided by the food as well as the amount of the different nutrients affect the microbiota. To try to figure out why the microbiota would differ between different individuals it would be interesting to find out what the wolves eat and if it differs between seasons. Scientists in Latvia have studied both the general food habits of the wolf and the winter diets. In the first study, feces from wolf as well as the stomachs of shot wolves were studied. The year was divided into two periods, one without snow (April-September) and one with snow (October-March), in the period with snow the majority of samples came from guts of shot wolves and the period without snow the examined samples were mostly feces samples. Samples were collected from three different areas in Latvia to look for any geographical differences in the diet. The feces or stomach material were washed through a sieve to collect hair, bones etc. which later could be used to identify what animal or plant source they came from. A total of 22 food categories were found, ungulates being the most common and beavers the second most. There were also a few seasonal differences e.g. the wolf consumed more beavers in the summer as well as small rodents and not surprisingly more berries and other plant material. During the winter time the wolves consumed more wild boars and livestock. There were also some geographical differences, in the west more cervids were consumed as well as more livestock. The intake of berries and small rodents did nevertheless not differ significantly between regions. Another conclusion that could be made was that it seems that wolves mostly consume wild animals and only consume livestock and other carnivores (such as dogs, raccoon dogs, red foxes and badgers) if there is a shortage of other food. (Andersone et al., 2004)

The same scientist that studied the general feeding habits of wolves during a full year in Latvia also studied wolves and lynx (*Lynx lynx*) in Latvia as well as Estonia. In conformity with the previous study they collected samples from both the gut of shot animals and feces, but this time the diet was only studied during the winter season (November-February). Macroscopic analysis of bones, teeth and hooves as well as microscopic analysis of hair, revealed the food sources of the wolf and lynx. Just as in the previous study the wolves had fed on a wide range of different food sources e.g. deer, moose, wild boar, beaver and domestic dog. One of the wolves stomach was also filled with grass, however no difference could be found between male and female wolves in terms of prey composition. The main prey for the wolves in both Latvia and Estonia appears to be wild boar. The reason for this is thought to be because wild boars are a relatively cost-effective prey and that some of the wolves have learned to ambush the wild boars at sites for ungulate supplementary feeding. A big portion (31.2 % males and 40.4 % female) of the intestine of the wolves were empty when examined, which indicates harsh feeding conditions during the winter season. (Valdmann et al., 2005)

In Poland the diet of wolves were examined by collecting scats all year round from the western and central parts of the country. The analysis was made in the same manner as previous studies and revealed that the wolves preyed almost entirely on wild ungulates, with Roe deer and wild boar being the most consumed. Wolves in Poland also appear to have a diverse diet with many different food sources, mainly other mammals but also birds, insects and plants. These last groups are nevertheless consumed in very low quantities. The consumption of other carnivores such as badgers, raccoon dogs, red foxes, domestic dogs and cats exist but their contribution to the diet is relatively low. Geographical differences in the consumption of domestic dogs could be seen though, with occurrence in scats from 0 -12.9 percent. The total occurrence were however determined to 2.3 percent and percentage of biomass consumed only to 1 percent. Even though scats were collected during the whole year, no seasonal difference was declared. (Nowak et al., 2011)

A study made in Sweden on the diet of the Scandinavian wolf examined scats taken from ten different territories. The samples were collected all year round from 1991 to 2006 and the season was divided into summer (May-September) and winter (October-April). Feces were examined as described in previous studies, by macroscopic and microscopic analyses and the prey items were divided into 15 different categories. Unlike the diet of wolves in Estonia and Latvia, the diet of Swedish wolves did not contain any wild boars. The main diet of these wolves seems to be moose, both during the winter and during the summer. Just like the wolves in Estonia and Latvia, the Scandinavian wolves seem to consume more rodents during the summer as well as more plant material. The wolves in Sweden did nevertheless consume fewer domestic animals during the winter than during the summer which is the complete opposite of the Estonian and Latvian wolves. Beside the fact that the wolves in Sweden had not consumed any wild boars and that the amount of each category of prey differed, it appears as if the wolves in Sweden, Estonia, Latvia and Poland all have a very diverse diet with many different preys. (Müller, 2006)

1.4 Diet of dogs

In a study made in Sweden the diet and activity of 460 Swedish dogs were analyzed by collecting data from mail and telephone questionnaires. According to the questionnaires the typical Swedish dog was given mostly (74% of the energy intake) commercial feeds. However smaller dogs (1-10 kg) only seemed to consume on average 62% commercial feeds while bigger dogs (30-40 kg) consumed up to 80%. The commercial feeds that were given to the dogs mostly consisted of dry feed and were given to 95% of all dogs. Only a low proportion (14%) of the dogs were given canned dog feeds. 93% of the dogs were given table foods and/or home-made diets, yet this was mainly given in addition to the dog feed and contributed only to a small portion of the energy intake. The most common table foods that were given to the dogs were vegetable oil, bread, meat/slaughter residues and sour milk. According to this study dry feeds mostly comprises of cereal grains (wheat, rice, maize, oats and barley) cereal by-products (wheat germ, com gluten meal and oat bran), soybean meal, animal products (meat meal, meat and bone meal and fish meal), fats and oils, and vitamins and minerals. The canned feeds on the other hand are mostly based on meat (beef, pig, lamb and/or poultry) and their by-products. Soy protein or soy flour was also used to give texture and the feeds were enriched with vitamins and minerals. (Sallander, 2001)

1.5 Importance of diet for the microbiota

As many dog owners might have realized from own experiences, what the dog eats highly effect the gut health of the dog. What they might not be aware of is that the growth of certain bacteria increase or decrease with certain diets or supplementations. In 1967 Albert S. Klainer and his colleagues performed an experiment on rats to see whether different diets could affect the microbiota in the feces. The rats were fed either chow pellets, ground lean beef or ground lean beef with added lactose, and these diets were kept for one month. Feces were collected and cultured on selective media and enumerations were done as CFU per gram sample. The group of rats that were given chow pellets had higher concentrations of lactobacilli than the rats given lean beef. There was however no difference in lactobacilli concentrations between rats fed with chow pellets and rats fed with lean beef with added lactose. (Klainer et al., 1967)

A study made on eight shepherd dogs in Yokohama in 1992, examined the effect of a dietary supplement of lactosucrose on the fecal microbiota. The dogs (four female and four male) were given the same amount of a fundamental diet as well as a specific amount of lactosucrose each day. This diet and supplementation were administrated for two weeks and three feces samples were collected from each dog. The feces samples were prepared and diluted before being inoculated unto four non-selective and 14 selective media. Even though some changes could be seen in the levels of *Clostridium* and *Bifidobacterium* with increasing amount of lactosucrose, no

other changes in microbial growth was observed. Lactobacilli levels seemed to be unaffected by supplementation of lactosucrose. (Terada et al., 1992)

Contrary to lactosucrose, a supplement of fructooligosaccharides and mannan-oligosaccharides seems to increase the levels of lactobacilli in the dog intestine and feces. An experiment on eight female dogs with hound bloodlines compared the bacterial enumeration between dogs given the supplement or a placebo (sucrose). The fecal samples were collected within 15 minutes of defecation and the ileal samples were collected using a surgically attached cannula. Samples were collected over a 14 day period and analyzed for bacterial enumeration of total anaerobes, total aerobes, *Bifidobacterium*, *Lactobacillus*, *C. perfringens* and *E. coli*. The analysis showed a significant increase in *Lactobacillus* counts in both ileal and fecal samples from dogs fed with fructooligosaccharides and mannan-oligosaccharides supplement compared to the placebo. Counts increased by more than one log unit in both fecal and ileal samples, while other bacterial counts were almost unaffected. (Swanson et al., 2002) In 2007 a comparison between six different diets was performed by Middelbos et al., one control diet and five diets containing different amounts of cellulose, fructooligosaccharides, mannanoligosaccharides (from yeast cell wall) and one diet with a supplement of beet pulp were given to dogs with hound bloodlines. The microbial analysis was done by serial dilutions that were plated out and by doing a quantitative PCR (qPCR) on extracted DNA. While the serial dilution and plating method did not show any difference in concentrations of lactobacilli between the different treatments, the qPCR did. The treatments with a supplement of cellulose plus fructooligosaccharides (1.5 %) and the combination of cellulose, fructooligosaccharides (0.9 %) and mannan-oligosaccharides (0.6 %) gave higher concentrations of lactobacilli. There also seemed to be a trend for increased lactobacilli concentrations in treatments with the beet pulp and cellulose/fructooligosaccharides (1.2 %)/mannanoligosaccharides (0.3 %) in comparison with a supplement of just cellulose. (Middelbos et al., 2007)

The effect of different protein contents in the diet has not been examined in dogs; an experiment on cats nevertheless gave some interesting results. This study took eight adult female cats and put them in individual cages and fed them with different diets. One diet contained 30 percent crude protein (moderate-protein diet, MP) and the other 60 percent crude protein (high-protein diet, HP). These diets were kept for eight weeks and fecal samples were collected after this period. DNA were extracted and isolated from the samples and *E. coli*, *Bifidobacterium*, *Lactobacillus* and *C. perfringens* were quantified via qPCR using specific primers. Pure cultures were then plated unto specific medium and the CFU was determined. The results showed that *Bifidobacterium* contents were higher in MP diet than in HP diet. The opposite was true for *C. perfringens*, where a HP diet gave higher concentrations than a MP diet. *Lactobacillus* and *E. coli* were not significantly affected by the diets and had only marginal changes in concentrations. (Lubbs et al., 2009)

Starvation and malnutrition can also have an effect on the GI microbiota. By depriving mice of food for three days or giving them a low-protein diet for up to 14 days containing by weight only 0.03% protein. After the treatment the cecum were removed, homogenized and made into different dilutions. These dilutions were plated unto different selective plates for strictly anaerobic bacteria, facultative anaerobic gram-negative enteric bacilli, gram-positive cocci and lactobacilli. Results were declared as populations (CFU) per gram of cecum. After only 24 hours on the starvation diet, a decrease of 1 log10 could be seen in the levels of lactobacilli and while these bacteria decreased an increase could be seen in gram-negative facultative anaerobic bacilli, but first after 3 days of starvation. The population levels of lactobacilli had however a steady decrease from day to day and ended up at approximately 2 log10 below the original levels. The low protein diet had similar results and also gave a 2 log10 decrease in lactobacilli counts. (Deitch et al., 1987)

1.6 History of probiotics

The birth of probiotics is supposed to have begun with a microbiologist named Élie Metchnikoff. Metchnikoff wrote a book called "Essaies optimists" in 1907 where he discussed philosophy, religion, folklore and science of ageing, and microbial gut ecology during human life. In 1920 Rettger and colleagues performed experiments which showed that lactic acid bacteria such as *Lactobacillus acidophilus* could very well survive in the human gut. They were also the first to do clinical trials on people with constipation in 1935, with promising results. Freter et al. made experiments on mice in the 1950s, which showed that broad-spectrum antibiotics could destroy their gut microbiota. These mice also became more susceptible to *Salmonella* and *Shigella* infections by a 1000-fold. In 1978 Collins did experiments on germ-free guinea pigs, which could be killed by only 10 cells of *Salmonella enteritidis* strain. To kill a guinea pig with complete gut microbiota, 10^9 cells were required. (Ljungh et al., 2009)

In the 1940s and 1950s studies in Japan discovered that *L. casei* and some *Bifidobacteria* species could protect young mice against gut infections after weaning. These effects and other health effects associated with probiotic bacteria were later confirmed and it was discovered that the unique cell wall constituents such as lipoteichoic acid (LTA) molecules and cell surface proteins (GSPs) were the reason for some of the effects. Amoureux et al. 2005 also realized that inflammation caused by peptidoglycan, DNA of many pathogenic bacteria and LPS of most Gram-negative bacteria, could be counteracted by probiotic lactic acid bacteria (LAB). The reason for this counteraction is that the probiotic bacteria prevent the gut epithelium tight junction from staying open. Some of the bacteria in the group of *L. casei/paracasei* appear to have cell components with anti-inflammatory properties. *L. reuteri* also seem to have positive effects on IBD induced by IL-10-/- *Helicobacter hepaticus* in a mouse model. (Ljungh et al., 2009)

1.7 Lactobacilli as probiotics

There are a number of different proposed mechanisms of LAB probiotics that both could be beneficial and detrimental for the host. The beneficial ones include the suppression of harmful bacteria by production of antibacterial compounds (e.g. reuterin), competition for nutrients and competition for colonization spots. LAB could also benefit the host by producing enzymes that support digestion of e.g. lactase. They could also improve the gut-wall function and decrease production of ammonia, amines or toxic enzymes. Studies have also shown that LAB might be able to improve the immune system of the host by increasing antibody levels and macrophage activity. It is also possible that they might contribute to the metabolism of substances such as cholesterol. In pigs a supplementation of *L. acidophilus* inhibited the increase of serum cholesterol when pigs were on a high-cholesterol diet. (Gilliland et al., 1984) It has also been suggested that LAB have a reducing effect on the production of harmful nitrogen compounds in the intestine. Once again it appears that a supplementation of *L. acidophilus* have positive effects on pigs, in this case the levels of produced intestinal amine were lower compared to untreated pigs. (Hill et al., 1970) Similar results have been shown on carcinogenic fecal enzymes β-glucuronidase, nitroreductase and azoreductase in humans. All these were decreased with a diet containing *L. acidophilus* by a 2- to 4-fold. (Goldin et al., 1984) *Lactobacillus* species may also aid in the digestion of certain substances e.g. lactose by producing and releasing enzymes such as β-galactosidase. This could be seen in rats that were fed yoghurt, which had increased β-galactosidase activity and it was thought that this activity were derived from bacteria. (Garvie et al., 1984)

Improving the immune system of the host is one of the previous mentioned beneficial mechanisms of probiotic LAB. This fact has been shown in studies comparing the immunoglobulin levels and phagocytic activity in conventional animals with a complete indigenous microbiota and germ-free animals. This has given the scientists the idea that the immune system might be enhanced even

further using probiotic strains of bacteria. *L. casei* and *L. acidophilus* have also been shown to activate macrophages and lymphocytes as well as increase the phagocytic function of macrophages in mice. An increase in the antibody production of e.g. IgG and IgA has also been shown after consumption of lactobacilli. (Nousiainen et al., 2004)

Before a probiotic can be accepted there are a number of different criteria that are needed to be fulfilled. Important traits for a probiotic, according to Nousianen, are that it should be a nonpathogenic strain found in the normal intestinal microbiota and it is preferable if it is specific to the host. This fact is however a subject to discussion within the field of microbiology. It should also be acid tolerant enough to survive the passage through the stomach and duodenum as well as being tolerant to bile in order to survive the passage of the upper small intestine. The bacteria should grow fast and metabolize quickly and be present in the gut at high concentrations. Ability to bind to the mucosa as well as ability to exclude other microbes from adhesion sites can be favorable as well. The probiotic should be easy to produce, survive at large-scale production and should also have high viability during storage. (Nousiainen et al., 2004) Another attribute that is of great importance before a strain of bacteria can be accepted as a probiotic is antibiotic resistance as well as the transferability of said resistance. Certain bacteria have an intrinsic resistance to an antibiotic e.g. most lactobacilli to vancomycin. Lactic acid bacteria in general seem to have a natural resistance to many antibiotics because of their structure or physiology. Most of these resistances are not transferable and these species also seem to be susceptible to the antibiotics in clinical use. (Donohue, 2004) Studies made on antibiotic resistance in lactobacilli have shown that 68.4% of the probiotic isolates were resistant to at least two antibiotics including intrinsic resistances. The antibiotics that the strains were resistant to included kanamycin, tetracycline, erythromycin and chloramphenicol. (Temmerman et al., 2003)

1.8 Lactobacillus reuteri as probiotic

Reuterin is a low molecular weight antimicrobial substance produced by *L. reuteri* when grown anaerobically with access to glucose, glycerol or glyceraldehyde. (Axelsson et al., 1989) *L. reuteri* produces this substance by dehydrating glycerol, and can subsequently be reduced to 1,3-propandiol by $NADH + H^+$ -dehydrogenase. (Veiga da Cunha et al., 1992) The reuterin cannot be produced during the log phase and starts instead to be accumulated during the stationary phase. (Axelsson, 1990) *L. reuteri* is not the only bacteria that dissimilate glycerol via this pathway, the accumulation and excretion of reuterin seems nevertheless to be a unique trait for *L. reuteri*. (Veiga da Cunha et al., 1992) Reuterin has been found to be not only antibacterial but also antifungal, antiprotozoal and antiviral. (Axelsson et al., 1989) It is not only other microorganisms that are sensitive to reuterin, *L. reuteri* and other lactic acid bacteria are also affected by it but seem to be more resistant than other microorganisms. Reuterin does not however seem to affect human cells in any negative sense. (Ouwehand & Vesterlund, 2004) The mechanism behind the antimicrobial effect of reuterin has been unsolved despite 20 years of investigation. According to Schaefer et al. (2010) the reason might be that reuterin induces oxidative stress in cells by modifying thiol groups in proteins and small molecules. *L. reuteri* also seem to be antibacterial against coliforms (e.g. *E. coli*) by lowering the pH in the gut. The lowering of pH is done by producing lactic acid and the same antibacterial affect could be seen when treating subjects with only lactic acid. (Ratcliffe et al., 1986)

Studies made on rats have shown that *L. reuteri* had positive effects on the mucosa in the gut, by maintaining the mucosal integrity. (Fabia et al., 1992) By inducing colitis in rats by the use of acetic acid, microbial changes could be seen similar to those in human patients with active ulcerative colitis. These changes involved reduction in the number of anaerobic bacteria and lactobacilli. An administration of exogenous lactobacilli appears to have had positive effects on rats with this acetic acid-induced colitis. By giving the subjects a rat-specific strain of *L. reuteri*, directly after the administration of the acetic acid, the development of colitis could be avoided.

The effects associated with the acetic acid treatment such as morphological damage and mucosal permeability were close to neutralized by treating the rats with *L. reuteri*. A delay of 24 hours before the treatment with *L. reuteri* did nevertheless lead to a less protective effect. This suggests that the lactobacilli prevents the colitis rather than treat it. (Casas & Dobrogosz, 2000) It has also been suggested that *L. reuteri* can have a protective role against colon cancer. Bacterial bile salt hydrolysis into harmful secondary bile salts is considered one of the factors that could contribute to the development of colon cancer. An addition of *L. reuteri* counteracted the negative effects and one plausible mechanism behind this might be a precipitation of the bile salts and physical binding of the salts to decrease the bioavailability of them. (De Boever et al., 2000)

1.9 Probiotics for dogs

Probiotics for companion animals is a relatively new area on the market and it does not seem to be that many products available at the moment. In Sweden for example a quick search on the web only gives two results. One product is supposed to increase the oral health of the dogs and another is for their bowel health. The latter product is meant to help animals with loose stool, diarrhea, stomach ache or animals which are unusually gassy. Reasons for these symptoms are many but can involve different kinds of stress, such as change in environment, diet or that the animal takes medicine (antibiotics, vaccines or anthelmintics). It also seems that both younger and older animals are extra sensitive for an imbalance in their gut microbiota, according to the manufacturers. The strains that are available at the market at the present are from the genera *Enterococcus* (*Enterococcus faecium* SF68) and *Streptococcus* which are from the same order (*Lactobacillales*) as *L. reuteri* but in other families (*Enterococcaceae* and *Streptococcaceae* instead of *Lactobacillaceae*).

A study on healthy adult dogs fed with a dry control food as well as *L. acidophilus* DSM13241 for four weeks, showed that the probiotic was able to survive the passage through the intestine. By surviving the passage these bacteria could in turn affect the colonic microbiota by increasing the numbers of lactobacilli and decreasing the numbers and percentage of clostridia. *L. acidophilus* also had positive systemic effects, e.g. increasing concentrations of neutrophils and monocytes, decreasing the fragility of erythrocytes and increasing the serum IgG concentrations. (Baillon et al., 2003) Another study with the same strain of *L. acidophilus*, made on dogs with non-specific dietary sensitivity (NSS), indicated an improvement in fecal consistency, fecal dry matter and defecation frequency. The fecal microbiota in this study was not affected significant in any way, neither by lowering the fecal concentrations of *C. perfringens* and *Escherichia spp.* or by increasing the *Lactobacillus spp.* and *Bifidobacterium spp.* (Pascher et al., 2008)

1.10 The canine gut microbiota

Examining the bacterial diversity in dogs was one of the goals with this study. The topic of bacterial diversity and quantity has been examined in a number of different studies on different dog species, dogs varying in age, difference between diets etc. A study made in 2001, looked at the microbiota in Labrador feces. By growing the bacteria on different selective substrates, the hope was to get a good overview of the bacteria present in the gut of the domestic dog. The target populations were total aerobes, total anaerobes, coliforms, *Bifidobacterium*, lactobacilli, Gram-positive cocci, clostridia and *Bacteroides*. The identification of the grown bacteria were done by 16S gene sequence determination and comparing these with available sequences in the GenBank/ EMBL database. Feces from four dogs were studied. From one of the dogs four samples were taken, whereas from the other three only one sample was analyzed. Between the four samples from the same dog, there were little deviation in bacterial counts, as well as between this dog and one of the other three. These two dogs were however from the same litter. The dog with the highest counts of all, except for coliform bacteria, was the youngest one. The lactobacilli count differed from 4.5 to 8.5 (CFU log10∕gram feces). The two dogs from the same litter had a count

of around 6. The 16S rRNA sequencing was done on the four samples from the same dog. This analysis revealed that the Rogosa agar was quite selective for lactobacilli, with the exception of *Streptococcus bovis* in one of the samples. The *Lactobacillus* species that were found were mainly *L. ruminus* and *L. murinus* but also *L. animalis* and *L. reuteri*. In two of the samples *L. murinus* was very dominant but in the other two it was absent. *L. reuteri* was found in two of the samples as well but at fairly low concentrations. (Greetham et al., 2002)

The impact of advances in age on the microbiota in the gastrointestinal tract of dogs is an interesting topic. To examine if there are any changes during the lifetime of dogs, Japanese scientists compared beagle dogs from two different age groups; less than 12 months old and more than 11 years old. All the subjects were male and none had a history of bowel disorders or antibiotic therapy. Samples from the stomach, duodenum, jejunum, ileum, cecum, colon and rectum as well as freshly voided feces were taken to analyze the microbiota. The samples were thoroughly mixed and a series of ten-fold dilutions were made ranging from 10^{-1} to 10^{-8}. Two to four dilutions were plated onto four non-selective and ten selective agar plates. Results showed no differences in the microbiota of the stomach, duodenum, jejunum and ileum between the two age groups. Nevertheless there were some differences in the microbial populations of the cecum, colon and rectum. Overall content of *Peptostreptococcus* and *Bifidobacteria* in the large bowel were higher in the younger dogs than in the older ones. The content of lactobacilli in the cecum and colon was also higher in the younger compared to the older dogs. The older dogs did however have larger populations of *Clostridium perfringens* and streptococci. Reasons explaining the lower content of lactobacilli in older dogs was suggested to be due to lower activities of intestinal functions e.g. intestinal motility, intestinal secretion and/or immune mechanisms. (Benno et al., 1992)

Small-intestine and fecal microbiotas have also been compared in a study made by Mentula et al.(2005) Samples from 22 male beagle dogs were collected and analyzed over a four week period. These samples were homogenized and made into ten-fold dilutions (10^{-1} to 10^{-7}) and plated onto several non-selective and selective agar media. Colonies were divided into different types and then isolated for identification. The total bacterial count of the small-intestine (10^2 - 10^6) was lower than the counts in the feces (10^8 - 10^{11}). In the jejunum the growth of anaerobic and aerobic was almost the same whilst in the feces the anaerobic bacteria dominated. Several bacterial groups or species found in most jejunal samples were seldom isolated from the corresponding fecal sample and vice versa. 25 percent of the bacteria found in the jejunum were not isolated from the corresponding fecal sample and 45 percent of the bacteria in the feces were not found in the jejunum. Lactobacilli were only found in one of 22 individuals in the jejunum and in seven of 22 in the feces. In the jejunum the CFU counts were mere 10^1 and in the feces the mean value was almost 10^7. The results also indicates that the composition of the jejunal microbiota was unique for every individual, that it was relatively simple with a few species at a time and that the microbiota changed over time with vastly fluctuating counts. The fecal microbiota on the other hand seem to be the opposite, with similar microbiota in the different individuals and with major bacterial groups that remained fairly constant during the whole study time.

Another comparing analysis of the microbiota was done in USA. The focus were on analyzing the bacterial diversity by extracting DNA from samples and identifying the bacteria by 16S sequencing. Samples were taken from six healthy Hound dogs evenly distributed between males and females with a mean age of 6.2 years. Intestinal fluid, parts of the intestine or solid intestinal content were taken from the duodenum, jejunum, ileum, colon and rectum, and genomic DNA were extracted individually from each sample. Comparative 16S rRNA gene analysis was done on each sample and the identification was done by comparing sequences with existing ones in RDP (Ribosomal Database Project). According to the result the most diverse phylum was *Firmicutes*

and *Clostridiales* the most diverse bacterial order, this order being most abundant in the duodenum and jejunum as well as being a major constituent in the microbiota in the ileum and the colon. The order *Lactobacillales* also had a high presence in the duodenum, jejunum and colon and even though it was observed in the ileum they only contributed to a small fraction of the identified bacteria here. Members of the *Lactobacillus* genus that were observed were *L. johnsonii* and *L. aviaries* as well as several isolates with less than 98 percent similarity to *L. murinus* and *L. reuteri* observed in the small intestine. (Suchodolski et al., 2008)

1.11 Bacterial diversity in wild wolves

One of the aims of this study was to find out how the microbiota in wolves differed from the microbiota in domestic dogs. This would be interesting to see since it might give clues to the reason behind problems the dogs have with their gut and intestinal tract. Have the dogs microbiota changed since before they were domesticated or is the reason for the deteriorating intestinal health of some dogs something else? The number of studies made on the microbiota of wolves are, to say the least, very few. One study made in 2009 by Chinese scientists has nevertheless identified bacteria from five major phylogenetic lineages in feces from captured wild wolves. Bacterial DNA were extracted from the feces and identified by 16S rRNA sequence analysis. The phylogenetic lineages that were found were *Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria* and *Actinobacteria*. Within the phylum *Firmicutes,* which were the most diverse and most abundant phylum, three different bacterial classes were identified, *Clostridia*, *Erysipelotrichi* and *Bacilli*. *Clostridia* were the largest subgroup with 35 phylotypes divided into five families, *Lachnospiraceae*, *Ruminococcaceae*, *Clostridiaceae*, *Peptostreptococcaceae* and *Peptococcaceae*. Three phylotypes belonging to the class *Erysipelotrichi* were found, and they all were classified within the family *Erysipelotrichaceae*. The last class was the Bacilli and there were only one phylotype found within this class and it represented the family *Planococcaceae*. No bacteria within the order of *Lactobacillales* were however found in this study. (Honghai Zhang et al., 2010)

1.12 Isolation and characterization of Lactobacillus

Isolating and characterizing *L. reuteri* was also one of the aims of this study. Exactly this has been done by the Oklahoma State University. Feces samples were taken from 20 healthy dogs and lactobacilli were isolated by pour plate technique using *Lactobacillus* selection (LBS) agar. All the isolated bacteria were kept alive in MRS broth stored at 2 to 5°C after 18 hour incubation at 37°C. In order to identify the different isolates six methods of characterizations were done, gram staining, catalase test, growth at 15 and 45°C, arginine hydrolysis test and carbohydrate fermentation. The results of the characterization tests for every isolate were compared to those for the lactobacilli in "Bergey's Manual of Systemic Bacteriology for identification". From four dogs a total of 22 lactobacilli were isolated, 18 out of these 22 were identified as *L. reuteri* and the remaining four were identified as *L. acidophilus*. In order to characterize and evaluate the isolates as potential probiotic products, a number of different analyses were done. The first analysis was bile tolerance and from the 18 original strains the ten most bile resistant were saved for further analyses. The four strains of isolated *L. acidophilus* were also analyzed for bile tolerance; these were nevertheless significantly less bile tolerant. Antagonist action toward *Salmonella typhimurium* was also analyzed for the ten remaining strains of *L. reuteri*. All of them showed an inhibitory effect towards *S. typhimurium* and ranged from 57.0 to 93.8 percent inhibition. However it does not seem that the decrease in pH due to the production of acids were the sole reason behind the inhibition, this since the samples with the lowest pH did not have the highest inhibition. The production of reuterin varied between the strains as well and only half of them produced any reuterin at all. As a final evaluation test the most promising strain which was among the most bile tolerant, most inhibitory and produced the most reuterin was tested for stability during frozen storage in dog food. The decrease in CFU per gram dog food was nevertheless

insignificant with only one-half log cycle decrease. This kind of stability during storage is essential when developing a new probiotic strain. Further experiment to see how viable the strain is during commercial production and storage is still needed before determining the potential of the strain as a probiotic product. (McCoy & Gilliland, 2007)

1.13 Aim of the project

The aim of this project was to find out if there is a difference in the microbiota of dogs and wolves, when it comes to total number of lactobacilli, as well as number of different species of *Lactobacillus*. The focus was nevertheless on the presence of *L. reuteri* in the gastro intestinal tract or feces and eventual differences between the bacterial strains in wolf and dog. Characterization of the isolated *L. reuteri* was also one of the aims of this study. These results could later contribute to the discovering of a probiotic product for dogs to overcome some of the gut problems associated with the modern domesticated dog.

2. Materials and methods

All recipes for substrates used in this study can be found in **Appendix 1**.

2.1 Study subjects and specimens

The fecal samples used in the analysis of the dog microbiota came from five different individuals. Three of them were beagles referred to as Beagle I, Beagle II and Beagle III. Two of them were male dogs (Beagle I; born 02/11/97 & Beagle II; born 15/04/99) and one was a bitch (Beagle III; born 06/05/04). These dogs have been used for education purposes for veterinaries etc. and were fed dry pebbles and had been known to eat feces from other dogs. Fecal samples were also taken from a mixed breed dog (bitch; born 19/10/04), as well as from a Springer Spaniel (bitch, born 04/12/00). The first samples from wolf (*Canis lupus*) referred to as VLT 115/12, came from a male (born 2011) that had been killed accidently by a train outside the city of Storvik (Gävleborg county). Three types of samples were analyzed, scrapings from the small and large intestine and visceral contents from the large intestine. The colon of this wolf contained plenty of feces and the feces itself contained hair, parts of plants, bones and more undeterminable contents. The second sample from wolf referred to as "Skansen", were fecal samples from three male wolves. These wolves were born and raised in captivity at Skansen in Stockholm and were fed on mostly pure meat, but sometimes also whole animals e.g. rabbits. They had no known diseases or intestinal problems. The feces from these wolves contained a lot of hair/fur and were generally very dry. No information on how fresh the feces were could be given. The last samples from wild wolves came from two adult males (V289/12 & V188/12) shot and killed (nuisance wildlife management) in Foskros (Dalarna county) and Stor-Grucken (Härjedalen county). Samples from the colon, small intestine as well as appendix were taken but only the colon samples and appendix samples were analyzed. The colon of both of the wolves was practically empty and mostly thick brownish mucus could be gathered from the colon.

2.2 Sample preparation and culturing of bacteria

The fecal sample from the Springer Spaniel was the first to be analyzed and was homogenized using a "stomacher" for 2 minutes at maximum velocity, to produce 10 % (w/v) slurry using phosphate-buffered saline (PBS) (pH 7.3). 10 ml of this product was then spiked with 10 µl of a bacteria suspension (*Lactobacillus reuteri* 5002 PTA 6127), this to test the analyzing methods and to ensure that there were lactobacilli present for the isolation. Serial tenfold dilutions of the fecal material were then prepared (up to 10^{-5}) and 100 µl of each dilution were plated out onto Rogosa agar and Rogosa agar with added vancomycin. Rogosa plates were incubated under anaerobic conditions at 37°C for two nights, and Rogosa plates with added vancomycin were incubated under anaerobic conditions at 45°C overnight (ON). The plates were then examined and colony forming units (CFU) were counted. Bacteria differentiated in size, color and shape, were collected onto two de man Rogosa Sharpe plate (MRS) with numbered grids and incubated overnight at 37°C.

Samples from the other four dogs and the four wolves were prepared in the same fashion as the first dog with the exception that the samples were homogenized by vortexing hard for 2-3 minutes and that PBS-tween 20 (0.05 %) were used. A series of tenfold dilutions were made with these samples as well, up to 10^{-6} and plated onto Rogosa and Rogosa/vancomycin agar. Colonies were collected according to previous criteria onto two MRS plates with numbered grids.

2.3 Reuterin assay

The Rogosa and the Rogosa/vancomycin agar plates used for the first sample from dog were also used for the reuterin assay. 10 ml of glycerol agar (500 mM, 1% agar; melted and preheated to 45-50°C) were added and the plate was incubated at 37°C for 35 minutes. Following the incubation, 5 ml of 2.4-DNPH solution (0.1% 2.4-dinitrophenyl hydrazine in 2 M HCl) was poured onto the

plate and left for 3 minutes. After pouring of the DNPH solution, 5 ml of 5 M potassium hydroxide (KOH) was added onto the plate. The presence of red-brown zones could be seen around a few colonies and these were counted as reuterin positive bacteria.

With all of the following samples a separate MRS plate with numbered grids was used for the reuterin assay. This to ensure that these reuterin positive CFU could be further analyzed. Changing from Rogosa agar to MRS agar also ensured a stronger visual result.

2.4 rep-PCR analysis

In order to group the bacteria into different genotypes, a genomic fingerprinting method called rep-PCR was performed. From a second MRS plate not used for the reuterin assay, a number of morphologically different colonies were chosen. Approximately 1 µl of these colonies were suspended in 100 µl of water (Mol Bio grade, DNase-, RNase- and Protease-free, (5PRIME)). From these suspensions, 0.5 µl was mixed with 9 µl of a solution made of 1 µl of primer (20 µM of 5'gtg gtg gtg gtg gtg 3'), 22.5 µl of water (Mol Bio grade) and one "puReTaq Ready-To-Go PCR-bead" (GE healthcare). The rest of the bacteria suspensions were stored in a freezer at -20°C. The following PCR-program was run: 98°C, 5 min; 30 x (90°C, 30 s; 95°C, 30 s; 40°C, 60 s; 65°C, 4 min); 65°, 16 min; 16°C, ∞. This was followed by visualization of the PCR product by gel electrophoresis. 2 µl of loading buffer were added to the samples and 10 µl were added to each well on the gel (1 % agarose gel). A 1 kb marker was also added to the gel to determine the base pair position of each sample. The gels were stained with ethidium bromide and run at 70 V for two hours before being analyzed using UV light.

2.5 16S rRNA gene sequencing

Frozen suspensions of bacteria used in the rep-PCR analysis were thawed and 0.5 µl of the suspension were mixed with 1 µl F8M primer (5'-AGAGTTTGATCMTGGCTCAG-3'; 10 pmol/µl), 1 µl 926r primer (5'-CCGTCAATTCCTTTRAGTTT-3'; 10 pmol/µl), 22,5 µl water (Mol Bio grade) and one "Ready-To-Go PCR bead". The following PCR-program was run: 95°C, 5 min; 30 x (95°C, 30 s; 55°C, 30 s; 72°C, 60 s); 72°, 16 min; 16°C, ∞. Some of the PCR products were checked by agarose gel electrophoresis to ensure a good preparation and that there were enough DNA present in the sample. The samples were sent away to be analyzed by Macrogen in the Netherlands and the achieved sequences could later be analyzed using the software FinchTV. The sequences were compared with sequences of already isolated bacteria in the GenBank database and the Ribosomal Database Project, to identify the isolated bacteria.

2.6 Freezing of isolated strains

All of the isolated bacteria that were sent away for 16S sequencing were also kept as frozen isolates. This was done by plating each of them unto a separate MRS agar plate and incubating them anaerobically for 48 hours at 37°C to achieve enough bacteria for a thick suspension. A blue (10 µl) inoculation loop was used to scrape colonies of the agar plates and then suspend them in freezing media. All samples were stored at -70°C and saved for future use.

2.7 Mucus binding capacity

2.7.1 Coating with mucus

Mucus from the small intestine of a pig was diluted 20 times in PBS before being added to the wells of a Greiner microtiter plate. The plate was then incubated at 2°C overnight while being slowly shaken. The mucus that had not bound was removed from the wells, and the wells were washed once with PBST (0.05%) and blocked with 200 µl PBST (1%) for an hour in room temperature while being shaken. As a last step the wells were washed twice with PBST pH 6.0 before the plate were ready to use.

2.7.2 Growing of bacteria

In order to determine the mucus binding capacity of the isolated strains of *L. reuteri* from the samples, the frozen isolates first had to be grown. The first test was done with eight strains of *L. reuteri*, four from dog, two from wolf and two controls, one that had been documented as a good mucus binder (*L. reuteri* ATCC PTA 4659) and one with only weak binding (*L. reuteri* DSM 17938). All of the strains were grown overnight at 37°C in tubes with MRS broth. The following day 10 µl of this bacterial suspension was added to two different tubes with MRS broth, one with added mucin (0.1%) and one without. This was done to check if bacteria grown with a supply of mucin would get an increase in mucus binding capacity. The suspensions were once again incubated at 37°C ON. After the incubation 100 µl of the suspensions were transferred to a eppendorf tube and 1.5 ml of PBST pH 6.0 were added before the samples were centrifuged for 6 minutes at 10 000 rpm. The supernatant were then removed and the pellet suspended in 1 ml of PBST pH 6.0. 100 µl of bacterial suspension was added to the wells which were left to incubate for four hours at 37°C. After the incubation the wells were washed four times with PBST pH 6.0, between each washing step the plate was shaken for five minutes.

2.7.3 Reading of results

Before being studied with an inverted microscope, 100 µl of PBST pH 6.0 were added to each well. Since it was hard to determine the presence of bacteria in the wells, the PBST were removed and the plate was left to dry in room temperature until the next day. The presence of bacteria was then determined by the presence of crystals, which indicates the binding of bacteria to the mucus. The results were evaluated by comparing the samples with the controls (ATCC PTA 4659 and DSM 17938)

2.8 Characterization of L. reuteri

2.8.1 RAPD

From the previously made bacterial suspensions, 0.5 µl were taken from each confirmed *L. reuteri* strain. A mixture of one "PuReTaq Ready-To-Go PCR bead" for every 24 µl of CB3-solution (5'-AGC AGG GTC G-3') were made and 9 µl were added to each 0.5 µl bacteria suspension. The following PCR-program was run: 98°C, 3 min; 20x (94°C, 1 min; 29°C, 1 min; slope 0,5°C/s; 72°C 2 min); 45x (94°C, 30s; 55°C, 30 s; 72°C, 30 s) 72°C, 30s.

Following the PCR-program, 2 µl of 6x DNA loading Dye were added to each sample before being analyzed by gel electrophoresis on agarose gel (3 %) in 0.5x TBE buffer. The gel was run at 120 V for 1 hour and 15 minutes before the results were visualized by Trans UV light.

2.8.2 Bile tolerance

In order to test the bile tolerance of isolated strains of *L. reuteri* MRS plates were made with 0.5, 1, 2.5 and 5 percent of porcine bile (SIGMA B8631). The frozen isolates of bacteria were grown for 16 hours in MRS broth at 37°C. The suspensions were then diluted in PBS to approximately 10^3-10^6 CFU ml^{-1}. 5 µl of each dilution were dropped onto the plates and let dry, on each plate a number of 8-10 drops were applied. A triplicate of each bacterium was analyzed and the plates were incubated for 72 hours at 37°C in anaerobic conditions. Colonies were counted and the bile tolerance (survival rate) was estimated by comparing the number of colonies on the bile plates in relation to the MRS plate without bile.

2.8.3 Detection of *ureC* and *pduC*

The bacteria suspensions that had been used in rep-PCR, 16S sequencing and RAPD were also used in this analysis. PuReTaq Ready-To-Go PCR beads were mixed with primers for *ureC* (ureCF – GAAAGTCTTTTTGGTGGTGG and ureCR - AACGTCGTCAGGAATCTTAG) and primers for *pduC* (pduCF –CCTGAAGTAAAYCGCATCTT and pduCR -

GAAACYATTTCAGTTTATGG) and water (Mol Bio grade). 23 μl of water were mixed with 1 μl of each primer (ureCF and ureCR or pduCF and pduCR) and one PuReTaq bead. 9 μl of this mix were added to each PCR tube as well as 0.5 μl of bacteria suspension before the following PCR program was run: 95°C, 5 min; 30x (95°C, 30 s; 53°C, 30 s; 72°C, 120 s); 72°C, 10 min. The products were visualized using agarose gel electrophoresis, the gel was run at 70 V for two hours before being visualized by UV-light.

2.8.4 Antibiotic susceptibility (Etest assay)

Another important factor for a future probiotic supplement is the fact that it should not harbor any transferrable antibiotic resistance gene. According to the method used in previous experiments done on the antibiotic susceptibility of *Lactobacillus* species (Egervärn et al., 2006), a total of six different antibiotics were tested. The antibiotic susceptibility was analyzed using Etest assay and the effect of six different antibiotics were analyzed, ampicillin, gentamicin, streptomycin, tetracycline, erythromycin and clindamycin. The same isolated *L. reuteri* strains used in previous experiment were analyzed. In order to get a better result, the susceptibility of the strains was analyzed after both 24 and 48 hours. All of the tested antibiotics had the same concentrations and the scale on each strip went from 0.016-256 MIC (micrograms per milliliter).

Frozen cultures of *L. reuteri* were grown on MRS plates for 20 to 24 hours. Colonies from these cultures were suspended in saline solution (0.9% sodium chloride, wt/vol) to a density of McFarland standard 1 (bioMérieux). These suspensions were swabbed evenly on a 4-mm-thick plate made on a mixture of Iso-Sensitest agar (90%) and MRS agar (10%). (Klare et al., 2005) Using a cotton swab the inoculum was swabbed in three directions and dried for approximately 15 minutes before applying two antibiotic strips on each plate. These strips were applied in opposite directions to avoid a synergy effect between the two antibiotics. The plates were incubated anaerobically for 24 hours in 37°C before the first reading and then another 24 hours before a second reading.

2.8.5 Growth temperature

Growth temperatures were for most of the isolates determined by the cultivation on Rogosa/vancomycin agar at 45°C. The isolates that were picked from the Rogosa agar incubated at 37°C were nevertheless further analyzed by suspending the frozen isolates in MRS broth and incubating them at 45°C ON. This was done in order to determine if these isolates could grow at higher temperatures as well.

2.8.6 Protein X gene sequence determination

Recently the discovery of a cell surface protein (referred to as "protein x") unique for *L. reuteri* was made. Certain parts of the protein are similar for all *L. reuteri*; one part however differs in its sequence between different strains. With the help of this protein it is possible to make primers for this variable segment and by using PCR the sequence of this segment for each strain can be determined. This gives us the opportunity to separate strains of *L. reuteri* from different sources and determine how closely these strains resemble each other by comparing the sequences.

Six strains of the isolated *L. reuteri* were analyzed by using this new method for comparing *L. reuteri* strains (Roos and Jonsson, personal communication); three strains from wolf (V13-1, V4-2 & V7-2) and three from dog (H3-2, H23-2 & H14-1). These samples were chosen after the first rep-PCR and were thought to be different. The sequences from these strains were compared with *L. reuteri* from cat, rodent, dog, human, pig and chicken. A phylogenetic tree for strains of *L. reuteri* could then be made to illustrate the conformation of the sequences from different sources.

2.9 Immunomagnetic isolation of L. reuteri

In order to try to develop a new method of isolating *L. reuteri* from environmental samples, the method of coating magnetic beads with specific antibodies for *L. reuteri* 1063 was used. These antibodies had been raised against a mix of cell surface protein from *L. reuteri* 1063 and the hope was that this could bind all the *L. reuteri* strains present in a sample. This method had previous been successful in isolating other types of bacteria. (Islam et al., 1992 & Lamoureux et al., 1997)

2.9.1 Antibody coupling

The first step of the method was to bind the antibodies to the Dynabeads (M-270 Epoxy, Invitrogen). Dissolving the Dynabeads in C1-solution (Invitrogen) ensured an easier way of weighing the beads. An amount of 6 mg of Dynabeads in 100 µl C1 were mixed with another 900 µl of C1and put on a magnet and the supernatant was removed. Antibodies against *L. reuteri* (purified 2001-09-14) that had been freeze dried were mixed with water to a volume of 100 µl (0.2 mg antibodies ml^{-1}). The antibodies were added to the Dynabeads and the mix were centrifuged for 3 minutes at 18 000 x *g*. 200 µl of C1 were added to the mix and then added to the Dynabeads. This solution was mixed by pipetting before 300 µl of C2-solution (Invitrogen) were added. The new solution was incubated on a roller (20 rpm) at 37°C overnight (ON). The following day the solutions were put on a magnet and the supernatant was removed. The pellet was then washed with 800 µl HB Wash (Invitrogen) and the supernatant was removed once again. A similar step was done with 800 µl LB Wash (Invitrogen) as well as with SB Wash (Invitrogen). The final washing step was adding SB Wash and incubating for 15 minutes on a roller before removing the supernatant once more. After this, the pellet was suspended in SB solution and incubated at 4-8°C. The same method was used for coupling IgG to the Dynabeads, with the exception that 300 µg of IgG were used (50 mg/ml).

2.9.2 Binding of isolated bacteria

The first strains and species of bacteria that were analyzed using this method of immunomagnetic isolation were *L. reuteri* (1063) and *L. rhamnosus* (CCUG 18011). 10 µl of each bacteria suspension was mixed with 5 µl of the Dynabead-solution and 500 µl of PBStween before incubation on a roller at RT for 30 minutes. The solution was put on a magnet and the supernatant was removed. The pellet was washed three times with 500 µl of PBStween and was put on a roller for 5 minutes between each wash step. After this the pellet was considered free of contaminants and was suspended in 50 µl of PBStween. The presence of bacteria and aggregates was investigated by microscopy. After analyzing another four samples in the same manner, it was decided to extend the incubation time to both 2 hours and ON as well as changing the temperature from RT to 37°C, in order to find the optimal settings. The amount of bacteria suspension that was used was also increased to 30 µl. Comparing the results from the 2 hour incubation and ON incubation gave the insight that no significant difference could be seen between them, hence the 2 hour incubation was used for the following samples. A total of 13 strains of *L. reuteri* as well as one strain of three other lactobacilli, *L. rhamnosus*, *L. murinus* and *L. plantarum* were tested.

2.9.3 Binding of lactobacilli in environmental samples

In order to see if the immunomagnetic isolation could be used in environmental samples, the coated Dynabeads were added to diluted samples from both dog and wolf. The dog sample used for testing this method was the feces sample from Springer Spaniel spiked with *L. reuteri*. 1 ml of the spiked slurry was mixed with 10 µl of Dynabeads coated with antibodies against *L. reuteri* 1063. This mix was incubated for two hours at 37°C before making a series of tenfold dilutions (10^{-1} to 10^{-5}). The dilutions were plated onto Rogosa and Rogosa/vancomycin and incubated at 37°C for 48 hours and 48°C ON. After the incubation the colonies were counted and isolated for identification by 16S sequencing and characterization by rep-PCR and reuterin assay.

3. Results

3.1 Bacterial enumeration

The total count of bacteria that grew on the Rogosa or Rogosa/vancomycin agar varied between samples but seemed to be higher in dog samples than in wolf samples, at least for bacteria grown at 37°C on Rogosa agar. For the dog samples the CFU count ranged from 10^6 to almost 10^8 on Rogosa and from 0 to 10^6 on Rogosa/vancomycin agar (**Fig. 1** and **Appendix 4**). Samples from wolf contained lower bacterial counts than samples from dog and only ranged from 10^3 to almost 10^5 on Rogosa and 0 to 10^3 on Rogosa/vancomycin. Generally there were less CFU g^{-1} on Rogosa/vancomycin agar incubated at 45°C than on Rogosa agar incubated at 37°C, and in samples from Beagle III, V188/12 and V289/12 there were no bacteria at all present on the Rogosa/vancomycin agar. The bacteria isolated from these samples also differed in colony morphology on the Rogosa plates compared to the other samples. Normally big and slightly yellowish colonies could be seen on the agar, but on these plates, smaller and very white colonies were the only colony type seen on the agar. These colony types were also present on the Rogosa agar from the other samples but in lower quantities when big and yellow colonies dominated. In lower dilutions the big yellow colonies were fewer, these small and white colonies however increased in quantity.
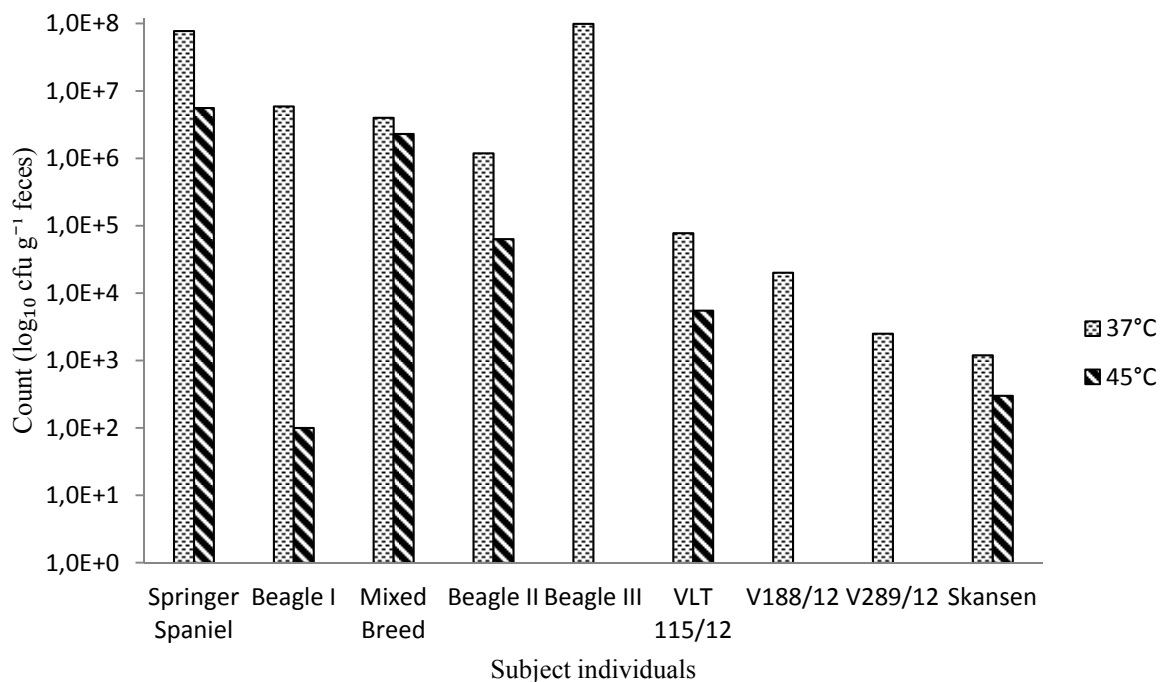
Fig. 1. Total counts for cultured fecal sample microbiota, on Rogosa (37°C) and Rogosa/vancomycin (45°C) agar. Single samples from each individual were analyzed on six plates with different concentration of bacteria (tenfold dilutions).

From the wild wolves, scrapings from the small intestine, the colon and the appendix were also taken to be analyzed. The scrapings from the colon contained more or less the same number of CFU g^{-1} as the colon content. Scrapings of the small intestine did nevertheless contain lower concentrations of bacteria than could be detected on the plates, and no bacteria did grow on Rogosa/vancomycin. Compared to the colon samples, the samples from the appendix from wolf contained lower concentrations of detectable bacteria that grew on Rogosa agar and there were no detectable bacteria on Rogosa/vancomycin agar (**Table 1**).

Table 1. CFU content of different samples from wolf, N/A = not available, since it was not analyzed. ND – not detected

Wolf	Colon (scraping) 45°C / 37°C	Colon (content) 45°C / 37°C	Small intestine (scraping) 45°C / 37°C	Appendix (content) 45°C / 37°C
VLT 115/12	9200 / $5.86 \cdot 10^4$	$5.5 \cdot 10^3$ / $7.78 \cdot 10^4$	ND / 3300	N/A
V188/12	ND / $1.6 \cdot 10^4$	ND / $2.01 \cdot 10^4$	N/A	ND / $2.4 \cdot 10^3$
V289/12	ND / 300	ND / $2.5 \cdot 10^3$	N/A	ND / 100

3.2 Bacterial identification by 16S rRNA gene sequencing

When determining which colonies that were supposed to be analyzed by 16S sequencing, the bacteria were first studied in a microscope to determine if they were rods or cocci. Colonies containing rods were picked to a MRS plate and grown ON. After reuterin assay and rep-PCR (to determine if the picked bacteria were the same or different types), one of each type were analyzed by 16S rRNA gene sequencing. A small number of colonies containing cocci were however analyzed by 16S sequencing since there exists *Lactobacillus* species that are in fact cocci, i.e. *L. equigenerosi*. (Endo, A. et al, 2008) Despite this fact the results showed that most cocci that had been analyzed by 16S sequencing were in fact *Streptococcus*. A small number of the isolated cocci were also *Pediococcus* who phylogenetically are lactobacilli.

There were a total of seven different species of *Lactobacillus* found in the samples from dog and wolf (**Table 2**). In dog there were only two species found in the feces, *L. reuteri* and *L. animalis*. The samples containing the highest number of *Lactobacillus* species came from the wolf referred to as VLT 115/12. From these samples a total of six different *Lactobacillus* species were cultivated. Sample cultivations from two of the wolves (V188/12 and V289/12) as well as the sample from one of the beagles (Beagle III) did not contain any lactobacilli. The samples that came from the wolf living in captivity (Skansen) contained the same *Lactobacillus* species that could be found in dog samples and one species that were not found in any other sample, neither from wolf nor dog.

Table 2. Different species of *Lactobacillus* found in the samples from dog and wolf. - = not present in any samples; + = present in at least one sample.

Bacteria	Dog					Wolf			
	Beagle I	Beagle II	Beagle III	Mixed breed	Springer Spaniel	VLT 115/12	Skansen	V188/12	V289/12
L. reuteri	-	+	-	+	+	+	+	-	-
L. animalis	+	+	-	+	+	+	+	-	-
L. johnsonii	-	-	-	-	-	+	-	-	-
L. murinus	-	-	-	-	-	+	-	-	-
L. gasseri	-	-	-	-	-	+	-	-	-
L. taiwanensis	-	-	-	-	-	+	-	-	-
L. plantarum	-	-	-	-	-	-	+	-	-

3.3 Characterization of *L. reuteri*

Different assays and methods were used to try to characterize the isolated *L. reuteri* found in dog and wolf. The first assay, reuterin assay, was in fact done before the bacteria had been identified. This in order to characterize the unknown bacteria a bit before choosing which bacteria was to be sent to Macrogen for 16S sequencing. The reuterin assay could give an indication that the bacteria might be *L. reuteri* since it is mostly these bacteria that produce reuterin. However, two more bacteria were discovered during the course of this study that were not *L. reuteri*, but nevertheless gave a positive result in the reuterin assay. These bacteria were later identified as *C. perfringens*. Since the reuterin assay done on the sample analyzed from the first dog (Springer Spaniel), were done on the same Rogosa agar as these bacteria were picked from, whether or not these specific bacteria were in fact reuterin positive could not be decided. When analyzing the rest of the samples the duplicate plates ensured information of which colony that was positive or negative. A very small number of bacteria were in fact positive in the reuterin assay and most of them were sent away for 16S sequencing.

In order to avoid duplicates of bacteria from being sent away for sequencing, rep-PCR genomic fingerprinting analysis were done. From the visualization on agarose gels, different types of bacteria could be determined. These results as well as the results from the reuterin assay results, made it possible to decide which bacteria to send away for sequencing. If two bacteria were in the same group of rep-PCR types, the reuterin assay could decide if both of them were to be sent away or just one of them. Colony morphology also had some impact on choosing bacteria. After identification of the bacteria by 16S sequencing, all the strains of *L. reuteri* were further characterized. From all the samples from both dog and wolf, a total of 20 strains of *L. reuteri* had been isolated. In order to minimize that number to allow an easier workload and to avoid duplicates, another round of rep-PCR analysis was done (**Table 3**). All of the 20 strains were analyzed on the same gel to allow them to be distinguished from each other easier. From this analysis the number of different strains was diminished to 13 (**Appendix 2**).

Table3. Characterization of *L. reuteri* found in dog (H) (1 = Springer Spaniel; 2 = Mixed breed; 3 = Beagle II) and wolf (V) (1 = VLT 115/12; 2 = Skansen), [a] = *L. animalis*, used as a negative. N/A = not available, since it was not analyzed. For reuterin, pduC and ureC assay, - = not present, + = present. GT = growth temperature, MBC = mucus binding capacity (- ≤ 5% plate covered; (+) = 10-25% covered; + ≈ 50 % covered; ++ ≈ 75% covered).

Culture	RAPD type	rep-PCR type	Reuterin	pduC	ureC	MBC	MBC (mucin)	GT	Sample
H5-1	1	1	N/A	-	-	N/A	N/A	45°C	Feces
H12-1	1	1	N/A	-	-	N/A	N/A	45°C	Feces
H14-1	1	3	N/A	-	-	N/A	N/A	45°C	Feces
H3-3	1	5	-	-	-	N/A	N/A	45°C	Feces
H5-3	1	10	+	+	-	N/A	N/A	45°C	Feces
H9-2	2	12	+	+	-	+	-	45°C	Feces
H6-2	3	1	-	-	-	-	-	45°C	Feces
H1-2[a]	4	11	-	-	-	N/A	N/A	45°C	Feces
H3-2	5	8	+	+	-	++	-	45°C	Feces
H8-1	6	4	N/A	-	-	N/A	N/A	45°C	Feces
H13-1	7	2	N/A	+	-	N/A	N/A	37°C	Feces
V3-1	1	7	-	-	-	(+)	-	45°C	Colon scraping
V1-1	2	9	-	-	+	N/A	N/A	45°C	Colon scraping
V7-2	2	6	-	-	+	N/A	N/A	45°C	Colon content

In order to try and minimize the number of *L. reuteri* strains even further, another genomic fingerprinting analysis called RAPD were performed. From this analysis six types were achieved,

however the morphology of some of the analyzed strains had been very different, this questions the methods potential for typing lactobacilli. These result were instead used to ensure that the typing from the rep-PCR were in fact correct.

Some *L. reuteri* strains, even though they do not produce reuterin, still might carry the gene for synthesizing it. In order to examine if this was true for some of the isolated bacteria and to see if the bacteria that had not been analyzed using the reuterin assay were positive, these 14 strains of bacteria (13 *L. reuteri* and 1 *L. animalis* used as a control) were analyzed using PCR and gel electrophoresis. According to the results, all of the strains that had been positive when analyzed by reuterin assay were indeed positive and those who had been negative were once again negative. The sample referred to as H13-1 that had not been tested using reuterin assay was also positive for this gene. Presence of the gene encoding urease was also tested. This enzyme catalyzes the hydrolysis of urea, leading to the production of ammonia (NH_3) and carbon dioxide (CO_2). (Burne & Chen, 2000) Strains of *L. reuteri* capable of producing this enzyme have almost exclusively been found in rodents but some urease producing strains have also been found in pig. (Walter et al., 2011) All of the 13 strains of bacteria were tested for this gene and only two of them were positive, sample V1-1 and V7-2.

Another way of characterizing *L. reuteri* strains is by analyzing their mucus-binding capacity (MBC), this is probably an important trait for future probiotics since the bacteria can more easily adhere to the GI mucosa if it has a high MBC. (Jonsson, H. et al, 2001) This analysis did work as expected but the results were hard to read and hence only a few strains of *L. reuteri* were studied. Observing the bacteria in the wells was very hard using the inverted microscope and the wells had to be dried before results could be seen. After the wells had been dried it was still hard to distinguish bacteria in the wells, so the presence of crystals was instead counted. This result may nevertheless not be entirely reliable and hence no more strains were tested for MBC. The ones that were tested differed in MBC and varied from no binding of bacteria to the mucus (H6-2) to covering almost three quarters of the well (H3-2). Introducing mucin to the bacteria while growing in MRS broth, did not increase the MBC of any strains instead it seem to have decreased the capacity.

3.4 Bile tolerance

One factor that is important when finding out if a specific strain of bacteria is suitable as a probiotic supplement is bile tolerance. 13 of the isolated *L. reuteri* strains that were considered different according to RAPD and rep-PCR analysis were also analyzed for bile tolerance. One strain of *L. animalis* that was found in dog and *C. perfringens* found in wolf were also analyzed to have something to compare the *L. reuteri* strains with. The results showed that the different strains of bacteria acted slightly different from each other when it came to survival rate at different bile concentrations (**Fig. 2**). Still most of them did not fall beneath 50% survival rate for any bile concentration. There were a few exception though, sample H5-3 and the sample with *L. animalis* had 0% survival rate even at 0.5% bile content. *C. perfringens* on the other hand was not affected at all by bile at these concentrations. At what level of bile content the strains reacted the most also seemed to differ a lot between strains, H13-1 was not affected by bile concentration down to 2.5% but at 5% the survival rate suddenly dropped to beneath 50%. Other strains e.g. H6-2 however was affected immediately and the survival rate dropped to 50% at a bile concentration of 0.5 %. After this it increased slightly to 70% before dropping to 50% again at 5% bile content, similar results was seen for isolate V7-2.
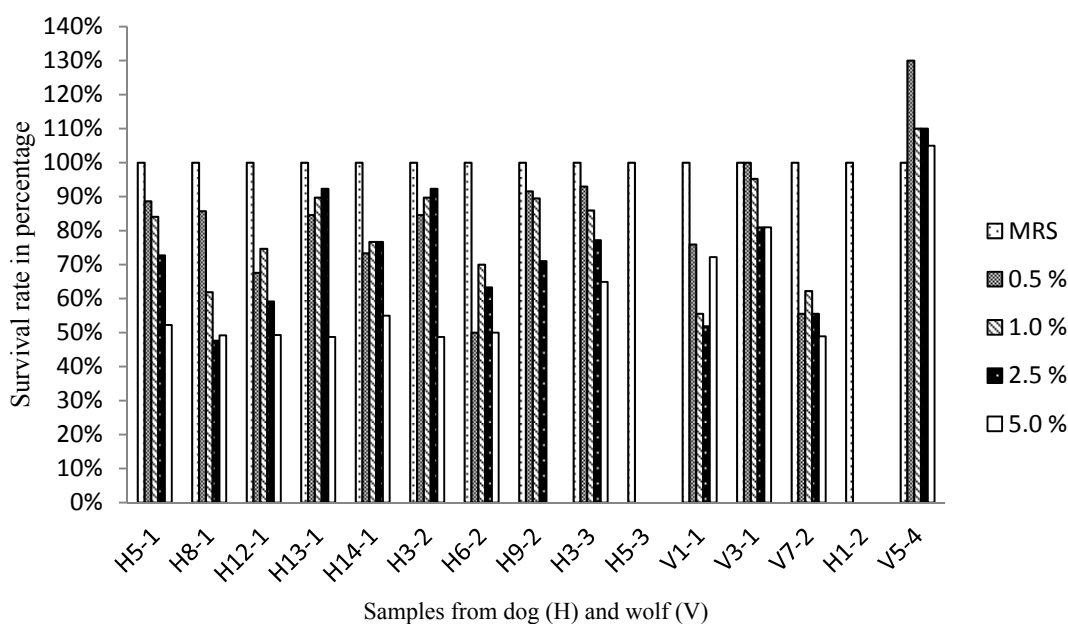
Fig. 2. *L. reuteri* strains tested for bile tolerance at five different concentrations, 0 %; 0.5%; 1%; 2.5% & 5%. H1-2 (*L. animalis*) and V5-4 (*C. perfringens*) were used to compare the *L. reuteri* with other species. Where no data can be seen, no bacteria had grown except for strain H9-2, were the lack of data from 5 % were due to difficulties in determining single CFU.

Most of the strains also had produced zones around their colonies that were very distinct at a concentration of 0.5% bile and became less clear with every increase in bile concentration. The only isolates that had not produced these zones were V7-2 and H3-3. H9-2 produced very obscure zones at 0.5% bile and no zones could be seen at higher concentrations.

3.5 Antibiotic susceptibility

The results showed that the strains of *L. reuteri* isolated from dog and wolf grouped together for most antibiotics but was a bit scattered for tetracycline (**Table 4**). Some of these results, especially after 48 hours, can be explained by difficulties in determining were the line between the full growth and no growth went. These lines were for half of the strains very fuzzy.

Table 4. Distribution of MICs of six antibiotics for *L. reuteri* isolated from dog and wolf as determined by Etest. Reason for one less strain tested for tetracycline was due to difficulties in determining inhibition zone.

Antibiotic	Time[b]	Distribution of MICs (µg/ml)										n[a]
		≤0.12	0.25	0.5	1	2	4	8	16	32	64	≥128	13
Ampicillin	24	1	8	4									13
	48	1	8	4									13
Clindamycin	24	9	4										13
	48	9	4										13
Erythromycin	24		2	11									13
	48			2	3	5	3						13
Gentamycin	24					1	11	1					13
	48					1	10	2					13
Streptomycin	24									5	8		13
	48									5	8		13
Tetracycline	24				1	2	3	2	4	1			13
	48						4	2	1	4	1		12

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According to the achieved results, some of the strains were more resistant to tetracycline than others, and the MICs varied between 1-64 µg/ml. For ampicillin and clindamycin the strains had MICs lower than 1 µg/ml. The MICs for erythromycin were also lower than 1 µg/ml after 24 hours but increased a bit after 48 hours and reached 0.5-4 µg/ml. Gentamycin susceptibility were very even between the strains and only varied from 2-6 µg/ml with only a slight increase for some strains after 48 hours. The strains were nevertheless resistant to relatively high doses of Streptomycin with results from 24-64 µg/ml. For specific MICs for all *L. reuteri* strain tested, see **Appendix 3**.

3.6 Protein X gene sequencing

The results from the protein x gene sequencing can be seen in the phylogenetic tree below (**Figure 3**).According to the results three of the strains (V4-2, V13-1 & H23-2) were 100% identical. Strain H14-1 was grouped together with LPA-1, a strain from pig. V7-2 was instead grouped together with R2LC and close to 420 and 2010, all of which were strains of *L- reuteri* from rodents. H3-2 on the other hand was not closely grouped together with any strains.
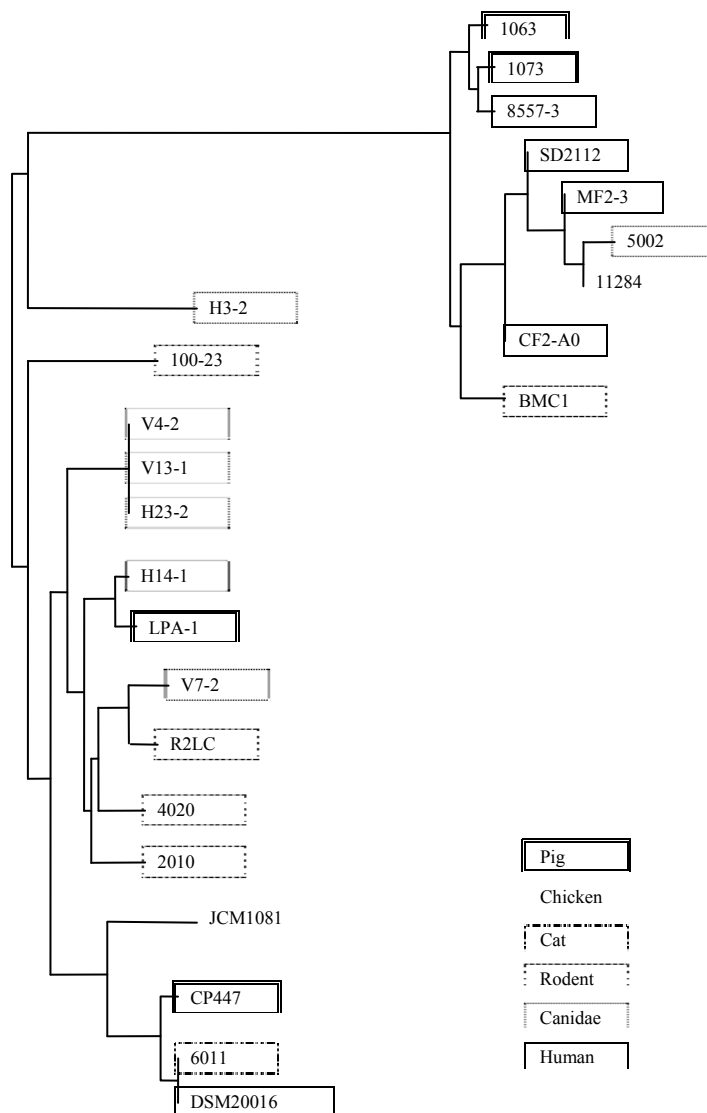
Fig. 3. Phylogenic tree for strains of *L. reuteri* from different sources based on the results from the sequencing of protein X.

3.7 Immunomagnetic isolation of L. reuteri

The binding capacity to both IgG and the specific antibodies differed much between the strains of *L. reuteri* i.e. strain 1013 bound very well to both IgG and the antibodies and formed big aggregates. Other strains like HW8 and 5010 bound neither to IgG nor the specific antibodies. The strain called 6010 did however only bind to the antibodies and not to IgG. Whether or not they aggregated differed a lot to between strains and variation between IgG and the antibodies could be seen here as well. Some strains formed aggregates only with the antibodies or the aggregates were bigger with the antibodies; others formed bigger aggregates with IgG. Of the other species of lactobacilli that were tested none bound to either IgG or the antibodies.

Table 5. Binding capacity of *Lactobacillus* strains to Dynabeads coated with antibodies (AB) and IgG. [a] = 6 µl bact. susp. µl^{-1} Dynabead solution, incubated for 2 hours at 37°C. [b] = 2 µl bact. susp. µl^{-1} Dynabead solution, incubated for 30 min at RT. - = no or very low binding capacity; (+) = low binding capacity; + = moderate binding capacity; ++ = high binding capacity. Size of aggregates are graded from 0-5; 0 = no aggregates and 5 = very big aggregates.

Bacteria	Strain	Binding capacity (AB)	Aggregates	Binding capacity (IgG)	Aggregates
L. reuteri	R2LC (rat)[a]	++	5	+	2
	2010 (rat)[a]	(+)	0	(+)	0
	6799jml (mouse)[a]	++	5	++	4
	LB54 (chicken)[a]	++	2	++	0
	HW8 (chicken)[a]	-	0	-	0
	5010 (dog)[a]	-	0	-	0
	5002 PTA 6127 (dog)[a]	++	4	++	4
	CP395 (pig)[a]	(+)	0	(+)	0
	1013 (pig)[a]	++	1	++	3
	1063 (pig)[b]	++	4	++	4
	6010 (cat)[a]	+	1	-	0
	DSM 17938 (human)[a]	(+)	0	(+)	0
	ATCC PTA 4659 (human)[a]	++	4	++	4
L. rhamnosus	CCUG 18011[b]	-	0	-	0
L. murinus	CCUG 33904[b]	-	0	-	0
L. plantarum	LP299V[a]	-	0	-	0

After testing the method on suspensions with single strains of lactobacilli, the method was tried on fecal samples, one dog sample (Springer Spaniel) and one wolf sample (VLT 115/12). This method was compared with samples that had been directly plated out on the agar (**Table 6**). The result indicates that the quota between the amount of bacteria grown on Rogosa and Rogosa/vancomycin agar was higher with the Dynabead method than the commonly used method in examined samples from dog. The quota for the Dynabead method were 0.38 $(1.3 \cdot 10^5 / 3.4 \cdot 10^5 = 0.38)$ and for the directly plated method 0.07 $(5.6 \cdot 10^6 / 7.76 \cdot 10^7 = 0.07)$ The bacteria in the samples from wolf on the other hand did not bind at all to the Dynabeads.

Table 6. Bacterial enumerations of samples from dog and wolf pretreated with Dynabeads or using normal plating methods

Sample	CFU count (37°C), Dynabeads	CFU count (45°C), Dynabeads	CFU count (37°C), directly plated	CFU count (45°C), directly plated
Springer Spaniel	$3.4 \cdot 10^5$	$1.3 \cdot 10^5$	$7.76 \cdot 10^7$	$5.6 \cdot 10^6$
VLT 115/12 (colon scraping)	0	0	$5.86 \cdot 10^4$	$9.2 \cdot 10^3$

4. Discussion

4.1 Contents of lactobacilli

4.1.1 Bacterial enumeration

The number of bacteria that grew on the Rogosa and Rogosa/vancomycin were considerably higher in the samples from dog than from wolf. Even though the presence of different colony types was noted, unfortunately the ratio of these was not. This makes it impossible to determine the count of lactobacilli in most of the samples. In some of the samples this ratio was nevertheless noted, when the number of colonies that grew on the agar was fairly low or if the presence of only one colony type could be seen. The samples from Springer Spaniel, VLT 115/12 and Wolf (Skansen) grown at 45°C and samples from V188/12 and V289/12 were examples of were such ratios had been noted. In these samples grown at 45°C exclusively bigger colonies grew which indicates the presence of only lactobacilli in these samples. The samples from V188/12 and V289/12 had no growth at 45°C and at 37°C only smaller white colonies grew which indicated that only streptococci and/or pediococci grew there. The presence of a few *Clostridium* species was also isolated from these samples, but these colonies were scarce and hard to find on the Rogosa agar. In the feces samples from wolf (Skansen) the majority (90%) of the colonies were bigger more yellowish ones. This also suggests that the majority of the bacteria present in these samples were lactobacilli. Pediococci were however only isolated from samples derived from the wolves living in captivity (Skansen).

Even if it is uncertain what the LAB count were in some samples, the conclusion that the samples from dog contained more LAB than the samples from wolf can still be made. The difference in concentrations of LAB in the dog and wolf samples was big enough to see a clear difference. This since the samples from dog contained 10-1000 times more lactobacilli than the sample from wolf containing the most bacteria. Even if the samples from dog only contained ten percent of LAB it would still be enough to contain higher concentrations than the samples from wolf. If we accept the premise that the samples from dog contain more lactobacilli than the samples from wolf, a few reasons for this can be discussed. One reason for this could be that the dog has a diet containing more carbohydrates than the wolf has which results in higher lactobacilli count. Both fructooligosaccharides and mannan-oligosaccharides have been shown to increase the lactobacilli count in the gut of dogs (Swanson et al., 2002 & Middelbos et al., 2007). Fructooligosaccharides can be found in cereals which are a major ingredient in dog food and some manufacturers also add extra fructooligosaccharides in their dog food. Mannan-oligosaccharides are also added to certain dog foods.

It could also be due to that the samples from the wolves were taken during the winter season when the wolves generally eat less and not as diverse as during the summer. The diet during the winter contains a lot of meat and studies have shown that a diet rich in protein could reduce the number of lactobacillus, at least in rats (Klainer et al., 1967). During the summer season they eat more berries and plants which could contribute to a higher concentration of lactobacilli. The lack of

lactobacilli in the samples from wolf V188/12 and V289/12 could be because of food deprivation, since the intestines did not contain much material. Starvation has been shown to decrease the concentration of lactobacilli, at least in rats (Deitch et al., 1987). The samples from two of the wild wolves examined in this study did not contain any detectable lactobacilli, this fact could strengthen the hypothesis that the wolf has lower concentrations of LAB in their intestinal tract than the dog.

4.1.2 Sample condition and preparation

Another factor for the varying concentrations of lactobacilli in some of the samples could have been the condition of the samples itself. The feces samples from Beagle I and III for example were a lot drier than the samples from the other dogs. Handling of the samples could have affected the bacterial counts in them, such as a freezing and thawing several times or keeping the samples at unfavorable temperatures. The feces samples from the wolves living in captivity (Skansen) were also quite dry and since the samples were not gathered shortly after defecation there is an uncertainty for how long they had been exposed to pending temperatures that could have affected the lactobacilli count negatively.

4.2 Bacterial identification

By comparing the samples from the wild wolf that did contain lactobacilli with the samples from the dogs, it could be suggested that the wolf may harbor a larger number of *Lactobacillus* species in their guts than dogs do. In one of the wolves (VLT 115/12) a total of six different species of *Lactobacillus* were found and in the Skansen wolf a total of three species were found. In dogs only two different species were found, *L. reuteri* and *L. animalis*. The concentration of each species might nevertheless be lower than in dogs. One fact that definitely could be stated is that *L. reuteri* is present in dogs as well as in wolves, both wild and wolves bred in captivity. According to results in this study it seems that *L. reuteri* is the dominating *Lactobacillus* species in both dog feces and the large intestine of wolf. This is in accordance with the result from the study made by McCoy and Gilliland, who found lactobacilli in 4 out of 20 dogs where *L. reuteri* contributed to 18 out of 22 isolated lactobacilli. Also Mentula et al. only found lactobacilli in few feces samples from dog (7 out of 22 individuals), but the species of lactobacilli were not however investigated. In Labrador dogs the dominating lactobacilli were *L. ruminus* and *L. murinus*. *L. animalis* and *L. reuteri* were also found but at low concentrations and only in two out of four dogs. These two dominating species in the gut of Labrador dogs were not found in any of the samples in this study, *L. animalis* were found in all but one dog and in both of the wolves containing lactobacilli. In contrast to the other studies done on bacterial diversity in dogs, the presence of lactobacilli in feces from dogs in this study was relatively high. Lactobacilli were found in four out of five examined dogs while the presence in other studies has varied from only four out of 20 to two out of four dogs. (Greetham et al., 2002 & Mentula et al., 2005)

4.3 Characterization of L. reuteri

4.3.1 Reuterin assay

Out of the 32 isolated bacteria identified as *L. reuteri* only six were positive according to the reuterin assay. Most of them were isolated from dog with the exception of one that was isolated from the Skansen wolf. This number could have been slightly higher if the isolates from the first dog had been analyzed individually. These were instead analyzed after colonies already had been picked to a new agar plate. Nevertheless it appears as if this number is slightly lower than results produced by for example McCoy and Gilliland, where five out of ten analyzed strains of *L. reuteri* were positive for reuterin. The presence of reuterin producing strains of *L. reuteri* in wolf is however an unexplored area then again so is the presence of *L. reuteri* in wolf. Even if *L. reuteri* were found in both wild wolves and wolves living in captivity, reuterin producing *L. reuteri* were

only found in the wolf living in captivity. More samples from wolves are still needed before such claims can be made.

4.3.2 Detection of *pduC* and *ureC*

Even though some strains of *L. reuteri* do not produce reuterin they might carry the gene for it. In order to analyze if this was the case for the reuterin negative strains in this study, detection of *pduC*, one of the genes encoding the enzyme involved in reuterin production, was performed. None of the previously reuterin negative strains were positive for the *pduC* gene. However, one strain that had not been analyzed with the reuterin assay was proven to be positive for the gene. It is safe to say that all strains that have the gene also expressed it in this study. In order to figure out if some of these reuterin negative strains might have originated from the diet, detection of the gene coding for urease production (*ureC*) were also performed. Urease positive strains of *L. reuteri* have mainly been found in rodents and occasionally in pigs. The reason for strains of *L. reuteri* being capable of producing urease is to protect them from the acid environment in the stomach. The stomach in rodents contains an area without any mucus (squamous epithelium) where bacteria can attach. In order to survive there they need to produce ammonia (NH_3). The ammonia shields them from the acid environment and allows them to grow. Since the stomach of e.g. humans, dogs and birds does not contain this mucus lacking area, it is possible that these lactobacilli are not present here. The strains of *L. reuteri* that are urease positive are generally reuterin negative and hence a presence of strains which are urease positive and reuterin negative in wolf and dog samples could indicate that they have received these strains by eating rodents. Two strains of the isolated *L. reuteri* that were reuterin negative (V7-2 & V1-1) were in fact urease positive. Both of these strains came from the wolf referred to as VLT 115/12. This could mean that this wolf have eaten rodents, which is not a long shot since studies of the feeding habits of wolves in Scandinavia has suggested that this occurs. (Müller, 2006) This could be true since this strain seems closely related to strains from rodents according to the phylogenetic tree from the protein x-typing.

4.3.3 RAPD & rep-PCR

According to the result from the first genomic fingerprinting method, rep-PCR, the 32 isolated *L. reuteri* strains could be decreased to 19 different types. A second rep-PCR analysis with all of the 19 different types on the same agarose gel further decreased this number to 11 different types. Some of the results from this analysis were however hard to read so a different genomic fingerprinting method (RAPD) was used. This method gave the results that only six different types existed. In order not to exclude strains that were in fact different despite the RAPD results, a total of 13 different strains were chosen to be further characterized. A few doubles from the rep-PCR analysis were also analyzed further to evaluate this method as well.

4.3.4 Mucus binding capacity

Analyzing mucus-binding capacity of bacteria is a method of characterizing different strains of *L. reuteri*. This method did not work out as planned and the results given were vague and the mucus binding capacity hard to determine. A total of six strains were tested for their capacity and four of these were used in further characterization. The result for these four strains varied quite a lot from binding very well (H3-2) to almost no binding at all to the mucus (H6-2). Incubating the strains with added mucin lowered the binding capacity of all of the strains to almost no binding. Because of the poor results and lack of time no further analysis of mucus binding capacity was performed.

4.3.5 Bile tolerance

Bile tolerance is another important factor for a potential probiotic strain that also was used for characterizing of strains of *L. reuteri*. The 13 strains chosen after rep-PCR and RAPD were analyzed for their survival rate at different bile concentrations. All of the strains survived the

highest concentration of bile except for one strain (H5-3), which did not even grow at the lowest concentration. Certain trends could be seen among the strains, some of them had a relatively high survival rate (70-90%) down to 2.5 % bile and at 5% bile there was a sudden drop down to around 50% survival rate. Other strains dropped quickly in survival rate at the lowest bile concentration but also ended up at approximately 50% survival rate at 5% bile. The two strains that seem to have been the least affected were isolate H3-3 and V3-1, who ended up at 65 and 81 % survival rate at 5% bile. There were also two strains that were almost identical in their bile tolerance profile, H13-1 and H3-2 with the same survival rate for every bile concentration except for at 5% where they only differed 1%. Two other strains (H3-3 and H9-2) were also very similar in survival rate and also shared another attribute, both of did not produce any zones around their colonies.

The *L. reuteri* strain with the most potential for use as probiotic according to these result would without a doubt be V3-1, a strain that was almost unaffected by bile down to 2.5% concentration. At 2.5 and 5 % the survival rate was as high as 81% which was high compared to the other strains. strain H13-1 and H3-2 had a higher survival rate at 2.5% bile but they dropped to just below 50% survival rate at 5% bile, why the overall survival rate were the best in strain V3-1.

4.3.6 Antibiotic susceptibility

Antibiotic susceptibility is very important feature when developing new probiotics, mainly because of the fear the resistant strains of lactobacilli might transfer their resistance to other bacteria (Nousiainen et al., 2004), worst case scenario to a pathogenic bacteria. Antibiotic susceptibility might also be used for characterizing lactobacilli by testing their resistance to different antibiotics. By comparing the results from this study to other studies (Egervärn et al., 2007) suggests that the results are fairly reliable. Most of the tested strains fell into the same range of MICs as the strains of *L. reuteri* that Egervärn et al analyzed. There were a few exceptions though, for tetracycline the strains from this study ranged from 1-64 MIC, while the strains studied by Egervärn et al ranged from 4-256 MIC. The results for erythromycin and gentamycin also differed slightly between the different studies, by gaining slightly higher (one extra MIC-value) results for both antibiotics in this study. For ampicillin, clindamycin and streptomycin though the results from both studies coincide relatively well. Resistance against an antibiotic (MICs ≥8) was only seen in samples tested for streptomycin and tetracycline. The strains were near the limit for resistance against gentamycin with the highest score being 6 MICs. The MIC score for the rest of the antibiotics were relatively low. Some of the strains varied quite a lot from other strains and where the biggest range of MICs could be seen was in the susceptibility against erythromycin and tetracycline. The reason for some of the results being fuzzy can be because of uneven swabbing of the plates or that the placement of the antibiotic strip was not correct.

When it comes to the individual results of the different strains it seems as if H13-1 were the least resistant strain with the lowest scores for all antibiotics except for tetracycline. Still H13-1 had a low MIC score for that antibiotic as well. V1-1 was also among the least resistant strains with lowest scores for streptomycin, tetracycline and clindamycin and low scores for erythromycin and gentamycin. The strain with the most resistance against the antibiotics was H8-1 with the highest scores for four out of the six antibiotics. For the remaining two antibiotics it had moderately high score compared to the other strains. Sample V3-1 also had among the highest scores for most of the antibiotics.

4.3.7 Protein X gene sequencing

According to the results isolate V4-2, V13-1 and H23-2 were 100% identical. These strains still differed slightly using the genomic fingerprinting method rep-PCR, where V4-2 had a clearly visible segment at 2500 bp while V13-1 and H23-2 had this segment at 2750 bp. Since these strains were not run on the same agarose gel when analyzing types with rep-PCR it cannot be

completely ruled out that these isolates indeed are the same strain of *L. reuteri*. Instead of using V13-1 and V4-2 for the following characterization steps, isolate V3-1 was chosen since this was considered as a purer isolate with more DNA present in the bacteria suspension. The other three strains (H14-1, H3-2 and V7-2) that were considered different using this method had already been considered different since they belonged to different rep-PCR and RAPD groups. V7-2 had been grouped together with strains from rodents, the fact that V7-2 might be derived from rodents had already been suspected since the isolate had been positive for urease and negative for reuterin. H3-2 was the isolate with the most differentiated sequence compared to the other isolates. It was also the only isolate that had been positive for reuterin.

4.4 Immunomagnetic isolation of L. reuteri

One of the goals of this study was to try to test a new method of immunomagnetic isolation for detecting *L. reuteri* in samples. By coating the Dynabeads with antibodies for a mixture of surface proteins of *L. reuteri*, the hope was to be able to bind strains of *L. reuteri* and no other lactobacilli. Even though the beads did not bind to any of the other species of Lactobacillus tested, the method was not successful. While some of the strains of *L. reuteri* bound very well to the Dynabeads and formed big aggregates others did not attach to the antibodies at all. Some of the strains formed small aggregates but this might not have been due to that the bacteria bound to multiple beads but rather that the beads somehow got stuck together. This phenomenon could be seen in samples that did not contain any bacteria or antibodies. Using this method on environmental samples was not a success either, even though it worked fairly well with the sample from the Springer Spaniel where the Dynabeads bound a relatively high number of bacteria that most likely would be lactobacilli since they survived both 45°C and presence of vancomycin. The ratio between the Rogosa agar and the Rogosa/vancomycin were for the Dynabead method a lot higher (0.38) than for the method of directly plating the samples (0.07). This suggests that the beads were more effective in excluding other genus of bacteria than the selective media were. The bacteria that the Dynabeads appears to have bound were also solely *L. reuteri* as seen in the experiment with already isolated bacteria. After the success with the feces sample from the Springer Spaniel, samples from a wolf (VLT 115/12) were also tested using this method. These results were however a letdown since no bacteria at all bound to the Dynabeads. Isolation of bacteria using normal plating techniques seems on the other hand to have isolated a number of different strains of *L. reuteri*. This method could have great potential for the isolation of *L. reuteri*, a different antibody might be needed though. This antibody would have to be able to bind to a protein present in all the different strains of *L. reuteri*.

4.5 Characterization of L. reuteri

According to the genomic fingerprinting method rep-PCR there were at least 11 different types of *L. reuteri* isolated from the dogs and wolves. The RAPD method on the other hand gave the results that there only were six different types. If one were to characterize the isolates by looking at the results from the bile tolerance test there would be at least 9 different types. The antibiotic susceptibility results made it slightly harder to divide the isolates into different groups since only three isolates had identical values, similar profiles could however be seen for other isolates. According to these results at least 9 different types existed. In an attempt to try and use these results as well as the results from the pduC and ureC detection to make a conclusion it appears that the only isolates that resemble each other are H5-1 and H12-1. These two were of the same RAPD and rep-PCR type, were urease and reuterin negative and got similar results in the antibiotic susceptible test except for erythromycin. The only thing that differed significantly between the two isolates was the bile tolerance. H8-1 was also similar to these two isolates but got a different result on the rep-PCR gel and different bile tolerance results. V7-2 and V1-1 were urease positive, were typed together in the RAPD test and had very similar results from the antibiotic susceptibility test. On the other hand they got very different results on the rep-PCR gel and different results in the bile tolerance test, V7-2 also did not produce any zones around its

colonies in the bile test. Taking all of the results into consideration would give the result that there could be 10-12 different strains among these 13 isolates. One thing is sure though, the genomic fingerprinting method RAPD is not the best method for dividing isolated strains of *L. reuteri* since it group together strains that are very different e.g. strains that are reuterin positive with reuterin negative as well as urease positive. It also group together strains that are bile resistant with strains that do not tolerate very low concentration of bile. With this in consideration it would be wiser to divide the isolates into different types using rep-PCR.

4.6 Potential as probiotic

Out of the total 31 isolated *L. reuteri* from dog and wolf, there were four strains that expressed the best potential for being a future probiotic strain for dogs. How big that potential is compared to other probiotics is still to be determined since only a handful of attributes have been discussed and analyzed in this study. Four more or less important attributes were still tested; mucus binding capacity, bile tolerance, production of reuterin and antibiotic susceptibility. The strain, isolated from wolf, that showed the greatest potential as a probiotic strain were isolate V3-1. This strain was the most bile tolerant among all of the analyzed strains of *L. reuteri*. It also had a relatively low resistance to the different antibiotics except for streptomycin and tetracycline. Unfortunately it had a low mucus binding capacity. From the dog samples there were three strains that showed very good potential as probiotics. Isolate H13-1 were not tested for mucus binding capacity but were reuterin positive and were among the most bile tolerant strains. It was also one of the strains with lowest MIC scores in the antibiotic susceptibility test and was only resistant to streptomycin. Another isolate (H3-2) had identical results as H13-1 in the bile tolerance test and were also reuterin positive. This strain was however slightly less susceptible to the antibiotics and was resistant to streptomycin as well as tetracycline. In the mucus binding capacity test it scored the highest though and were almost as capable as the positive control in binding to the mucus. The last isolate that had potential as a probiotic were H9-2. This strain had good mucus binding capacity, was reuterin positive and was only resistant to streptomycin. It was quite tolerant to bile down to a concentration of 2.5% bile, the results at 5% bile were unfortunately too hard to read so no results at this concentration could be achieved.

Among these four strains of *L- reuteri* the last three from dog (H3-2, H13-1 and H9-2) would be the top candidates from this study for further analyses of their probiotic potential. Such further analyses could be antagonist action toward different pathogens, stability during storage and production. (McCoy & Gilliland, 2007)

5. Conclusion

The lactobacilli contents in the large intestine of dogs were higher than in wolves according to the results in this study nevertheless in half of the samples from wolf lactobacilli could not be isolated. Some wolves tend to have periods of starvation during the wintertime and that might explain the lower contents of lactobacilli in the gut of these individuals, the lack of material in the large intestine of these wolves also points to this conclusion. The differences between the dogs, where samples from one dog did not contain any lactobacilli are thought to be a result of inadequate handling of the feces sample. Differences could also be seen in the bacterial diversity in the gut of wolf and dog. The wolf appears to have a greater number of different *Lactobacillus* species when fed properly with three times as many species. The number of different species in the wolf in captivity on the other hand seems to be similar to the dog which may be caused by a less diverse diet compared to the wild wolf. According to the results there are a few similarities between wolf and dog as well, the gut of both species appear to contain *L. reuteri* and *L. animalis*. Some of the strains of *L. reuteri* also appear to be the same in wolf and dog, while other strains seem to be present only in one e.g. the lack of reuterin producing *L. reuteri* in wild wolves.

Some of the isolated strains of *L. reuteri* were also analyzed for their potential as a future probiotic strain for dogs. Among the total of 13 analyzed strains, three strains appear to have the best potential according to the methods used. These strains (H3-2, H13-1 and H9-2) all produced reuterin, had good tolerance to bile and were among the isolates with the lowest resistance to the six tested antibiotics. H3-2 and H9-2 both bound well to porcine mucus, with H3-2 being slightly better at binding (H13-1 were not tested for mucus binding capacity). More methods for testing the probiotic potential of these strains is however needed to evaluate them further.

In addition to further analyses of the probiotic potential of the different strains of *L. reuteri* the bacterial content and diversity should be studied further. It would be interesting and perhaps necessary to examine the microbiota in wolves during the whole year the get a better picture of the difference between domesticated dogs and wild wolves. This since some wolves has a very restricted diet during the winter and even has periods of starvation. Fresh feces samples from the analyzed dogs would also be preferred to avoid the bacterial contents being affected by environmental factors such as fluctuating temperatures.

6. Acknowledgments

7. References

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Appendix 1

Recipes for substrates

MRS agar
- 6.2 g of deMan-Rogosa-Sharpe (MRS) agar (OXOID?)
- 100 ml Water

Mix until fully solved then autoclave at 125°C.

MRS agar with bile (0.5; 1; 2.5; & 5 %)
- 6.2 g of MRS agar
- 100 ml of water
- 0.5; 1; 2.5; 5 g of porcine bile (Sigma B-8631)

Mix until fully solved then autoclave at 125°C.

Rogosa agar & Rogosa agar with vancomycin
- 7.2 g of Rogosa Agar (Merck)
- 100 ml of water
- 0.13 ml Acetic acid
- With vancomycin:

Mix until fully solved then boil in microwave until completely clear. Vancomycin is added just before pouring the agar unto plates.

MRS broth
- 5.9 g of MRS broth (OXOID)
- 100 ml of water

Mix until fully solved then autoclave at 125°C in individual tubes with 9 ml of MRS broth in each.

IST/MRS agar
- 2.83 g of Iso-Sensitest (IST) agar (OXOID)
- 0.62 g MRS agar
- 100 ml of water

Mix until fully solved and correct pH to 6.7, then autoclave at 125°C.

Freezing medium
- 0.082 g K_2HPO_4
- 0.012 g KH_2PO_4
- 0.059 g Na-citrate
- 0.025 g $MgSO_4$ x 7 H_2O
- 17.2 ml glycerol (87%)
- Water to 100 ml

The solution were mixed until fully solved and filtrated through a sterile filter (Sarstedt)

Appendix 2

Results from the genomic fingerprinting methods of RAPD and rep-PCR
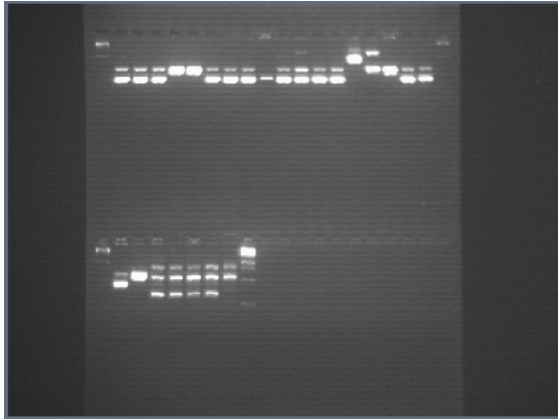

Figur 3. Results from RAPD of *L. reuteri* visualized by gel electrophoresis
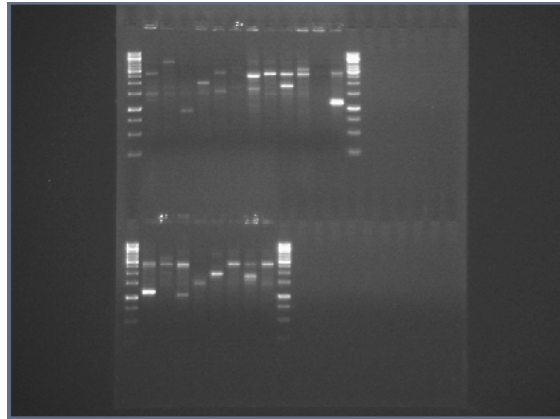
Figur 5. Results from rep-PCR of *L. reuteri* visualized by gel electrophoresis

Table 7. Showing placement of the isolates on the agarose gel, nr. 1 being the first sample at the top from the left. H1-2 = *L. animalis*; V1-4 = *C.baratii*; V2-4, V3-4, V4-4 & V5-4 = *C. perfringens*.

RAPD number	Isolate	rep-PCR number	Isolate
1	H7-1	1	H5-1
2	H5-2	2	H13-1
3	V3-1	3	H14-1
4	V1-1	4	H8-1
5	V1-2	5	H6-2
6	H3-3	6	H9-3
7	H6-3	7	V7-2
8	H5-1	8	H7-1
9	H6-2	9	H3-2
10	H8-1	10	H3-3
11	H13-1	11	V1-3
12	H14-1	12	V1-1
13	H12-1	13	V1-2
14	H1-2	14	H6-3
15	H3-2	15	H5-3
16	H9-2	16	H12-1
17	H5-3	17	H1-2
18	H9-3	18	V3-1
19	V1-3	19	H9-2
20	V7-2	20	H5-2
21	V1-4		
22	V2-4		
23	V3-4		
24	V4-4		
25	V4-5		

Appendix 3

Table 8. Antibiotic susceptibility of *L. reuteri* strains in MIC after 24 hour incubation (48 hour incubation), x = the inhibition zone around the strip were too vague to be read correct

Antibiotic	Culture												
	H5-1	H8-1	H12-1	H13-1	H14-1	H3-2	H6-2	H9-2	H3-3	H5-3	V1-1	V3-1	V7-2
Ampicillin	0.38 (0.38)	0.38 (0.38)	0.25 (0.25)	0.125 (0.125)	0.25 (0.25)	0.25 (0.25)	0.25 (0.25)	0.25 (0.25)	0.25 (0.25)	0.38 (0.38)	0.25 (0.25)	0.38 (0.38)	0.25 (0.25)
Streptomycin	48 (48)	64 (64)	48 (48)	24 (24)	32 (32)	48 (48)	64 (64)	24 (24)	48 (48)	48 (48)	24 (24)	64 (64)	24 (24)
Erythromycin	0.5 (3)	0.5 (1)	0.5 (0.75)	0.25 (0.38)	0.5 (3)	0.5 (1.5)	0.5 (2)	0.25 (0.5)	0.5 (2)	0.5 (2)	0.38 (0.75)	0.5 (3)	0.5 (1.5)
Tetracycline	24 (32)	16 (64)	16 (32)	3 (4)	16 (x)	4 (8)	6 (24)	2 (4)	12 (32)	4 (8)	1.5 (3)	8 (16)	1 (4)
Clindamycin	0.19 (0.19)	0.19 (0.19)	0.125 (0.125)	0.047 (0.047)	0.094 (0.094)	0.19 (0.25)	0.125 (0.125)	0.047 (0.064)	0.064 (0.064)	0.064 (0.125)	0.047 (0.047)	0.19 (0.19)	0.064 (0.094)
Gentamycin	3 (6)	6 (6)	3 (3)	2 (2)	3 (3)	3 (3)	4 (4)	3 (4)	4 (4)	3 (4)	3 (3)	4 (4)	3 (3)

Table 9. Bile tolerance of *L. reuteri* strains, CFU [survival rate], [a] = third tenfold dilution, [b] = fourth tenfold dilution, [c] = *L. animalis*, [d] = *C. perfringens*

Bile content	Culture														
	H5-1	H8-1	H12-1	H13-1	H14-1	H3-2	H6-2	H9-2	H3-3	H5-3	V1-1	V3-1	V7-2	H1-2[c]	V5-4[d]
0%	44[a]	63	71	18	60	39[b]	30[a]	95	57	57	54[a]	21	45	84	20
0.5%	39[a] [89%]	54[a] [86%]	48 [68%]	9 [50%]	44 [73%]	33 [85%]	15 [50%]	87 [92%]	53 [93%]	0	41[a] [76%]	21 [100%]	25 [56%]	0	26 [130%]
1%	37 [84%]	39 [62%]	53 [75%]	10 [56%]	46 [77%]	35 [90%]	21 [70%]	85 [90%]	49 [86%]	0	30 [56%]	20 [95%]	28 [62%]	0	22 [110%]
2.5%	32 [73%]	30 [48%]	42 [59%]	21 [117%]	46 [77%]	36 [92%]	19 [63%]	67.5 [71%]	44 [77%]	0	28 [52%]	17 [81%]	25 [56%]	0	22 [110%]
5%	23 [52%]	31 [49%]	35 [49%]	8 [44%]	33 [55%]	19 [49%]	15 [50%]	N/A	37 [65%]	0	39 [72%]	17 [81%]	22 [49%]	0	21 [105%]

Appendix 4

Table 10. LAB content in dog and wolf feces, counts reported as CFU g^{-1} feces, ND = not detectable

Dog	CFU count (37°C)	CFU count (45°C)	Wolf	CFU count (37°C)	CFU count (45°C)
Springer Spaniel	$7.76 \cdot 10^{7}$	$5.6 \cdot 10^{6}$	VLT 115/12	$7.78 \cdot 10^{4}$	$5.5 \cdot 10^{3}$
Beagle I	$5.9 \cdot 10^{6}$	100	V188/12	$2.01 \cdot 10^{4}$	ND
Mixed Breed	$4.01 \cdot 10^{6}$	$2.3 \cdot 10^{6}$	V289/12	$2.5 \cdot 10^{3}$	ND
Beagle II	$1.19 \cdot 10^{6}$	$6.33 \cdot 10^{4}$	Skansen	1200	300
Beagle III	$9.89 \cdot 10^{7}$	ND			

Appendix 5

Populärvetenskaplig sammanfattning

Laktobaciller i mag- och tarmkanalen hos hund och varg

Hunddjuren (Canidea) är en familj av rovdjur som består av 38 olika arter. Denna familj innefattar både ensamjagande arter som räven, samt flockdjur som vargen, jackalen och präriehunden. Den domesticerade hunden härstammar från vargen och tros ha hållits som sällskapsdjur i över 14 000 år. Under dessa år har hundens biologi påverkats avsevärt, både när det gäller utseende och beteende. Vissa egenskaper var efterfrågade men allt eftersom djuren avlades efter dessa har det även uppstått en del problem. Vissa hundar fick problem med andningsvägar, leder, hjärta, mag- och tarmkanalen osv. Även om vissa av dessa problem helt tros bero på avlingen, kan sjukdomar som drabbar mag- och tarmkanalen även till viss del förklaras av den ändring som skett i hundens levnadsmiljö. Stress av olika slag som förknippas med den moderna livsstilen tros påverka tarmhälsan på ett negativt sätt, även dieten tros ha sin påverkan. Vargen å andra sidan sägs ha en väldigt bra tarmhälsa med få problem. Eftersom probiotika idag är något som marknadsförs flitigt och dessutom har visat sig ha positiva effekter på människor, har fokus även flyttats till våra sällskapsdjur och däribland hunden. Är det möjligt att den sviktande tarmhälsan hos våra hundar beror på ändringar i mikrobiotan? Kan vargens mer stabila tarmhälsa bero på att den har fler "goda" bakterier eller kanske bara annorlunda arter av bakterier som är bättre på att skydda mot dessa sjukdomar? Detta var frågor som låg till grunden för denna studie, där skillnaden i antalet laktobaciller i tarmprov från varg och träckprov från hund analyserades. Både det totala antalet laktobaciller samt antalet olika arter av laktobaciller studerades. Fokus låg på isoleringen av en speciell art av *Lactobacillus*, nämligen *Lactobacillus reuteri*, och eventuellt hur isolerade stammar skiljer sig åt mellan varg och hund. Totalt analyserades avföringsprover från fem hundar (en springer spaniel, en blandras och tre beagles) samt hos fyra vargar (tre vilda och en från Skansen). Som ett första steg för att uppskatta bakteriernas potential som probiotika undersöktes bland annat hur väl de binder till tarmslem samt deras galltolerans. Detta är viktiga egenskaper för att eventuella probiotiska bakterierna ska kunna överleva och konkurera med andra bakterier i mag- och tarmkanalen. Resistens mot antibiotika av olika slag studerades även hos de olika bakteriestammarna. Här eftersöks en låg resistens eftersom man bland annat är rädd för att denna resistens ska kunna spridas vidare till mindre hälsobringande bakterier. Enligt resultaten från den här studien, hade hunden ett större antal laktobaciller än vad vargen hade. Anledningen till varför antalet bakterier skiljer är svårt att veta men andra studier har visat att bland annat svält kan minska antalet laktobaciller i tarmen. Eftersom tarmarna från två av vargarna var näst intill tomma skulle detta kunna vara en förklaring. Ett högre intag av kolhydrater har däremot visat sig öka antalet laktobaciller i tarmen. Till skillnad från vargen som främst äter kött, får de flesta hundar foder som innehåller mycket kolhydrater. Även om vargen verkar ha ett lägre totalt antal laktobaciller, innehöll proverna från varg emellertid fler olika arter av *Lactobacillus* än hunden. Av de olika arter som isolerats från både hund och varg, verkar *L. reuteri* vara den mest dominerande. Ett antal olika stammar av arten hittades, några som var gemensamma för både varg och hund samt andra som bara fanns hos varg eller hund. De flesta stammar liknade varandra i antibiotikaresistens och galltolerans, medan bindningen till tarmslem skiljde sig markant mellan olika stammar. En annan egenskap hos *L. reuteri* som anses viktig för vissa probiotiska produkter, är produktionen av en antimikrobiell substans (reuterin). Produktion av reuterin observerades bara hos några få av de isolerade stammarna och verkar vara vanligare hos stammar från hund än från varg. Från hund isolerades ett antal stammar med denna egenskap medan det i varg endast påträffades en sådan stam och den kom från vargen på Skansens djurpark. Även om analyserna i denna studie kan bidra till framtagningen av en probiotisk produkt till hundar, behövs ytterligare studier. Först och främst bör mikrobiotan hos hundar som har mag- och tarmproblem studeras för att ta reda på eventuella skillnader gentemot friska hundar och vargar. De isolerade stammarna av *L. reuteri* bör också analyseras vidare för att hitta en probiotisk kandidat som sedan skulle kunna användas i kliniska försök på sjuka hundar.