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How the antimicrobial Protein, Lipocalin 2, affects the Establishment of Microbiota in the Gut of Mice.

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Nora Hersoug Nedberg

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ABSTRACT

Several studies have suggested that the host genetics may influence the composition of gut microbiota, but few genes involved in host control have been proposed (Sekirov *et al.*, 2010). Lipocalin 2 (Lcn2) prevents growth of bacteria that rely on catechol type siderophores for iron acquisition (Goetz, *et al.*, 2003; Flo, *et al.*, 2004). It was hypothesized that Lcn2 may impart a selection pressure on establishment of the gut microbiota and thus influence the commensal diversity. The aim of this study was to find out whether the antimicrobial protein, Lipocalin 2, has a determining effect on the colonization of the gut microbiota in mice. Two factors were investigated: genotype (Wt, Ht and Lcn2 KO) and habitation (single-housing and co-housing). The naturally developing gut microbiota of wild type mice (Wt), heterozygote mice (Ht) and lipocalin 2 deficient mice (Lcn2 KO) were studied, as well as re-established microbiota after antibiotic perturbation, by collecting stool samples. Microbial community profiles were generated by the use of PCR and denaturing gradient gel electrophoresis (DGGE). The gels were analysed by the software program gel2K (Norland, 2002) and band intensity profiles were compared by statistical analysis.

The study of mice gut microbiota revealed differences in the microbial profiles between Wt-, Ht- and Lcn 2 KO-mice. The result showed that both the factor of genotype and habitation were significant factors for the observed differences.

For the single-housed mice (mice of same genotype), a significant difference of gut microbiota was found between Wt/Ht-mice and Lcn 2 KO-mice, indicating that the genotype was the main factor for the observed differences. Lcn 2 thus seems to influence the natural colonization of the mice gut, as well as the re-establishment of the microbiota after a perturbation with antibiotics treatment.

For the co-housed mice (mice of mixed genotypes) both the effect of genotype and maternity seemed to influence the composition of the microbiota, although the factor of maternity was not taken into account in the analysis. The experimental set-up was designed with the intention of comparing littermates in order to minimize other effects than the knockout of Lcn2. Unfortunately this design meant that the effect of genotype and maternity could not be differentiated.

ABBREVATIONS

ANSOSIM	Analysis of similarity
ANOVA	Analysis of variance
APS	Ammonium persulfate
bp	Base pair
2D	2 dimensional
3D	3 dimensional
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
GI	Gastrointestinal tract
GM	Gut microbiota
H’	Shannon diversity index (Shannon index)
Ht	Heterozygote
IBD	Inflammatory bowel disease
J’	Pielou’s evenness index (Evenness)
K’	Band richness
Lc2	Lipocalin 2
Lc2 KO	Lipocalin 2 knock-out mice
NM-MDS	Non-metric multidimensional scaling
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
TMED	Tetramethylethylenediamine
Wt	Wild type

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1. INTRODUCTION

In the past several decades, most research involving the study of the gut microbiota revolved specific pathogens and how they may cause disease. Today however, there is an increasing interest in studying how the composition of the gut microbiota affects the mammalian gut and the immune system (Sekirov, 2010). While it was thought that most infectious diseases in the gut are under control in the western countries, inflammatory conditions however, have risen dramatically. The reason why is still unknown, although it has been speculated that an imbalance of the gut microbiota may be the underlying cause of diseases such as Ulcerus colitis and Krohn's disease (MacDonald & Monteleone, 2005). Research on how the gut microbiota is colonized is therefore an important field of research in order to better understand the underlying mechanisms that may cause imbalances of the gut microbiota that may lead to diseases.

The aim of this project was to find out whether the mammalian protein, lipocalin 2, has a determining effect on the colonization of the gut microbiota in mice. This idea arose from the discovery that lipocalin 2 binds to the siderophore enterochelin that is produced by many members of the *Enterobacteriaceae* family, thereby preventing the growth of bacterial strains that rely on the production of this siderophore to satisfy their iron demands (Flo *et al.*, 2004; Goetz *et al.*, 2003; Raffatellu *et al*., 2009). Lipocalin 2 is produced from macrophages and epitileal cells in response to inflammatory signals, but also in response to rapid cell growth (Kjeldsen *et al*., 2000; Berger *et al*., 2005; Nairz *et al*., 2010), as occur during colonization of the gut microbiota. It was therefore speculated that the antimicrobial protein, Lipocalin 2 might have a role in selecting which bacteria may establish in the gastrointestinal tract of mice.

This project was performed in collaboration between the Faculty of Biotechnology and Institute for Cancer Research and Molecular Medicine, Norwegian University of Science and Technology (NTNU).

1.1 THE MICROBIOTA AND THE IMMUNE RESPONSE

The collection of microorganisms that coexist with their host is referred to as the microbiota, microflora, or normal flora (Sekirov, 2010). Colonization of the human gut with microorganisms begins at birth. Upon passage through the birth canal, neonatals are exposed to a complex microbial community (Moal & Servin, 2006). One of the major factors influencing the initial establishment of the gut microbiota (GM) is the mode of delivery. Infants who are born vaginally are first colonized by fecal and vaginal bacteria originating from the mother, whereas infants born through cesarean section become immediately exposed to bacteria from the hospital environment and health care workers. Other circumstance that may influence the composition of the GM is the environment during birth, prematurity, sanitation, type of infant feeding and treatments with antibiotics (Adlerberth, 2008; Penders *et al*., 2006). Host genotypes are thought to influence inter-individual variation in the GM, given that some immune related genes are highly polymorphic (Dethlefsen, *et al.*, 2006).

The first bacteria to establish in the neonatal gut are usually aerobic or facultative anaerobic bacteria, like *Enterobacteria, enterococci* and *staphylococci* (Adlerberth, 2008). Facultative anaerobes can perform either aerobic or anaerobic metabolism, while strict anaerobes cannot utilize oxygen and many of them lack enzymes that detoxify oxygen (Adlerberth, 2009). During the growth of aerobic and facultative bacteria, they consume oxygen and change the intestinal environment, making it more suitable for anaerobic bacteria. *Bifidobacterium, Clostridium* and *Bacteroides* are among the first anaerobes to establish in the microbiota. As more oxygen-sensitive species establish and the complexity of the microbiota increases, the population size of aerobic bacteria decline as a result of oxygen depletion. After the initial establishment of the intestinal microbiota and during the first year of life, the microbial composition of the mammalian intestine is fairly simple and varies widely between different individuals (Sekirov, 2010). The complexicity of the GM changes with age, and with adulthood it becomes more versatile. The established human GM is dominated by two bacterial phylums: the *Bacteroidetes* and the *Firmicutes,* whereas *Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria,* and *Cyanobacteria* are present in minor proportions (Adlerberth, 2008).

Establishing a stable gut microbiota is highly beneficial, providing the host with essential nutrients, such as vitamin K, metabolizing indigestible compounds (e.g. fiber) and defending

the host against colonization by pathogens (Penders *et al*., 2006). The stable bacterial colonies create a physical barrier that helps exclude incoming pathogens by occupying the attachment sites, consuming nutrients and producing antimicrobial compounds. They also stimulate the host to produce various antimicrobial molecules, such as lysozyme (Sekirov, 2010). Studies of germfree mice have shown that the presence of a microbiota also affects gut morphology and function. In response to colonization of the gut, the mucosa became thicker (Alam, 1995), the expression of intestinal epithelial cell is induced, the biosynthesis of mucus is turned on (Chowdhury *et al*., 2007) and antimicrobial compounds is secreted from Paneth cells (Midtvedt, 1999). In this way the microbiota provides the stimulation for the development of the immune system (Penders *et al*., 2006). Figure 1.1 shows a draft of the mucosal barrier in the gastrointestinal system, with microvilli, mucus layer and epithelial cells.

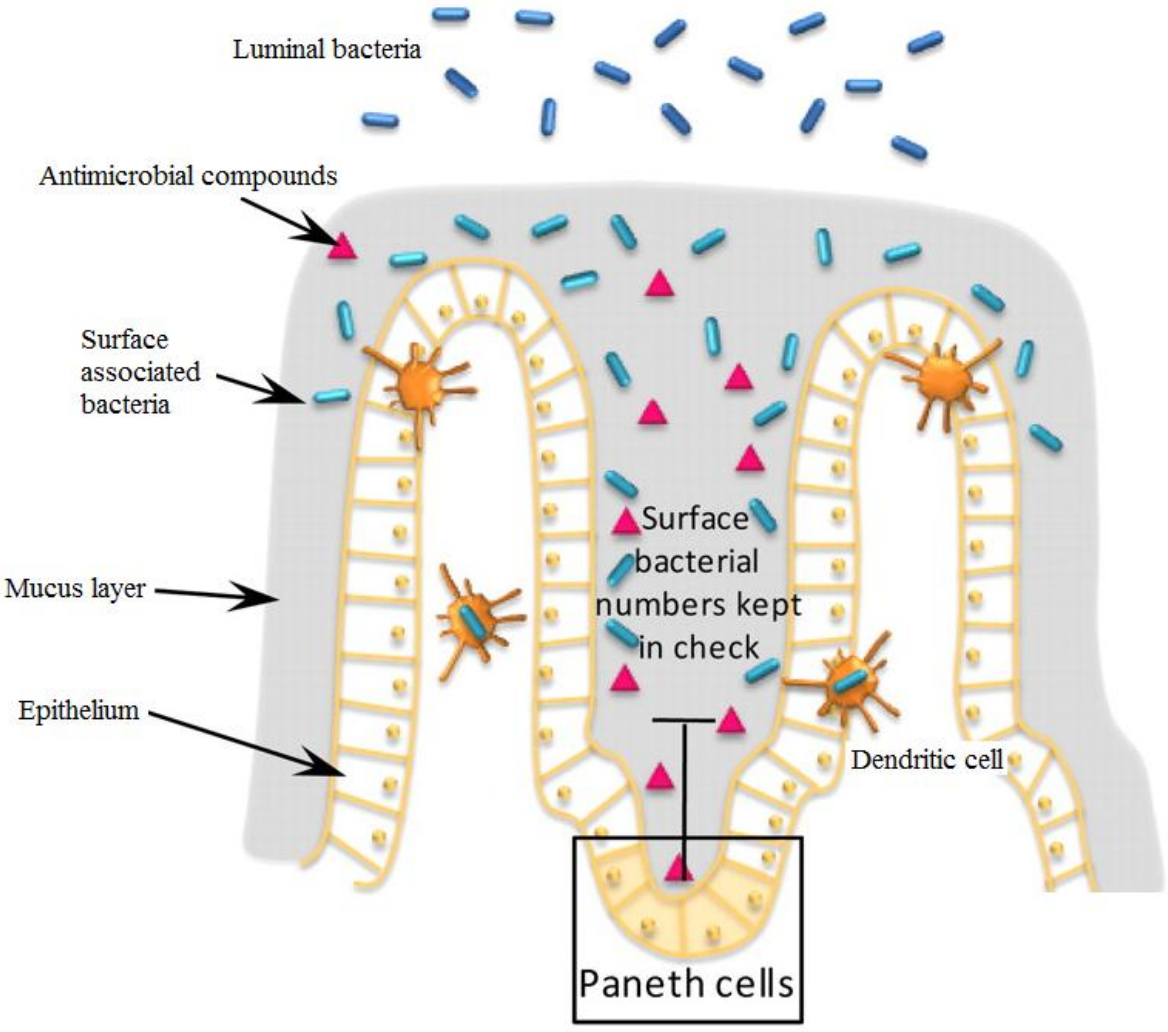

Figure 1.1: Structure of the gut environment showing cellular components that act to maintain the gut architecture and function. Redrawn from; Vaishnava, *et al*., (2008).

A healthy GM is crucial for maintaining the health of the host and conversely, disturbances of the composition of the microbiota have the potential to promote illness and cause various diseases. A number of studies have linked an imbalance of the microbiota to chronic diseases such as inflammatory bowel disease (IBD) (Frank, *et al.*, 2007; Sartor, 2008), diabetes (Larsen, *et al*., 2010), colorectal cancer (Sobhani, *et al*., 2011) and obesity (Turnbaugh, et *al*., 2009). These observations raise the question whether the association is the cause or the consequence of the diseases. Vaginal candidiasis and *Clostridium difficile* colitis often follow a course of antibiotics treatment that disrupts the balance in the GM. This is in favor of the hypothesis stating that the imbalance is the causative agent of the pathology (Sekirov *et al*., 2008), however this result cannot be transmitted to account for all disorders, as very little is known about perturbation in the GM on host susceptibility to invading pathogens.

1.2 THE IMPORTANCE OF IRON

Iron is an essential requirement for nearly all living organisms. Either alone or incorporated into clusters, the iron atom serves as the catalytic centre of enzymes. These enzymes are central to cellular processes such as electron transport, peroxide reduction, amino acid and nucleoside synthesis, DNA synthesis and photosynthesis (Wandersman & Delepelaire, 2004).

Nutrients such as nitrogen from amino acids and nucleotide bases, phosphate, potassium and magnesium are all freely available in the fluid of the host for bacteria to utilize. Iron, however, is not (Ratlegde & Dover, 2000). One reason why iron metabolism needs to be tightly regulated is that free iron (Ferrous Fe^{2+}) catalyses the formation of free radicals that will damage the components of cells (Andrews, 2000). In order to avoid this problem most of the iron in the body is bound to various proteins that help keep the iron in a ferric state (Fe^{3+}), making it less reactive to initiate free radical reactions (Fraga & Oteiza, 2002). The majority of iron is bound either intracellular by heme and ferritin or extracellular by transferrin and lactoferrin (Berger *et al*., 2005). Ferritin is the body's major iron storage protein, while transferrin and lactoferrin functions as iron transport protein in the serum and reversibly binds iron with high affinity.

Because the reduced form of iron is highly toxic to biological systems, and the oxidized form is insoluble, the bioavailability of iron is extremely low in most microenvironments

(Wandersman & Delepelaire, 2004). To satisfy their requirements for iron, many bacteria produce iron-chelating molecules called siderophores that help scavenge iron from the surroundings (Fischbach *et al*., 2006). Siderophores often display higher affinities for iron than mammalian proteins (Bachman *et al.*, 2009) and facilitate bacterial uptake of iron through receptor mediated mechanisms (Berger *et al*., 2005). While some bacteria only secrete one type of siderophore, others produce multiple types. Production of multiple siderophores may be advantageous for microbes to survive and grow in different environments (Sandy & Butler, 2009). Because excess iron increases the virulence of organisms such as *Escherichia, Klebsiella, Listeria, Neisseria, Pasteurella, Shigella, Salmonella, Vibrio* and *Yersinia* (Raymond *et al*., 2003), a general mammalian strategy to limit bacterial growth is to up-regulate expression of lactoferrin receptors and ferritin, which decreases the concentration of extracellular free iron in serum (Fischbach *et al*., 2006). These mammalian proteins participate in the iron-depletion pathway and anti-bacterial effector system of the innate immune system (Dunn *et al.*, 2006).

1.3 SIDEROPHORES

Siderophores are low molecular weight iron chelators, produced by virtually all bacteria, fungi and some plants (Drechsel & Jung, 1997). They serve to solubilize, capture and deliver Fe (III) into microbial cells (Sandy & Butler, 2009). For a secondary metabolite to be classified as siderophore, three conditions must be met: The siderophore must 1) only be produced under conditions of iron scarcity, 2) show ferric ion chelating capability and 3) pursue active transport through the cell membrane (Drechsel & Jung, 1997). Siderophores can generally be classified according to their functional group: catecholates (catechols, $C_6H_4(OH)_2$, hydroxymates (hydroxamic acids, R-CO-NH-OH) and carboxylates (hydroxyl-carboxylic acids, $RCOO^-$). Many siderophores, however, contain more than one type of functional group, e.g. aerobactin having two hydroxymates and one α–hydroxy carboxylate ligand (Sandy & Butler, 2009).

Many different siderophores and several types of transport systems are known, although enterochelin is perhaps the best understood (Raymond *et al*., 2003). Enterochelin is a catecholate-type ferric siderophore, produced by many members of the *Enterobacteriaceae*

family (Raffatellu *et al*., 2009). O'Brien, *et al*., (1970) isolated the compound from *Salmonella typhimurium* and named it enterochelin, whereas that same year Pollack & Neilands, (1970) isolated the compound from *Escherichia Coli* (*E.coli*) and termed it enterobactin. Since then, enterochelin has been isolated from numerous enterobacteria, such as *Shigella*, *Klebsiella*, *Enterobacter* and *Serratia* (Berner *et al*., 1990 & Raymond *et al*., 2003).

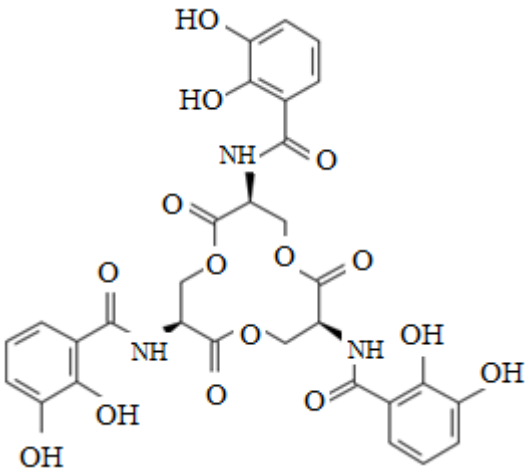

Figure 1.2: Structure of enterochelin. Redrawn from Flo, *et al*., (2004).

Some bacterial strains of the *Enterobacteriaceae* family, such as *Proteus, Providencia, Morganella* and *Yersinia* do not seem to produce any catecholate siderophores (Berner *et al*., 1990). As the *Enterobacteriaceae* family falls in to the category of gram- negative bacteria, enterochelin was thought unique to this group. This hypothesis however was rejected by Fiedler *et al*., 2001, reporting to have isolated enterochelin from two gram positive Streptomyces species. This suggests that the use of the enterochelin siderophore may be wider than previously assumed (Raymond *et al*., 2003).

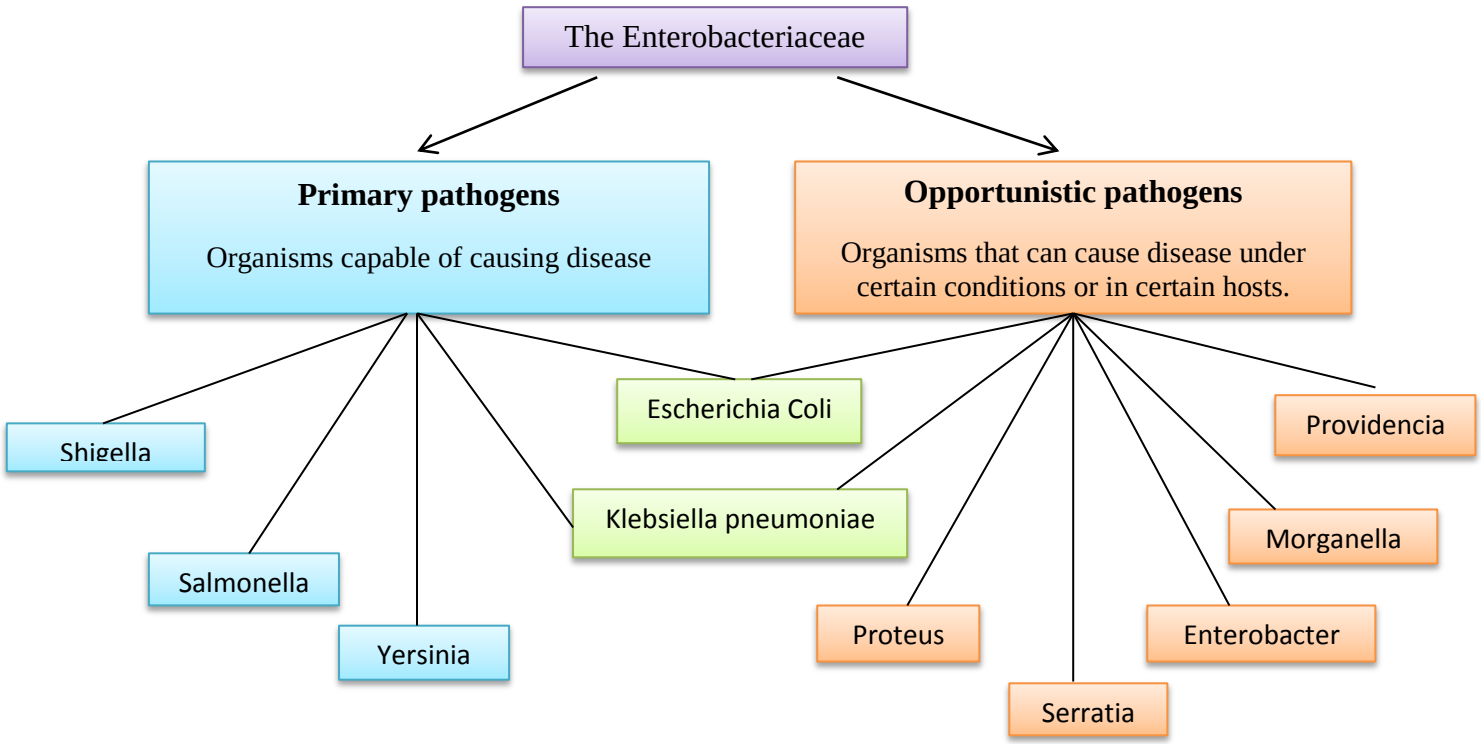

Figure 1.3: Tree structure of the enterobacteriaceae family. Redrawn from; Testore (2011).

1.4 LIPOCALIN 2

Lipocalin 2 (Lcn2), also known as neutrophil gelatinase- associated lipocalin (NGAL), siderocalin and uterocalin, is a 25 kDa protein that belongs to the family of lipocalins (Kjeldsen *et al*., 2000). This family of proteins share a similar tertiary structure comprising an eight-stranded antiparallel β-barrel (Zhang, 2007). Lipocalins has high binding affinity for lipophilic molecules such as fatty acids, cholesterol, retinoids and prostaglandins and was originally characterised as transporter proteins (Berger *et al*., 2005). The eight-stranded antiparallel β-barrel functions as a ligand binding pocket and enables the proteins to bind and transports low molecular weight molecules. These ligands are thought to define the biological activity of the lipocalins (Goetz *et al*., in 2002 & Schmidt-Ott *et al*., 2007).

Lcn 2 was originally identified as a component of neutrophil granules and later shown to be expressed in macrophages, adipocytes and epithelial cells in response to inflammatory signals. It is known to be an acute phase protein highly up-regulated in serum during infection and inflammation (Kjeldsen *et al*., 2000; Berger *et al*., 2005; Nairz *et al*., 2010). Early after the

discovery of Lcn 2, the protein was shown to take part in many diverse cellular processes, although no ligand for the protein had yet been found. The molecular basis of these functions remained unknown until a ligand of lcn 2 was discovered by Goetz *et al*., (2002). The researchers observed that when recombinant lcn 2 was expressed in bacteria, the protein appeared either colorless or slightly red, depending on the bacterial strain used to express the protein. This reddish color was found to be caused by the presence of iron and enterochelin (Schmidt-Ott *et al*., 2007). Under conditions of iron scarcity, such as during infection, bacteria synthesize siderophores to scavenge iron from the host (Berger *et al*., 2005). In response to inflammatory signals or rapid cell growth, the Toll-like receptors on immune cells stimulate the transcription, translation and secretion of lcn 2 to sequester bacterial ferric siderophores. Lcn 2 acts by binding to enterochelin, thereby preventing the growth of bacterial strains that rely on the uptake of this siderophore to satisfy their iron demands. In this aspect, lcn 2 acts as an antimicrobial defence protein, participating in the iron-depletion strategy of the innate immune system (Flo *et al.*, 2004; Goetz *et al.*, 2003; Raffatellu *et al*., 2009). While other components of this system, such as lactoferrin, which binds to and sequesters free iron, lcn 2 on the other hand binds to iron that is bound specifically to siderophores (Goetz *et al.*, 2003). The discovery that lipocalin 2 has high binding affinity for enterochelin suggested a role for Lcn 2 in infection. A study by Flo *et al.*, (2004) demonstrated that genetically modified mice, lacking the gene coding for lipocalin 2 (Lcn2 KO-mice), were more sensitive to Gram-negative bacteria that relied on enterobactin as their sole siderophore for iron uptake and more readily died of sepsis than normal mice (Flo *et al.*, 2004; Schmidt-Ott *et al*., 2007).

Some pathogens have evolved strategies to avoid Lcn2 binding by attaching glucose to Enterobacterin or by making alternative siderophores (Bachman *et al.*, 2009). *Salmonella* and *Klebsiella* glycosylate enterochelin to salmochelins, whereas *M. tuberculosis* modifies its carboxymycobactins. These modifications sterically impairs the ability of lcn 2 to bind these siderophores (Nairz *et al*., 2010; Raffatellu *et al*., 2009).

1.5 MICE AS ANIMAL MODEL

There has been a considerable increase in the use of transgenic rodents in recent years. Transgenic animals are characterized by the presence of a stably introduced foreign (in vitro recombined) DNA sequence into their germline. One explanation for this increase is that the genomes of man, mouse and rat are completely sequenced. This has stimulated the development of models to investigate the biological function and regulation of genes, as well as models for the study of human diseases. To generate transgenic rodents, viral vectors can be used to introduce short DNA sequences via *in vitro* or *in vivo* infection of rodent embryonic stem cells. At present however, only stem cells from mice have successfully been isolated, cultured, modified and shown to contribute to germline, and these are made from only a few inbred strains (Rülicke *et al.*, 2007).

Mice are the most widely used mammalian model for microbiome studies (Spor, *et al*., 2011). They are coprophagous, which means that they tend to eat other mouse faeces. The ingestion of faeces have been reported to serve as a source of some vitamins, aid in fermentation of other nutrients and help the digestion of the animals natural diet (Bugle & Rubin, 1993). Despite the fact that their overall body size is vastly different from humans, the caecum is larger and the diet is different (Spor, *et al*., 2011), they are highly comparable to humans when it comes to organ systems, tissue, physiological systems and behavioural traits. Mice carry nearly all the genes that function in the human body. The use of mice as animal models has many advantages. They are easy to breed and have short generation times, but most important, the possibility to make gene modifications is fairly easy in mice. A knockout mouse is a genetically engineered mouse in which one gene is inactivated (Hofker & Deursen, 2003). Knockout models allow a specific gene to be studied under specific conditions.

Outbreed mouse stocks are often regarded as representatives of human genetic diversity. Unfortunately, random and uncontrolled genetic variation increases the sample size necessary to detect differences in characters of interest (Seong *et al*., 2004). Inbred strains, however, provide a way to control and investigate genetic variation over time (Festing *et al*., 2004). Choosing the right mouse strain is essential in order to retrieve the maximum amount of information yet using the least number of animals (Rülicke *et al.*, 2007). C57BL/6 mice are often used as a standard genetic background for the generation of congenic mice. Congenic mice are two mice that differ in one locus (The Jackson laboratory, 2012). C57BL/6 mice was also the first mouse strain to be sequenced and thus, a lot of information such as molecular

libraries is derived from this strain (Rülicke *et al.*, 2007). However, it is important to remember that inbred strains vary in characteristics that may indirectly influence experimental results. Phenotypic differences of the background strain thus need to be taken into account when designing experiments (Beck *et al*., 2000). Another thing which is important to think about when

1.6 MICROBIAL METHODS FOR BACTERIAL COMMUNITY COMPOSITION

The gut microbiota (GM) is a complex microbial community with high density and diversity. Most of the bacteria here are obligate and facultative anaerobic and thus culture dependent techniques are largely restricted to cover the aerobic heterotrophic fraction of the total bacterial population. Culture independent approaches are therefore more suitable for characterizing the GM (Hufeldt *et al.*, 2010).

Polymerase chain reaction (PCR) made it possible to detect unculturable microorganisms in virtually any environment and has thus become more and more used in the study of microbial diversity (Baker *et al.*, 2003). In microbial diversity studies, a universal primer pair is often used in order to amplify a conserved DNA region from several different bacterial taxa. Prokaryotic ribosomes consist of three ribosomal RNA (rRNA) subunits, classified as 23S, 5S and 16S (Fabrice & Didier, 2009). Of these three, the 16S rRNA gene has become a standard in bacterial taxonomic classification because it is more easily and rapidly sequenced (Spiegelman *et al*., 2005). The Bacterial 16S rRNA gene contains nine hypervariable regions (V1 – V9) that demonstrate considerable sequence diversity among different bacteria. No single region can differentiate between all bacteria, therefore, systematic studies have been performed that compare the relative advantage of each region for specific diagnostic research. Results from studies performed by Chakravorty *et al.*, (2007), showed that the V2 and V3 were more suitable for distinguishing all bacterial species to the genus level, except for closely related enterobacteriaceae. Despite the many advantages of using the 16S rRNA gene, it does entail the drawback that multiple and heterogenous 16S genes in a single microbial genome are not rare, and can lead to an overestimation of the abundance and bacterial diversity using culture-independent techniques (Fabrice & Didier, 2009).

To monitor and compare changes in microbial community structure over time, Denaturing gradient gel electrophoresis (DGGE) has been the preferred method. DGGE was developed by Fisher and Lerman (1983), and introduced in molecular ecology by Muyzer *et al*., (1993). The technique allows separation of PCR products of the same size that differ in their denaturing abilities due to differences in their base-pair sequences (Madigan & Matinko, 2006). The DGGE method is beneficial as many samples can be analysed simultaneously, and by using universal primers, the method permits analysis of bacterial communities without any prior knowledge of the species present. The technique is a combination of gel electrophoresis and PCR product denaturation. Electrophoresis utilizes the fact that the PCR products are negatively charged (DNA is a negatively charged molecule), and will wander towards a positively charged electrode in an electric field. In DGGE the PCR products are also exposed to a gradient of increasing denaturation as the molecules wander through the gel, with formamide and urea constituting the denaturing agents. The PCR products migrating through the gel will remain double stranded until they reach the concentration of denaturants that causes the lower melting domain of the fragment to melt. The branching of the molecule leads to a decrease of the fragment mobility in the gel. PCR products, diverging as little as a single base substitution, will melt at slightly different denaturing concentrations because of differences in interaction strengths between adjacent nucleotide bases. In order to avoid complete strand separation, as this would cause a loss of sequence-dependent gel migration, a GC-clamp is attached to one side of the DNA fragments. The GC-clamp is a sequence rich in the bases guanine and cytosine that show a higher melting domain compared to A-T base pairing due to a stronger bond linkage (Muyzer & Smalla, 1998; Sheffield *et al*., 1988). The DNA band pattern can be visualized using SYBR gold staining where each band on the DGGE gel represents different variants of the 16S rRNA gene that vary in their sequence (Madigan & Martinko, 2006). The band pattern reflects the genetic profile of the microbial community. It is the GC-clamp that makes the fragments stop migrating at different positions in the gel. An outline of the PCR/DGGE procedure to make microbial profiles is shown in figure 1.4.

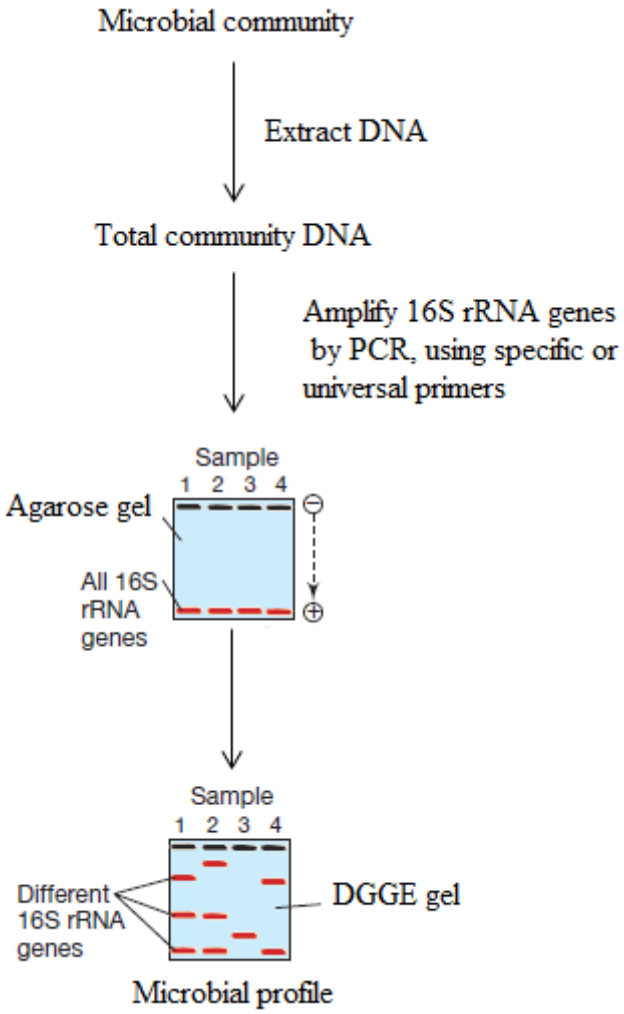

Figure 1.4: PCR/DGGE procedure. Redrawn from Madigan & Matinko (2006).

1.7 HYPOTHESIS, AIM AND OBJECTIVES

The aim of this project was to find out whether the antimicrobial protein, Lipocalin 2 influences the colonization of the gut microbiota in mice. Two variables were taken into account for this study. These were genotype and habitation. The hypotheses for this study were defined to be:

1) *Lipocalin 2 deficient mice (Lcn 2 KO-mice) establish a different gut microbial profile than Wt/Ht-mice.*
2) *The dominant genotype in cages of mice with mixed genotypes influences the minority in respect to the composition of the microbiota.*

The hypotheses would be tested by analysing and comparing microbial profiles of Wild-type mice (Wt), Heterozygote mice (Ht) and Lcn2 KO-mice (Lcn2 KO). The project was divided in two parts with the following objectives:

1) Study the naturally developing gut microbiota of Wt-, Ht- and Lcn2 KO-mice by collecting stool samples, taken when the mice were 3 and 11 weeks old. Use the samples to make profiles of mouse gut microbiota using the fingerprinting method DGGE and compare the analysed profiles of the different genotypes.
2) Study the reestablishment of the microbiota after antibiotic perturbation. Investigate how the gut microbial profiles change over time by collecting stool samples and analyse them 0, 2 and 6 weeks after the antibiotics treatment using the fingerprinting method, DGGE.

2. MATERIAL AND METHODS

2.1 EXPERIMENTAL DESIGN

To assess the role of Lcn2 in the establishment of the gut microbial profile in vivo, mice were used as an animal model. Male chimaeric C57BL/6 mice were mated with C57BL/6 female mice at the vivarium in St. Olavs Hospital in Trondheim, and the heterozygote F_1 progeny were intercrossed to generate an inbred strain of wild type mice (Wt), heterozygote mice (Ht) and lipocalin-2-deficient mice (Lcn2 KO) (Flo *et al*., 2004). The latter group (Lcn 2 KO-mice), were not able to produce the protein lipocalin 2. Both the heterozygote mice and the Lcn2 KO- mice were phenotypically normal.

To test the hypotheses, a two factor experiment that involved the two variables: genotype and habitat, was designed. The first variable (genotype) had 3 levels as there were three different genotypes: Wt, Ht and Lcn2 KO. The second variable (habitat) had 2: Co-housing and single housing. In order to differentiate between the impact of genetics and habitation, the cage set-up was designed to have some cages with only Lcn2 KO-mice, wild type mice and heterozygous mice (single housing), and a few cages with mice of mixed genotypes (co-housing) (figure 2.1).

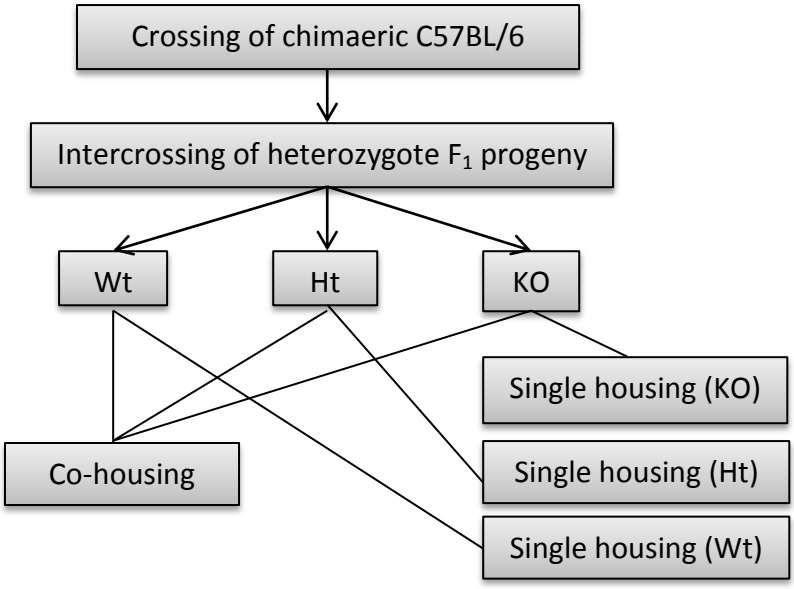

Figure 2.1: Flow chart showing the experimental set-up of the mice experiment.

The offspring were weaned at the age of 3 weeks and separated according to sex. Ear punches were taken to determine the genotype of each mouse with PCR. In order to distinguish individuals, the mice were inserted with a chip that read an individual code when scanned. When the genotype of each mouse was known, 10 Lcn 2 KO mice, 10 Ht mice and 9 Wt mice were chosen to be a part of the experiment. The mice were distributed to 9 cages in total, with 2 cages reserved for Lcn 2 KO-mice, 2 cages for Wt-mice, 2 cages for heterozygote mice and the remaining 3 cages for mice of mixed genotypes. To study the genetic influence on the composition of the GM, one of the cages of mixed genotypes contained more Lcn 2 KO-mice than normal mice, the majority of the second cage consisted of Wt-mice and the last cage was dominated by Ht-mice (figure 2.2).

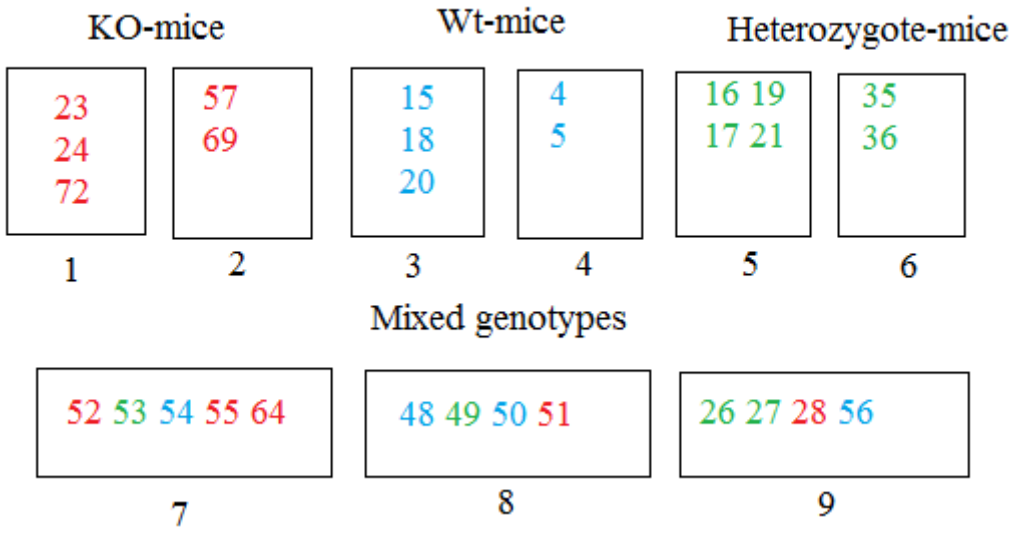

Figure 2.2: Cage set-up after the mice had been separated from their mothers. KO-mice are shown in red, Wt-mice in blue and heterozygote in green. There is one male and one female cage for each genotype. The first row represents the mice living in single-housing, while the second row represents the co-housed mice of mixed genotypes. The numbers inside the boxes represent the mice, marked with identification numbers and the cages are marked with numbers from 1-9.

As explained in chapter 1.7, the experiments were divided in two stages according to the objectives. The first part involved comparing the naturally developing microbial profile of the gastrointestinal tract of wild type mice (Wt), heterozygote mice (Ht) and Lipocalin 2 knock-out mice (Lcn 2 KO). This was done by collecting and analysing stool samples, taken when the mice were 3 and 11 weeks old. A microbial community profile was generated by the use of PCR and denaturing gradient gel electrophoresis (DGGE). The gel was analysed by the

software program gel2K (Norland, 2002) and band intensity profiles were compared by statistical analysis (figure 2.3).

In the second part of the study the same mice were given antibiotics in the drinking water for 4 weeks in order to kill most of the bacteria in the gastrointestinal tract (GI). This mixture consisted of ampicillin (1g/L), vancomycin (500 mg/L), neomycin sulphate (1 g/L) and metrondiazole (1 g/L). The objective was to study how Lcn2 impacts the reestablishment of the microbiota after perturbation with antibiotics, and see how the gut microbial profiles changed over time. Stool samples were collected and analysed 0, 2 and 6 weeks after the antibiotics treatment. The profiles of mice microbiota were made using DGGE.

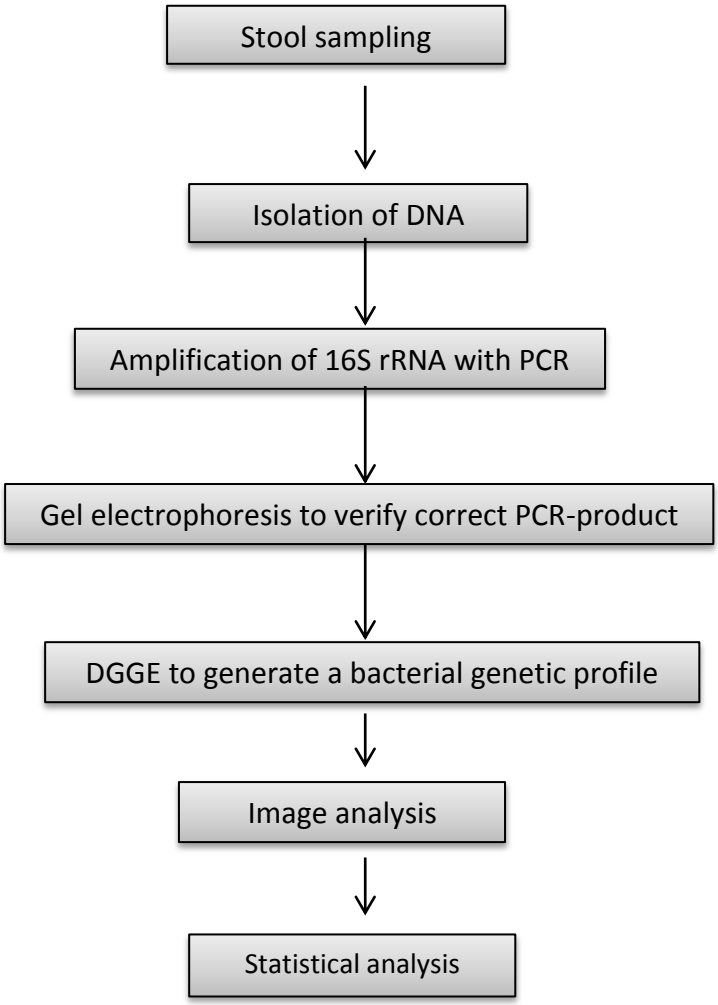

Figure 2.3: Flow chart showing the different procedures and methods during the research.

The mouse experiments were approved by the Norwegian Animal Research Authority (Forsøksdyrutvalget FDU, Norway*)*. The candidate attended a training programme approved by the Food Safety Authorities called FELISA C, which included theory (35 hours), home work (24 hours), training (80 hours) and a final exam.

Table 2.1: Cage set-up showing the mice identification numbers, which litter they come from (mice with the same number are siblings), gender, genotype and the cage number.

	Mice #	Litter	Gender	Genotype	New cage
KO mice only	23	4	F	K	1
	24	4	F	K	1
	72	12	F	K	1
	57	20	M	K	2
	69	11	M	K	2
Wildtype only	4	5	M	W	4
	5	5	M	W	4
	15	13	F	W	3
	18	13	F	W	3
	20	13	F	W	3
Heterozygote only	35	14	M	H	6
	36	14	M	H	6
	16	13	F	H	5
	17	13	F	H	5
	19	13	F	H	5
	21	13	F	H	5
Mix of genotypes	52	20	F	K	7
	53	20	F	H	7
	54	20	F	W	7
	55	20	F	K	7
	64	11	F	K	7
	48	19	M	W	8
	49	19	M	H	8
	50	19	M	W	8
	51	19	M	K	8
	26	4	M	H	9
	27	4	M	H	9
	28	4	M	K	9
	56	20	M	W	9

2.2 SAMPLING OF STOOL

The sampling of stool took place at the animal facility at St. Olavs Hospital in Trondheim. The pellets were placed in microtubes with a pair of tweezers rinsed with 96 % ethanol before they were put in a thermos containing liquid nitrogen. The thermos was carried to the laboratory at the Norwegian University of Science of Technology (NTNU) and immediately stored at -80 º, so that the DNA would not degenerate. At the end of the experiment the mice were sacrificed with the use of CO_2.

Table 2.2: Schedule explaining the main procedures throughout the experiment.

Part	**Age of the mice**	**Weeks after antibiotics treatment**	**Stool sampling**	**Notification**
1	3		Yes	The mice were separated from their mothers according to sex.
	6			After genotyping, 29 mice were chosen to be a part of the project. These were distributed to new cages (figure 2.2).
	11		Yes	
	16			Mice number 4 (Wt) and 20 (Wt) died. Mice 27 (Ht) and 56 (Wt) were moved to separate cages.
2	17			The mice started on antibiotics.
	21	0 weeks	Yes	The mice were given normal drinking water
	23	2 weeks	Yes	
	27	6 weeks	Yes	The mice were sacrificed.

* The week in which the mice were born is set to week zero. At the first sample taking the mice were approximately 3 weeks old.

2.3 ANALYTICAL METHODS

2.3.1 GENOTYPING OF MICE

When working with wild-type- and transgenic knockout mice it is necessary to check which ones are genetically modified and which ones are not. PCR can be used to determine if a gene is present or absent in a specific DNA sequence, in this case the gene coding for Lipocalin2.

With the help of a small ear punch devise, an ear sample was taken to obtain mice DNA. This was done under anaesthesia by an animal caretaker at the hospital. Kapa mouse Genotyping kit (Kapa biosystems) was used to perform the genotyping (see appendix x for detailed description of solutions). The ear samples were mixed with PCR- grade water, Kapa express extract enzyme and 10 * Kapa express extract buffer. The tubes containing the ear sample and the reaction mixture was incubated in a thermocycler at 75 º C for 10 minutes. During this step the cells were lysed, nucleases and proteins were degraded and the DNA was released. The samples were exposed to heat denaturation for 5 min at 95 º C to inactivate the thermostable Kapa express extract protease. The reaction product was vortexed and centrifuged for 1 minute to obtain pellet debris. The supernatant containing DNA was transferred to a clean tube where 1 µl of extract was used as template for PCR. The DNA was amplified with KAPA2G Fast Genotyping Mix (2X), which contained all the components for PCR except primers. The primer lcn2 wt was used together with lcn2-extra for amplifying the wild type lcn2 allele and lcn2-neo1500 was used together with Lcn2-extra to amplify the lcn2 knockout allele (Appendix B). The PCR protocol can be seen in table 2.2.

Table 2.3: PCR program for the amplification of the Lipocalin 2 gene.

Step	**Temperature (C)**	**Time**	**Procedure**
1	95	3 min	Denaturation
2*	95	15 sec	Denaturation
3*	55	15 sec	Annealing
4*	72	20 sec	Elongation
5	72	10 min	Elongation

*Step 2-4 was repeated 35 times.

The PCR-products were detected and visualized on an agarose gel. The presence of two bands on the gel implied that the mice were normal, one band that the mice were heterozygote and

the absence of bands implied that the mice were Lcn 2 KO-mice. Genotyping of 80 progeny of crossed heterozygote C57BL/6 mice identified 61 heterozygote (+/-), 9 wild-type (+/+) and 10 lcn2 KO-mice (-/-).

2.3.2 ISOLATION OF DNA FROM STOOL

Three different DNA isolation kits were tested in order to see which gave the highest yield of DNA, the purest sample and the best band separation on a DGGE gel. Stool samples from six different mice were first weighed. DNA from the first two samples was extracted using a kit provided by QIAGEN called QIAamp DNA stool handbook. The next two samples were extracted using a kit by Zymo Research called ZR Fecal DNA MiniPrep instruction manual. The last two samples were extracted using a kit by MolBio called Ultra clean faecal DNA isolation Kit. DNA from a piece of mice liver was also purified in order to see if the primers amplified mice 18S rRNA. The kit was called DNA extraction with Dneasy modified protocol. It was desirable to amplify the 16S ribosomal RNA gene that is present in all prokaryotes, not eukaryotes. The purified DNA from the mice liver was therefore used as a control to verify that the primer pair was amplifying the correct product.

To quantify the DNA concentration (ng/µl) in the extracts, and to see how pure the DNA was, Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) was used. The instrument measures the absorbency of light at a given wavelength, which according to Beer Lamberts law, is proportional to the concentration of the absorbing material. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as "pure" for DNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm (Thermo Scientific). Nuclease free water was added to initialize the spectrophotometer before eluation buffer S5 (MO BIO) was used as a blank. Of the isolated DNA, 2 µl was applied to the spectrophotometer for concentration measurements.

As stated in chapter 1.7, the bacterial 16S rRNA gene contains nine hypervariable regions (V1-V9). Although no single region can differentiate between all bacteria, Chakravorty *et al.*, (2007), showed that the V2 and V3 are most suitable for distinguishing all bacterial species to the genus level, except for closely related enterobacteriaceae. For this reason, the V3 region of

the 16S rRNA gene was considered as the most suitable for this project. The V3-region of the 16S rRNA gene was amplified with PCR using the universal primer pair 338F-GC and 518R as described by Bakke *et al*., (2010).

UltraClean Fecal DNA isolation kit provided by MO BIO was used for isolating DNA from mice stool (Appendix A), for reasons explained in the Results.

2.3.4 POLYMERASE CHAIN REACTION

For amplification of the V3-region, the GC- clamp primer 338F-GC and 518R was used. The purified DNA-samples were first diluted to a concentration of 15 ng/µl. 1 µl template was added to each PCR- tube with 24 µl of reaction mixture with 2 mM $MgCl_2$, 0.2 Mm of each dNTP, 0.3 µM of each primer, reaction buffer (QIAGEN) and Taq polymerase (QIAGEN) (Bakke *et al*., 2010) (Table 2.3). The PCR- reaction was run for 30 cycles. The theoretical product length was approximately 200 bp, as verified by gel electrophoresis.

Table 2.4: PCR program for 16S rRNA amplification of the V3-region.

Steps	**Temperature (C)**	**Time**	**Procedure**
1	95	3 min	Denaturation
2*	95	15 sec	Denaturation
3*	53	15 sec	Annealing
4*	72	20 sec	Elongation
5	72	10 min	Elongation
6	4		Store

*Steps 2-4 were repeated 30 times.

PCR-products were verified and visualized on agarose gel. The gel was casted by adding 1 % agarose in TAE buffer (Tris-HCl, acetic acid and EDTA). The solution was heated until the agarose particles were completely dissolved. When the solution had cooled, 20 µl Gelred/100 ml buffer was added. The solution was poured in a mould and was left standing for 15 min to polymerize. For each sample 5 µl of PCR-product was mixed with 1 µl of 6 x DNA loading

dye and loaded to each well. A negative control was included to check for contamination in the PCR mixture. On each side of the gel 5 µl of the standard 1Kb plus ladder was applied in order to measure the band size of the products. The gel was run at 140 volts for 45 min before it was visualized under UV-light (G: BOX, Syngene). The intensity of bands was used to determine how much PCR-product would be applied when running DGGE. The results from gel electrophoresis are not presented.

2.3.5 DENATURING GRADIENT GEL ELECTROPHORESIS

INGENY phorU system (Ingeny, Netherlands) was used for running denaturing gradient gel electrophoresis (DGGE). The gels contained acrylamide (8 %), with formamide and urea as denaturing agents. The denaturing gradient was initially 30-60 %, later optimized to 35-55 %.

Before casting the gel, two glass plates were carefully washed in warm water and wiped off with ethanol to avoid dust particles. Dust particles light up and disturb the picture when the gel is to be photographed. The plates were assembled with a spacer and put in a gel cassette with a comb mounted on the top. In casting the gel, 3 tubes were prepared for the DGGE solutions: one for the lowest denaturing concentration, another for the highest denaturing concentration and the last tube for the stacking gel. The content and amount of solutions is shown in Table 2.3. The first two tubes were prepared by mixing 80 % and 0 % denaturing acrylamide to a total volume of 24 ml in each tube. The 80 % solution was sterile filtrered and the solutions were sucked up by Falcon tubes. Tetramethylethylenediamine (TMED 16 µl) was added to each tube.

Table 2.5: Solutions for casting the 8 % acrylamide gel for DGGE.

Denaturing %	**0 %**	**80 %**	**TMED + 10 % APS**	**Total volume**
30	15 ml	9 ml	16 µl + 87 µl	24ml
35	13.5 ml	10.5 ml	16 µl + 87 µl	24ml
55	7.5 ml	16.5 ml	16 µl + 87 µl	24ml
60	6 ml	18 ml	16 µl + 87µl	24ml
0 (stacking gel)	8 ml		10 µl + 40µl	8ml

The DGGE system consists of a gradient mixer with two chambers that are connected with a tube leading to a pump and a stylus. Before the solutions were added to the chambers the

tubes were rinsed with MilliQ-water in order to avoid blockage of gel remnants. Ammonium persulphate (APS, 10 %, 87 µl) was added shortly before the lowest denaturing percentage was poured in the left chamber of the gradient mixer, and the highest denaturing percentage was poured in the right chamber. The syringe was placed in the middle of the comb. When the gel reached one centimetre from the comb, the stacking gel was added to the top. The comb was pressed down to form the shapes of the wells and the gel was left to polymerize for two hours.

Before running the gel, 20 l of 0.5 x TAE buffer (Tris-HCl, acetic acid and EDTA) was added to the buffer tank, and the buffer was heated to 60 º C. To avoid air bubbles, the gel cassette was carefully placed in the buffer tank. All the wells were rinsed with buffer by using a syringe. Samples (5-15 µl) and 6 x DNA loading dye (2-4 µl) were mixed and applied to the wells on the polymerized gel. A standard DGGE standard was applied to the outermost wells. The gel was run at 100 V for 10 minutes without circulation of the buffer, and 16 hours and 50 minutes with circulation. The gel was stained with SYBR Gold (3 µl) mixed with MilliQ-water (30 ml) and 50 x TAE (600 µl) for 1-2 hours. After the gel was rinsed with water, it was photographed under UV-light (G: BOX, Syngene). Images were captured at various exposure times.

2.3.6 ANALASIS OF THE DGGE GELS

The banding patterns, so called profiles, were analysed with the software gel2K (Svein Norland, Department of Microbiology, University of Bergen, Norway). Gel2K uses a band searching algorithm to recognize bands on the gel. Each band will form a peak with an area relative to its intensity of fluorescence, creating densitometric curves. The area under the curves was calculated by the program, giving each band a measured value. These values were transferred to an Excel spread sheet where the fractional peak area, p, was calculated using formula 2.1, where n is the peak area and T is the sum of all the peak areas in the densitometric curves for a specific sample (Santos, 2006).

$$p = n_i / T \qquad [2.1]$$

The fractional peak area was used to calculate band richness (K), Shannon diversity index (H') and Pielou's evenness index (J'). Microbial diversity, is by Bull, (1992) defined as: *The*

variety of microorganisms at the genetic, species and ecosystem levels; the ecological complexes in which they occur, and the ecological processes of which they are part.

Because it is almost impossible to determine the complete composition of species in a community, richness is often used to measure the number of species in samples of a constant chosen size (Peet, 1974). H' and J' are both diversity indices. The Shannon diversity index (H') represent not only the number of species but how the abundance of the species is distributed among all the species in the community. A community with only one bacterial species will have an H value of 0 because P_i would equal 1 and be multiplied by ln P_i which would equal zero. High values of H therefore represent a more diverse and even community (Mazeyose, 2011). H' was calculated by using the formula 2.2.

$$H' = \Sigma\, p_i * \text{In}\, p_i \qquad [2.2]$$

The observed diversity (H') is usually compared with the maximum Shannon diversity (H'_{max}), expressed by formula 2.3. H'_{max} occurs when all the species are of equal abundance (Kahn)

$$H'_{max} = \text{In}K \qquad [2.3]$$

Pielou's evenness index (J) expresses how evenly the individuals are distributed among the species present in a sample numerically (Kahn). A community in which each species is equally abundant has high evenness values, whereas a community with an unequal abundance has low evenness values (Smith & Wilson, 1996).

J' was calculated by using the formula 2.3, where H' is the Shannon diversity index and In (K) is the natural logarithm to the number of bands, e.g. species in the sample.

$$J = H' / H'_{max} \qquad [2.3]$$

2.3.7 STATISTICAL ANALYSIS

Non-Metric Multidimensional scaling (NM-MDS) method with Bray- Curtis distance measure was used to visualize the similarity of the mice gut microbiota profiles. The analysis was based on square root transformed data obtained from the DGGE gel. Square root transformations balance the advantages of using untransformed data, which preserve relative abundance information, and binary data, which down-weigh abundant groups (Thorne *et al*., 1999). NM-MDS is an ordination method that is based on any distance measure. The distances between the samples are converted to ranks that are plotted in a coordinate system so that similar objects will appear near each other in the plot, while dissimilar objects are placed further apart. In this way the distance between the samples is proportional to the similarity between them (Holland, 2008).

NM-MDS starts with a matrix of data consisting of n rows of samples and p columns of unique bands. From this, a n*n symmetrical matrix of all pairwise distances among samples is calculated with an appropriate distance measure, in this case the Bray-Curtis distance. The MDS ordination is then performed on this distance matrix where the method tries to preserve the rank similarity in 2 and 3 dimensions (Holland, 2008) (figure 2.4).

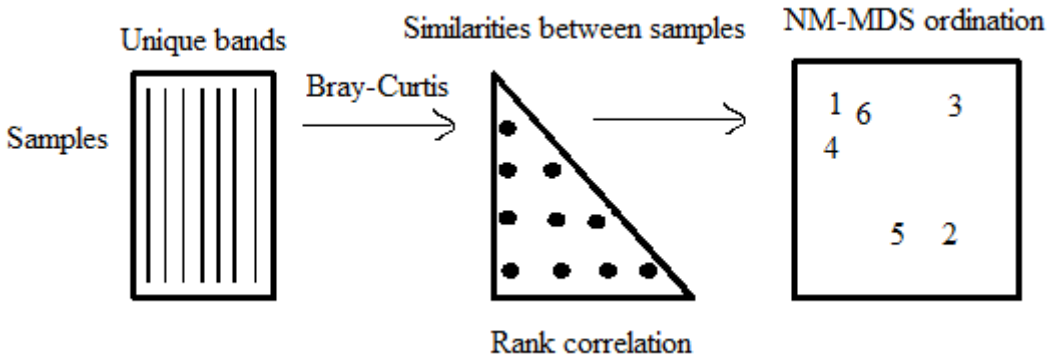

Figure 2.4: The procedure of non-metric multidimensional scaling ordination. Redrawn from Clarke & Ainsworth (1993).

The NM-MDS produces a stress value that explains how well the rank similarity is preserved in the plot. If the MDS plot reproduces the input data perfectly, the stress value will be zero. Thus, the smaller the stress value, the better the ordination. As a general rule, results should not be interpreted unless stress values are < 0, 2 (Clark, 1999; Bray and Curtis, 1957).

Analysis of similarity (ANOSIM) was used to determine if there were significant differences of the gut microbiota between Wt-mice, Ht-mice and Lcn2 KO-mice. The null hypothesis states that there are no differences between the groups (H_0), while H_1 states that there is a significant difference between the three groups. The method produces an R-value and a p-value. The ANSOIM statistic *R* is based on the difference of mean ranks between groups (*r_B*) and within groups (*r_W*) (Clarke & Ainsworth, 1993).

$$R = (r\_B - r\_W) / (N^* (N-1) / 4) \quad [2.4]$$

The R-value produced lies between -1 and 1, where R-values > 0 indicate that the computational dissimilarities are bigger between the groups than within each group. A high positive R-value therefore indicates a large difference between the groups while an R-value of zero implies that there are no differences between the groups. To reject H_0 the p-value should be <0.05 (Clark, 1993; Warwick & Clark, 1995; Bray & Curtis, 1957). To assess the significance of the ANOSIM statistics, the analysis was performed with 10 000 permutations.

Two-way Analysis (two-way ANOVA) was used to test whether the two variables: Genotype and habitat affects the band richness (K), Shannon diversity index (H') and Pielou's evenness index (J'). The first variable had 3 levels (Lcn2 KO, Wt and Ht), while the second variable had 2 (co-housing and single housing). This made 3 x 2 = 6 different combinations of genotype and habitat. Three hypotheses were tested. The null hypotheses of these are stated below:

1) H_0: The population means of the three genotypes (Lcn2 KO, Wt, Ht) are equal.
2) H_0: The population means of the mice living in co-housing and single housing are equal.
3) There is no interaction between the two variables.

The H_0 hypotheses were rejected if the p-value was < 0.05. The ANOVA statistical analysis assumes normal distribution.

All the multivariate analysis were performed using the PAST software package (Hammer, 2005) and ANOVA was performed in Excel using the Microsoft analysis Toolpak.

3. RESULTS

3.1 GENOTYPING OF MICE

The experimental set-up was designed in a way that allowed comparison of littermates in order to minimize other effects than the knockout of Lcn2. When crossing heterozygote mice for the Lcn2 knock out genotype, it was necessary to genotype each mouse to identify the participants for the experiment. Genotyping of 80 progeny of crossed heterozygote C57BL/6 mice identified 61 heterozygous (+/-, Ht), 9 wild-type (+/+, Wt) and 10 KO-mice (-/-, Lcn2 KO). The genotypes of the mice that were chosen for this project are given in table 2.1.

3.2 EVALUATION OF DNA ISOLATION KITS

To analyse the microbial flora, DNA isolation and PCR amplification were important methods to evaluate. Several methods and kits exist and the results obtained with them vary between applications. Three different DNA isolation kits: QIAamp DNA stool handbook (QIAGEN), ZR Fecal DNA MiniPrep instruction manual (Zymo research) and Ultra clean faecal DNA isolation Kit (MolBio), were tested to see which gave the highest yield of DNA, the purest sample and the best band separation on a DGGE gel. Results from Nano Drop showed that the Zymo Research kit gave the highest average concentration of DNA from the three parallels that were taken. The Qiagen kit gave the purest DNA-samples closely followed by MolBio as number two. The PCR-products, however, were best separated on an acrylamide gel with the MolBio kit. The ZymoResearch kit on the other hand showed a poor band separation. It was decided to use the MolBio kit for the isolation of DNA from the experiments because it showed an acceptable purity of DNA and the kit showed the best band separation on the DGGE gel. None of the results are included.

3.3 STUDY OF NATURALLY COLONIZED MICROBIOTA

Lipocalin 2 prevents growth of bacteria that rely on catechol type siderophores for iron acquisition (Goetz et al; Flo et al). It was hypothesized that Lcn2 may impart a selection pressure on the establishment of gut microbiota, and thus influence the commensal diversity. In part 1 of the project the genetic influence on the naturally established gut microbiota of Wt-, Ht- and Lcn2 KO-mice was studied. This involved collecting and analysing faecal samples, taken when the mice were 3 and 11 weeks old.

3.3.1 MICE 3 WEEKS OLD

Figure 3.1 shows the DGGE gel from the GI microbiota when the mice were 3 weeks old. At this stage the mice had only been weaned by their mothers and were not yet separated by sex. The gel contained a total of 60 different bands, but no bands were present in all the faecal samples.

The average band richness varied between 20 and 26.67, where the Lcn 2 KO-mice showed the lowest variation with a standard variation of 5.2 and the Wt-mice showed the largest variation with a standard deviation of 10.3 (Table 3.1). Results from One-way ANOVA showed that there was no significant difference ($p < 0.05$) in band richness between the three genotypes. The Shannon index and the evenness index were fairly similar, and there was no significant difference between the Lcn2 KO-mice, Wt-mice and Ht-mice.

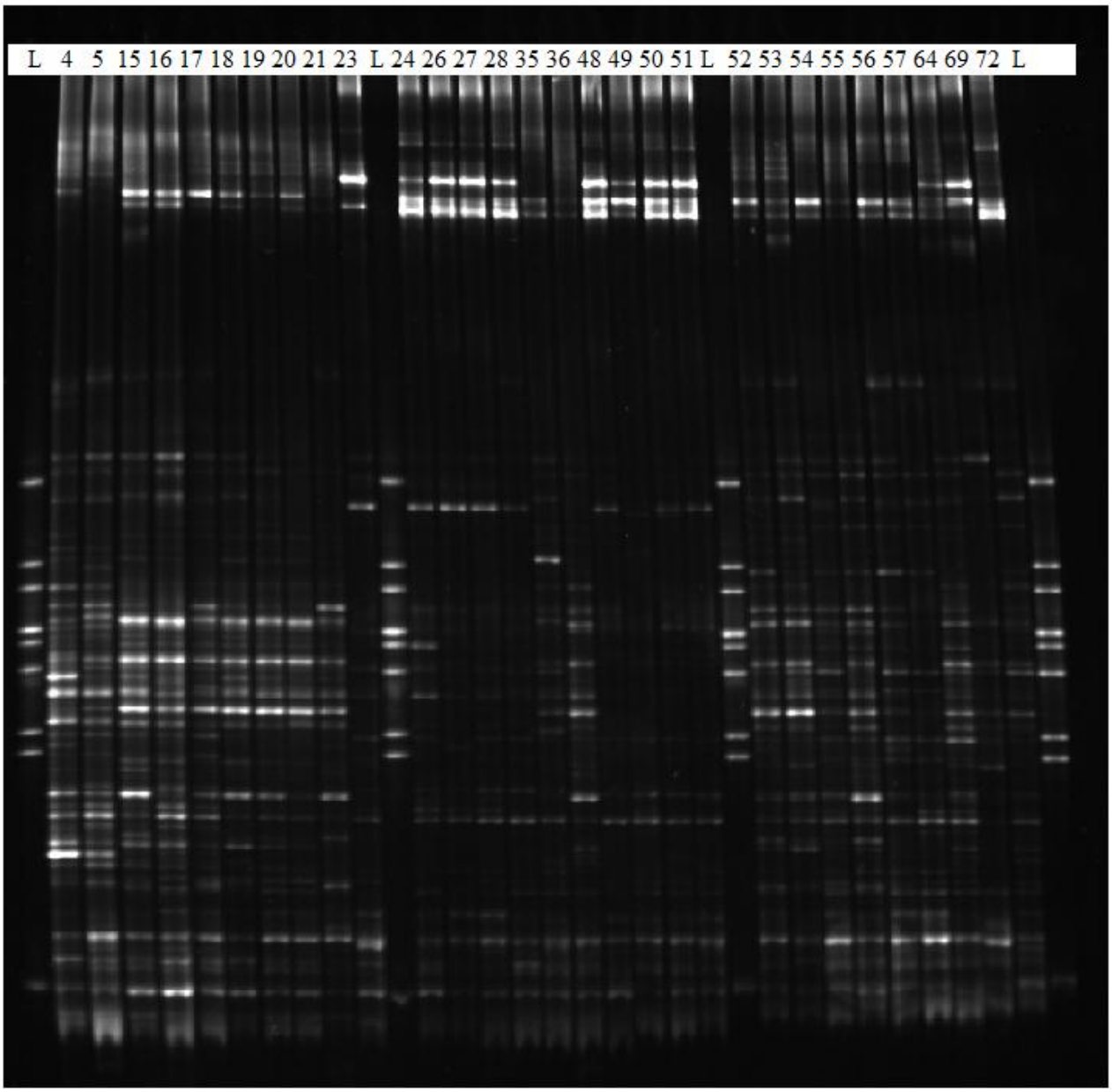

Figure 3.1: DGGE gel (35-55 % denaturing gradient) obtained from stool samples taken when the mice were 3 weeks old. The various lanes are marked with the mice identification numbers and lanes marked with L are the standard.

Table 3.1: The diversity indices obtained from the DGGE gel of mice gut microbiota (3 weeks old). The sample names refer to the mice identification number. The average and the standard deviation were calculated for each group, with the Lcn 2 KO-mice shown in red, the Wt-mice in blue and the Ht-mice in green.

	Mouse id	Band richness (K)	Shannon index (H')	Evenness index (J')
KO-mice	23	16	2.00	0.721
	24	17	2.15	0.760
	72	24	2.18	0.684
	57	22	2.43	0.787
	69	16	1.88	0.678
	28	17	2.20	0.776
	51	11	1.62	0.676
	52	25	2.67	0.831
	55	26	2.89	0.887
	64	26	2.89	0.887
	Average	20±5.2	2.29±0.42	0.769±0.080
Wt-mice	4	37	2.81	0.778
	5	41	3.20	0.862
	15	35	2.87	0.809
	18	29	2.74	0.813
	20	28	2.49	0.748
	48	10	1.64	0.710
	50	14	1.73	0.657
	54	23	2.54	0.809
	56	23	2.75	0.876
	Average	26.7±10.3	2.53±0.52	0.785±0.070
Ht-mice	16	38	2.96	0.813
	17	29	2.73	0.812
	19	29	2.59	0.769
	21	20	2.47	0.824
	35	19	2.59	0.881
	36	21	2.57	0.845
	26	12	1.68	0.678
	27	14	1.83	0.692
	49	10	1.68	0.732
	53	30	2.88	0.847
	Average	22.2±9.1	2.40±0.48	0.789±0.069

The NM-MDS analysis produced a stress value of 0.1406 (< 0.2) and thus the results are reliable to be interpreted. Figure 3.2 shows a clear overlap between the Lcn 2 KO-mice, Wt-mice and Ht-mice, indicating no differences in the microbiota between the genotypes. The one-way ANOSIM analysis confirmed the observations (Table 3.2), indicating no differences in the microbiota between the different genotypes: Lcn2 KO-, Wt- and Ht-mice. However, by observing the plot, there seems to be a link between similarity of gut microbiota and siblings that had sheared the same cage together. Siblings from litter number 4 are clustered together, and also siblings from litter 13 and 19 are clustered together.

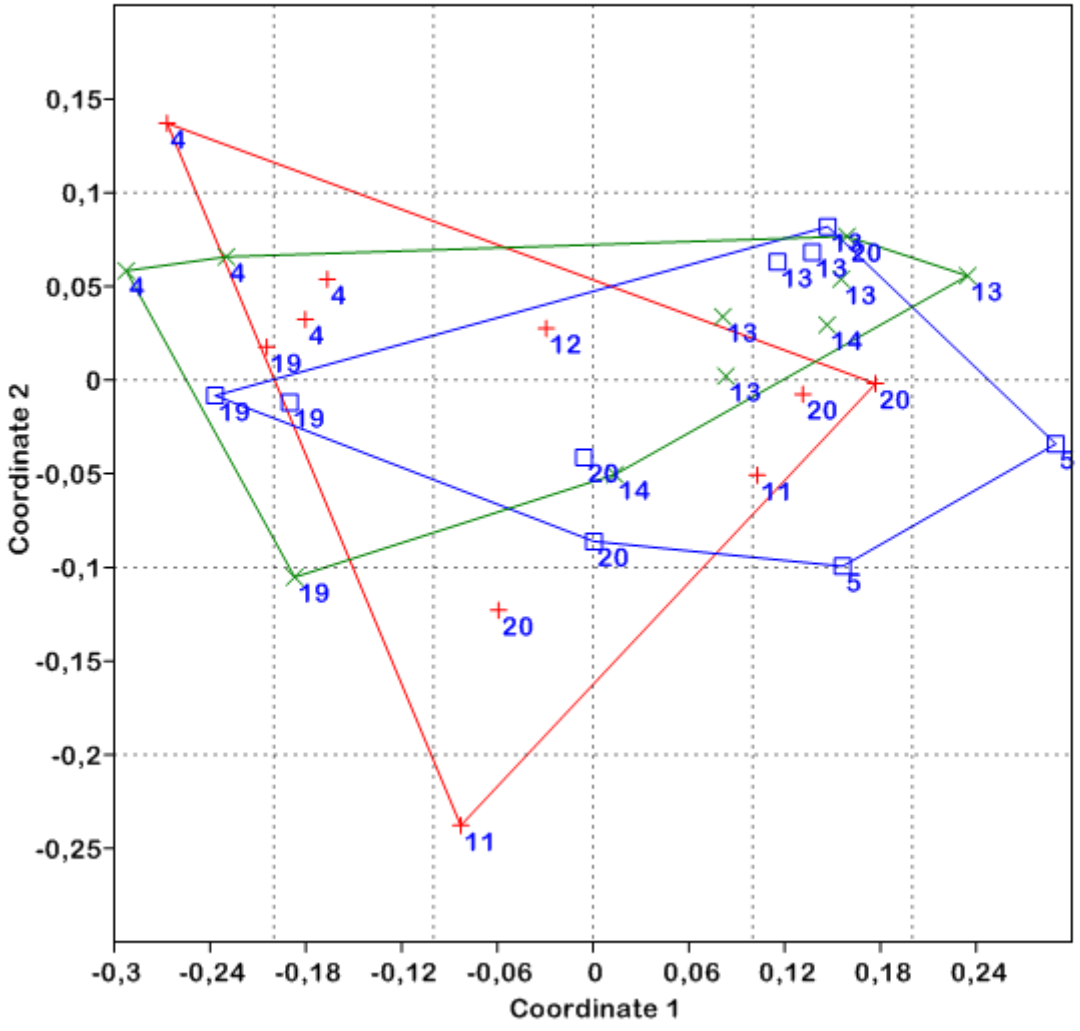

Figure 3.2: NM-MDS 2D-plot based on Bray-Curtis distance measure from when the mice were 3 weeks old and had not yet been separated from their mothers. Lcn2 KO-mice are shown as red crosses, Wt-mice as blue squares and Ht-mice as green multiplication marks.

The outer-most points in each group are marked with a line. The mice that were siblings and thus shared the same cage together are marked with a number representing the litter (Table 2.1). The mice that took part of the experiment were taken from 8 different litters.

Table 3.2: One-way ANOSIM analysis with sequential Benferroni significance, for the comparison of mice gut microbiota (3 weeks old) between the three different genotypes: Lcn2 KO-, Wt- and Ht-mice.

	Groups	**ANOSIM R-value**	**p-value**
Total	-	-0,02426	0.6007
Between groups	KO and Wt	-0.00823	0.4344
	KO and Ht	0.006667	0.3344
	Wt and Ht	-0.07517	0.9058

3.3.2 MICE 11 WEEKS OLD

In order to differentiate between the impact of genetics and habitation, the mice were moved to new cages (week 6, described in table 2.1) where they were either placed in single housing (same genotype) or co-housing (mixed genotype) (figure 2.2). At 11 weeks of age the mice had been living with their new cage mates for 5 weeks. Figure 3.3 shows the DGGE gel from the GI microbiota when the mice were 11 weeks old. The gel contains 45 different bands.

For the single housed mice, the average band richness (K), Shannon diversity index (H') and Evenness index (J') were all observed to be lower for the Wt-mice compared to the other two groups (Table 3.3). Results from one-way ANOVA however showed no significant difference of band richness. The average H'-values were similar between the Lcn2 KO-mice and Ht-mice (H'=2.95) and significantly higher than the Wt-mice (H'=2.74). The J' -values were also significantly different between the three groups ($p<0.05$).

For the co-housed mice the diversity indexes were observed to be fairly similar between the Lcn 2 KO-mice, Wt-mice and Ht-mice and one-way ANOVA confirmed that there were no significant differences of neither K'-, H'- or J'-values with regards to genotype.

When comparing the single housed mice with the co-housed mice in respect to band richness, both the single housed Lcn2 KO mice and the Ht-mice were observed to have slightly higher average band richness compared to the mice of the same genotype that had been living co-

housed. The average band richness was similar for the Wt single-housed mice and the Wt co-housed mice. The observations were confirmed by the results from two-way ANOVA, which showed that the effect of habitation was a significant factor ($p<0.05$) of band richness, but not genotype alone and the interaction between genotype and habitation. This result indicates that the mice that had lived single-housed, had significantly higher band richness compared to the co-housed mice. No significant difference of H'-values and J'-values were found.

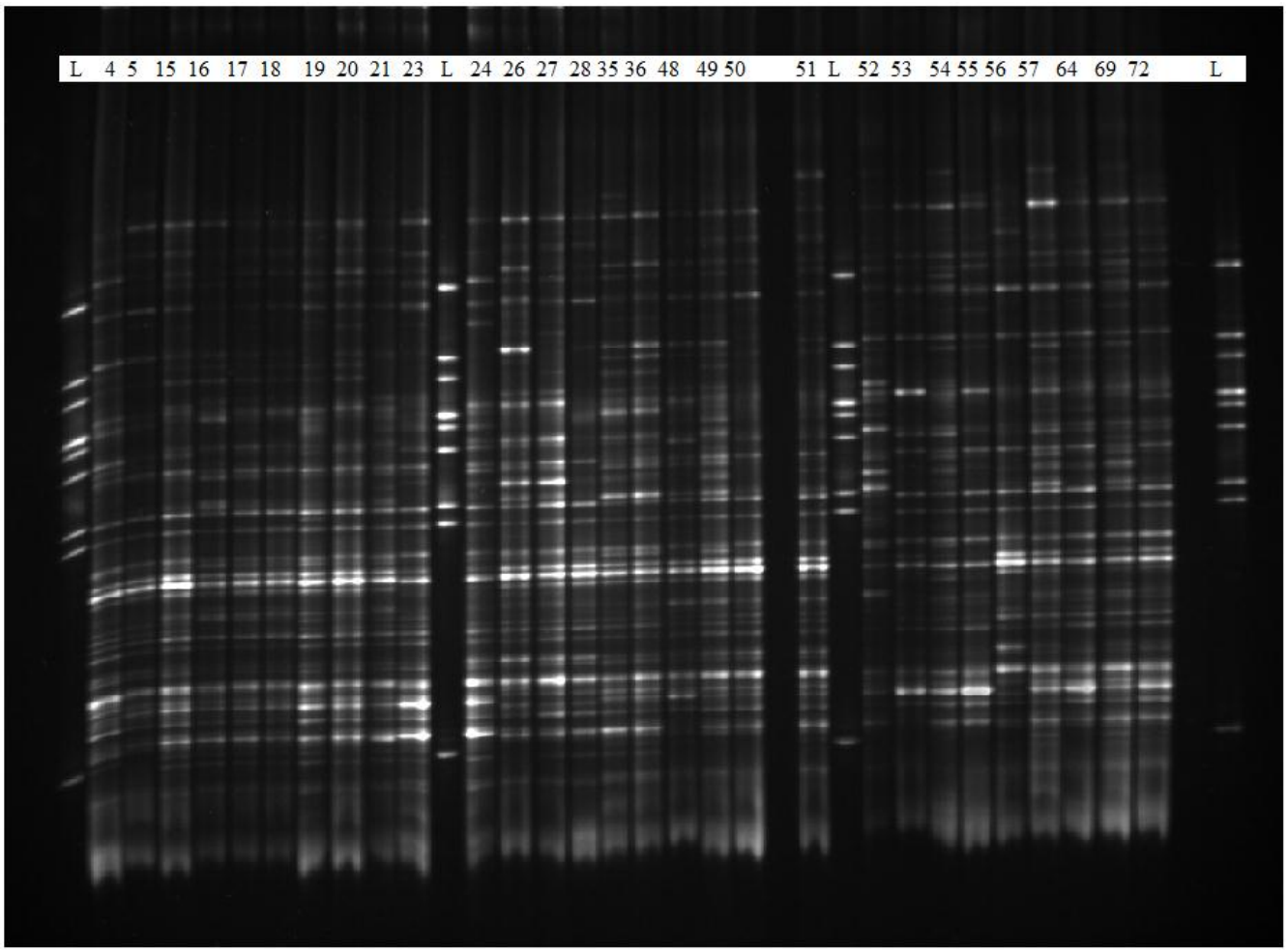

Figure 3.3: DGGE gel (30-60 % denaturing gradient) obtained from stool samples taken when the mice were 11 weeks old. The various lanes are marked with the mice identification numbers and lanes marked with L are the standard.

Table 3.3: Diversity indices obtained from the DGGE-gel of mice gut microbiota (11 weeks old). The average and standard deviation was calculated for the co-housed (CH), the single-housed (SH) and the two groups combined (all). Lcn2 KO-mice are marked in red, Wt-mice in blue and Ht-mice in green.

	Mouse id	Band richness (K)	Shannon index (H')	Evenness index (J')
KO-mice	23	29	2.85	0.847
	24	26	2.86	0.878
	72	27	2.93	0.890
	57	33	3.12	0.892
	69	32	2.99	0.862
	28	24	2.61	0.822
	51	28	2.72	0.816
	52	25	2.76	0.857
	55	24	2.68	0.844
	64	27	2.78	0.845
	Average all	27.5±3.1	2.83±0.15	0,855±0.026
	Average CH	25.6±1.8	2.71±0.07	0.837±0.017
	Average SH	29.4±3.0	2.95±0.11	0.874±0.019
Wt-mice	4	28	2.77	0.832
	5	23	2.60	0.829
	15	27	2.73	0.829
	18	26	2.65	0.813
	20	30	2.94	0.863
	48	26	2.80	0.858
	50	26	2.59	0.796
	54	29	3.09	0.919
	56	24	2.51	0.789
	Average all	26.6±2.2	2.74±0.18	0.836±0.040
	Average CH	26.3±2.1	2.75±0.26	0.840±0.061
	Average SH	26.8±2.6	2.74±0.13	0.833±0.018
Ht-mice	16	29	3.00	0.890
	17	27	2.74	0.831
	19	32	2.98	0.859
	21	30	2.85	0.839
	35	33	3.06	0.875
	36	32	3.10	0.895
	26	30	3.01	0.885
	27	29	2.93	0.870
	49	30	2.98	0.876
	53	19	2.37	0.804
	Average all	29.1±4.0	2.90±0.21	0.862±0.029
	Average CH	27.0±4.0	2.82±0.31	0.859±0.037
	Average SH	30.5±2.3	2.95±0.14	0.865±0.027

The NM-MDS analysis of both the single-housed- and the co-housed mice, produced a stress value of 0.1703 (< 0.2) and thus the results are reliable to be interpreted (figure 3.4). At first glance, the genotypes seem to be overlapping each other, although four samples from the Wt-mice, four samples from the Lcn 2 KO-mice and three samples from the Ht-mice stand out and are not part of the overlapping area. By studying the graph further, there seems to be a clustering of the Wt-mice in the upper left corner with one outlier. The Ht-mice are also more or less clustered in one area having also here one outlier. The Lcn 2 KO-mice are mostly found at the bottom part of the graph with three mice that seems to diverge from the rest.

Results from the hypothesis testing with two-way ANOSIM showed that both genetics and habitation were significant factors ($p<0.05$). *The result indicate that there is a significant difference in microbiota between the three different genotypes, and also that there is a significant difference in microbiota between the single housed mice and the co-housed mice.* The R-values were all > 0 although quite low, reflecting a weak difference between the groups (Table 3.4).

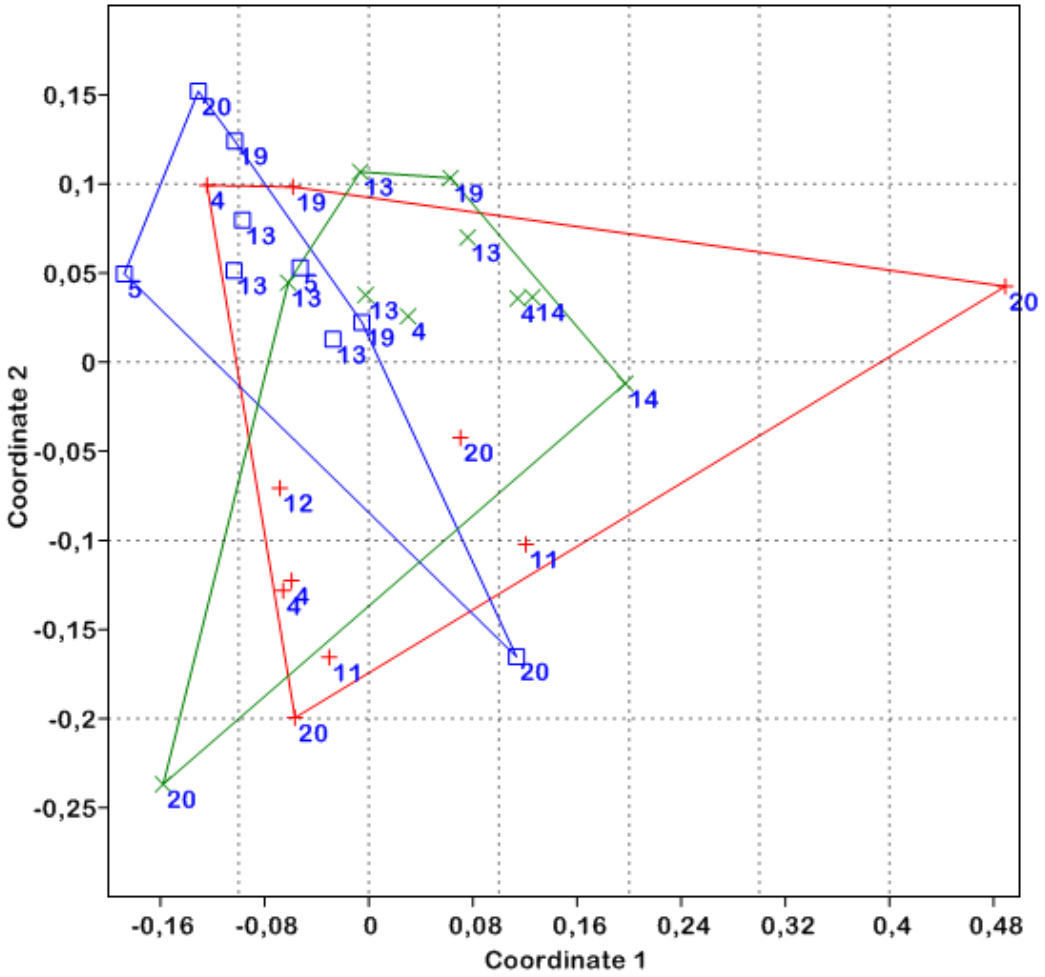

Figure 3.4: NM-MDS 3D plot based on Bray-Curtis distance measure. Results from the DGGE gel of mice gut microbiota (11 weeks old), where both the single-housed mice and the co-housed mice are plotted. Lcn 2 KO-mice are marked in red, Ht-mice in green and Wt-mice in blue and in addition the outer-points for each group are marked with a line. The points on the graphs are marked with the mice litter number (Table 2.1).

Table 3.4: Two-way ANOSIM of the gut microbiota of co-housed mice and single housed mice (11 weeks old), with sequential Benferroni significance.

Factor	**ANOSIM R-value**	**p-value**
Genetics	0.17871	0.0181
Habitation	0.22945	0.0053

The NM-MDS analysis of the co-housed mice produced a stress value of 0.09748 (< 0.2) and the results are therefore reliable to be interpreted (figure 3.5). The plot does not show a clear pattern of cage clustering. By studying the cage set-up (Table 2.2), the mice in cage 7 (from litter 20 and 11) are widely spread. Results from one-way ANOSIM showed no significant differences of gut microbiota between the three different genotypes (Table 3.5).

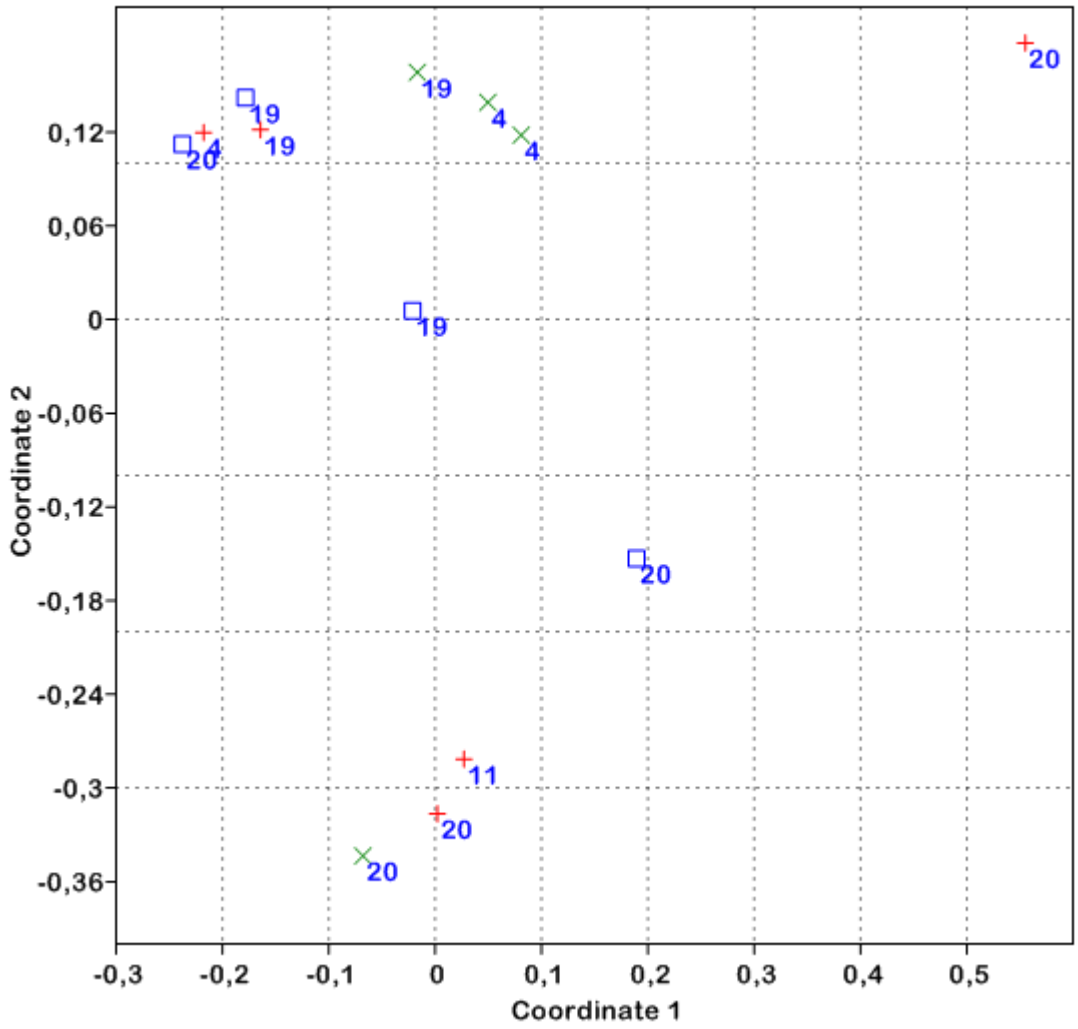

Figure 3.5: NM-MDS based on Bray-Curtis distance measure from the DGGE gel (mice 11 weeks old). Only results from the co-housed mice are shown. Lcn 2 KO-mice are marked in red, Ht-mice in green and Wt-mice in blue. The points on the graphs are marked with the mice litter numbers (Table 2.1).

Table 3.5: One-way ANOSIM of gut microbiota, (mice 11 weeks old). The analysis was only performed on the co-housed mice with sequential Benferroni significance.

	Groups	ANOSIM R-value	p-value
Total	-	-0.06981	0.7127
Between groups	KO and Wt	-0.09375	0.7259
	KO and Ht	-0.1	0.6943
	Wt and Ht	0.1146	0.2833

The NM-MDS of the single-housed mice (Figure 3.6) clearly separates the three groups, with a small overlap between the Wt-mice and the Ht-mice. The stress value was 0.1788 (< 0.2). The results from one-way ANOSIM, comparing the Lcn 2 KO-mice with Wt-mice (R= 0.5) and Lcn 2 KO-mice with Ht-mice (R= 0.5467), showed a significant difference between the composition of the gut microbiota. The R-value produced for the comparison of the Wt-mice and Ht-mice is 0.2773 and thus reflects a weaker difference (Table 3.5).

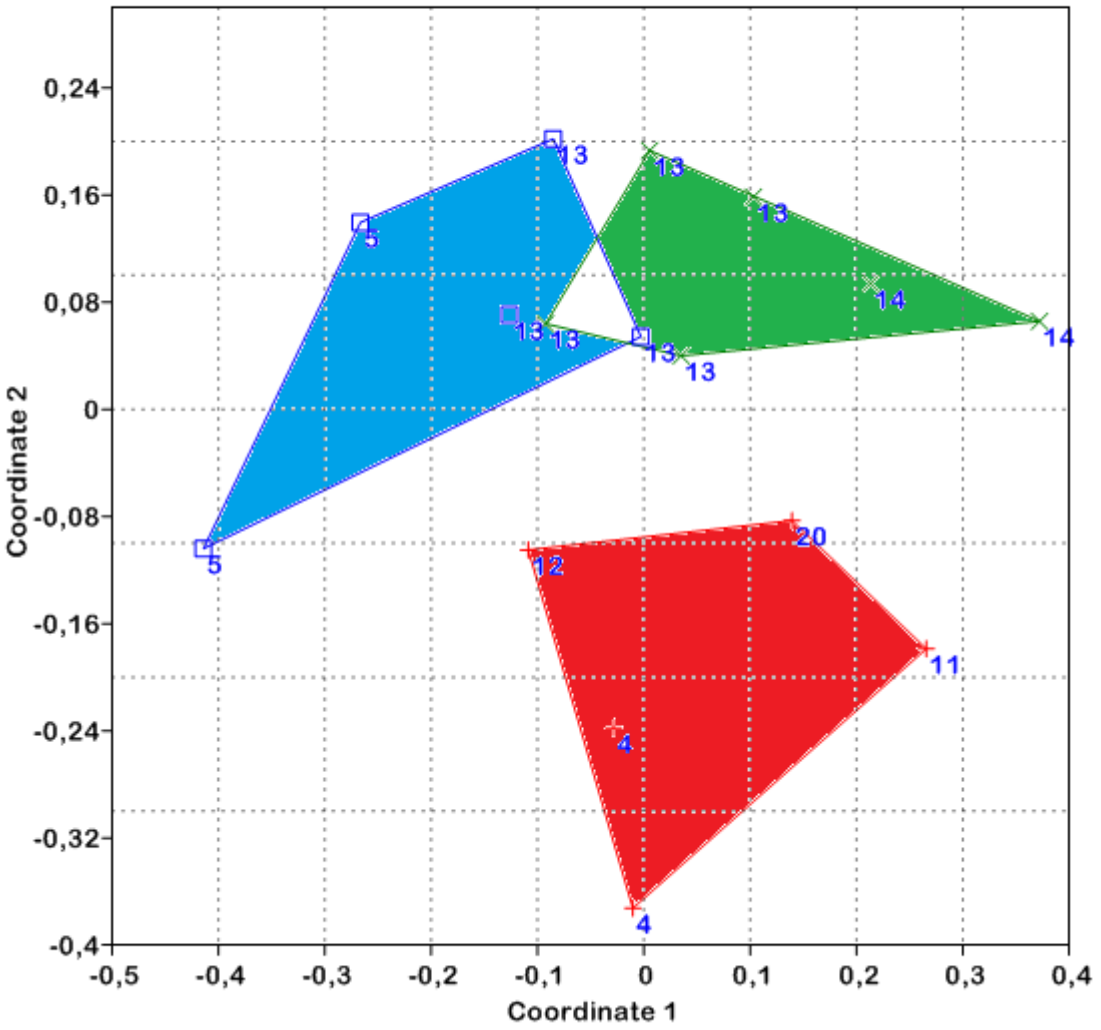

Figure 3.6: NM-MDS 2D plot based on Bray-Curtis similarity. Results obtained from the DGGE gel of gut microbiota, showing only the single-housed mice (11 weeks old). Lcn 2 KO-mice are marked in red, Ht-mice in green and Wt-mice in blue and in addition the outer-points for each group are marked with a line. The points on the graphs are marked with the mice litter number (Table 2.1).

Table 3.6: One-way ANOSIM of the gut microbiota of the single-housed mice (11 weeks old), with p sequential Benferroni significance.

	Groups	**ANOSIM R-value**	**p-value**
Total	-	0,4275	0,0003
Between groups	KO and Wt	0,5	0,0077
	KO and Ht	0,5467	0,0016
	Wt and Ht	0,2773	0,04

3.4 STUDY OF RE-ESTABLISHED MICROBIOTA AFTER PERTURBATION

The objective of part 2 of the study was to study the re-establishment of the microbiota and see if lcn2 influenced the gut microbial profiles changed over time. The mice were given a mixture of antibiotics in their drinking water for 4 weeks in order to perturb the microbiota. Stool samples were collected and analysed 0, 2 and 6 weeks after the antibiotics treatment.

27 mice participated in part 2 of the study, with 10 of these being Lcn2 KO-mice, 10 Ht-mice and 7 Wt-mice (Table 2.1). Mice number 4 (Wt) and 20 (Wt) died before the mice began the antibiotics treatment and therefore had to be left out. Because of fighting between males in cage no. five, which contained mice of mixed genotypes, mice no. 56 (Wt) and 27 (Ht) were moved to live in separate cages. This meant that only mice number 26 and 28 lived in the same cage together throughout the whole experiment of part 2.

3.4.1 MOUSE MICROBIOTA 0 WEEKS AFTER ANTIBIOTICS TREATMENT

Figure 3.7 shows the DGGE gel from the GI microbiota when the mice had just finished the antibiotics treatment (mice 21 weeks old). The gel shows considerably fewer bands and has a clearly visible band pattern reflecting the cage set-up. Cage 8 (48, 49, 50 and 51) all lack two of the most common bands that are found in the gut microbiota of all the other mice.

Of the single-housed mice, the Wt-genotype showed the highest average band richness of 25.0±1.7, while the Lcn 2 KO-mice showed the lowest average band richness with 16.8±2.4 (Table 3.6). Results from one-way ANOVA showed that genetics is a significant factor on band richness ($p<0.05$). This indicates that Wt-mice had significantly higher band richness compared to Lcn2 KO-mice. The Wt mice and the Ht-mice showed similar average diversity (H'=2.73) and was found to be higher compared to the Lcn2 KO-mice (H' =2.41). This observation was also confirmed by one-way ANOVA, which showed a significant difference of H'-values according to genotype. The evenness values were similar between the Wt-mice, Ht-mice and Lcn 2 KO-mice.

For the co-housed mice the diversity indexes were observed to be fairly similar between the Lcn2 KO-mice, Wt-mice and Ht-mice and one-way ANOVA confirmed that there were no significant differences of neither K'-, H'- or J'-values with regards to genotype.

The average band richness for the co-housed mice, single-housed mice and both combined (all), were generally lower than before the antibiotics treatment. Generally, the single-housed mice were observed to have a higher average band richness compared to the co-housed mice. This result was confirmed by two-way ANOVA confirming that the habitat of mice was a significant factor for band richness ($p<0.05$), as well as the interaction between genotype and habitat, but not the factor of genotype alone. This means that single-housed mice had a significantly higher average band richness compared to co-housed mice. The single-housed mice generally also showed a higher average diversity index (H') and evenness index (J'), compared to the mice that had been living co-housed. Results from two-way ANOVA showed that habitation was a significant factor ($p<0.05$) for both the diversity index and the evenness index, but not the factor of genotype alone, nor the interaction between genotype and habitat.

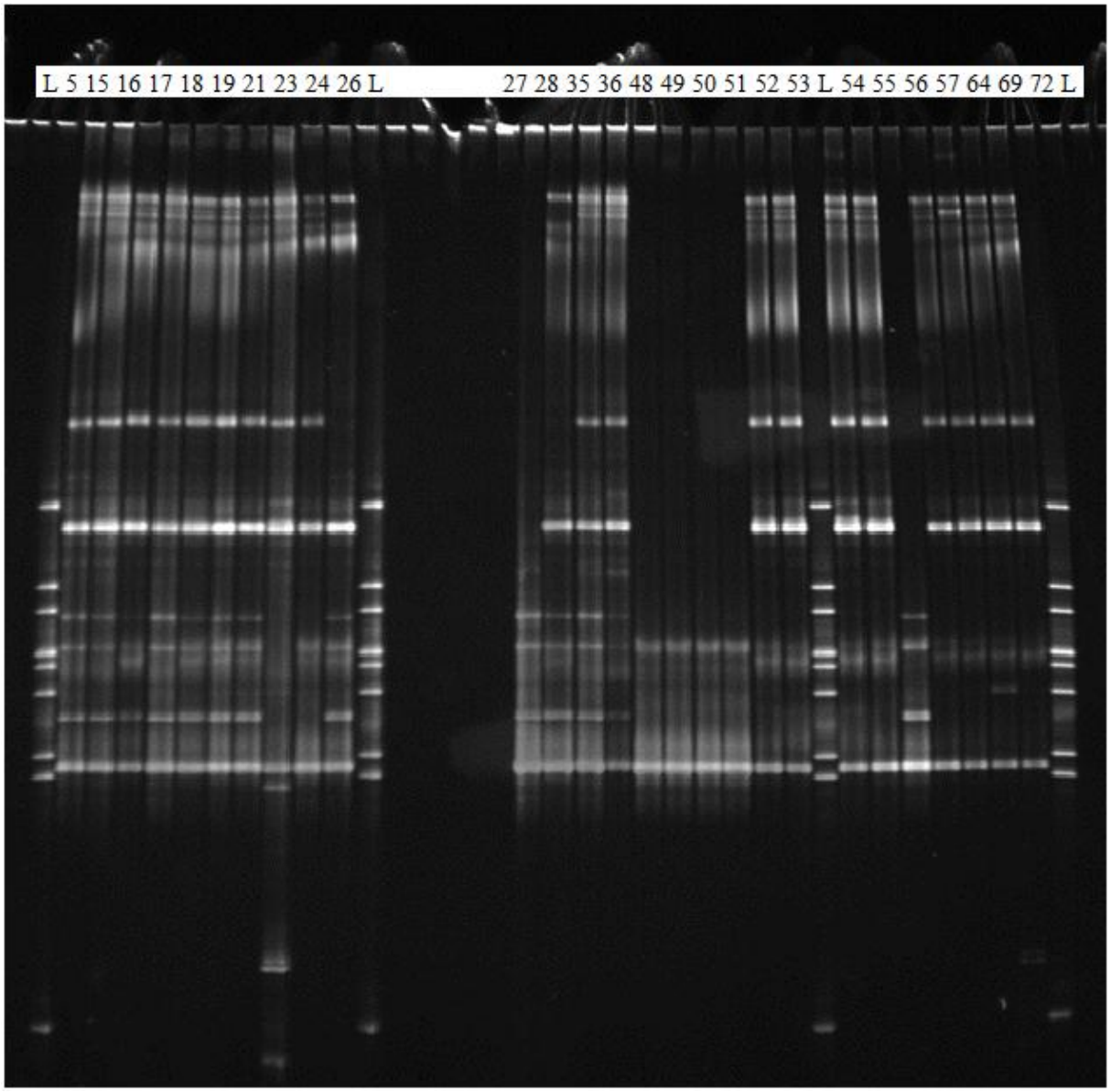

Figure 3.7: DGGE gel (35-55 % denaturing gradient) obtained from stool samples taken immediately after the mice had been treated with antibiotics. The various lanes are marked with the mice identification numbers and lanes marked with L are the standard.

Table 3.7: Diversity indices obtained from the DGGE gel of mice gut microbiota 0 weeks after antibiotics treatment. The sample names refer to the mice identification number. The average and standard deviation was calculated for the co-housed (CH), the single-housed (SH) and both of them together (all). Lcn 2 KO-mice are shown in red, Wt-mice in blue and Ht-mice in green.

	Mouse id	Band richness (K)	Shannon index (H')	Evenness index (J')
KO-mice	23	20.0	2.68	0.894
	24	18.0	2.55	0.882
	72	15.0	2.30	0.848
	57	14.0	2.13	0.808
	69	17.0	2.39	0.844
	28	19.0	2.52	0.857
	51	7.0	1.51	0.776
	52	20.0	2.49	0.830
	55	19.0	2.56	0.868
	64	13.0	2.13	0.830
	Average all	16.2±4.1	2.33±0.34	0.844±0.035
	Average CH	15.6±5.5	2.24±0.44	0.832±0.035
	Average SH	16.8±2.4	2.41±0.21	0.855±0.034
Wt-mice	5	23,0	2.61	0.833
	15	26,0	2.75	0.843
	18	26,0	2.84	0.871
	48	6,0	1.30	0.723
	50	6,0	1.33	0.742
	54	21,0	2.58	0.847
	56	10,0	1.73	0.751
	Average all	16.9±9.2	2.16±0.68	0.801±0.060
	Average CH	10.8±7.1	1.73±0.60	0.766±0.056
	Average SH	25.0±1.7	2.73±0.11	0.849±0.020
Ht-mice	16	24.0	2.77	0.871
	17	27.0	2.81	0.852
	19	25.0	2.81	0.874
	21	22.0	2.64	0.854
	35	22.0	2.71	0.878
	36	23.0	2.62	0.837
	26	19.0	2.54	0.864
	27	13.0	2.11	0.824
	49	8.0	1.52	0.733
	53	18.0	2.30	0.795
	Average all	20.1±5.8	2.48±0.41	0.838±0.045
	Average CH	14.5±5.1	2.12±0.43	0.804±0.055
	Average SH	23.8±1.9	2.73±0.08	0.861±0.016

The NM-MDS analysis of both the single-hosed mice and the co-housed mice produced a stress value of 0.1703 (< 0.2) and thus the results are reliable to be interpreted (figure 3.8). It is difficult to see any pattern in the coordinate system as there seemed to be major overlaps between the three different genotypes. The hypothesis testing with two-way ANOSIM however, showed that both genetics and habitation were significant factors ($p<0.05$). This result indicate that there is a significant difference in microbiota between the three different genotypes, and also that there is a significant difference in microbiota between the single housed mice and the co-housed mice. The R-values were all > 0 although quite low, reflecting a weak difference between the groups (Table 3.8).

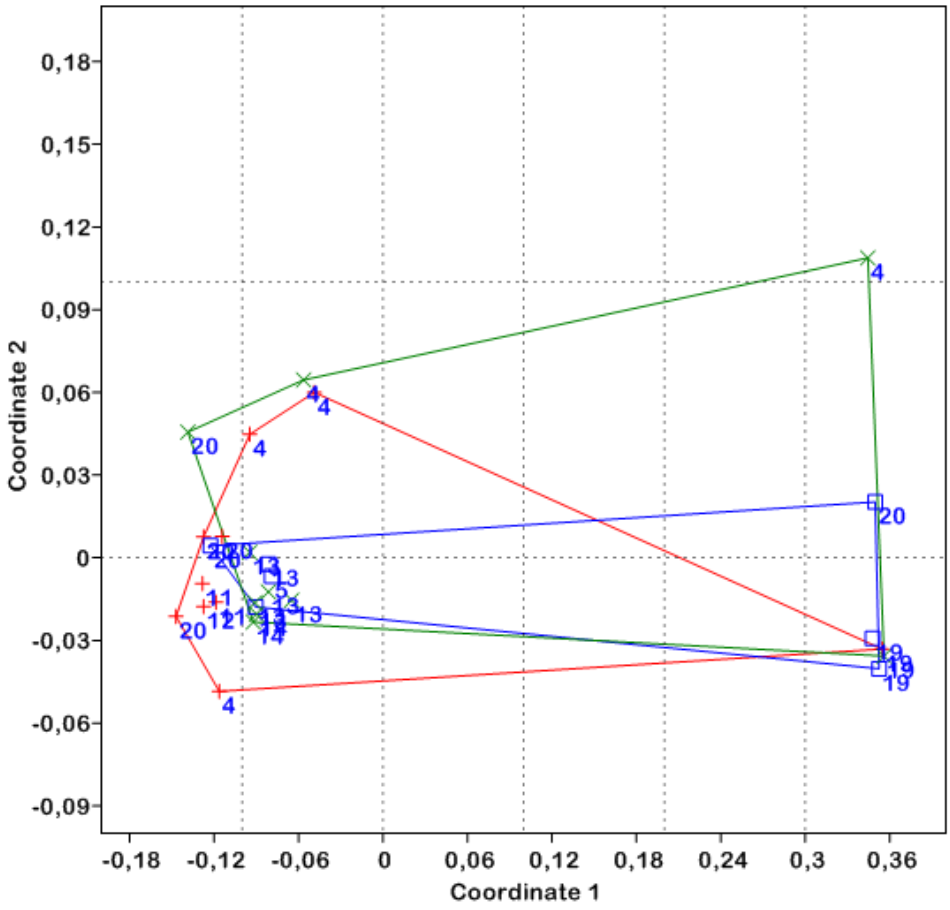

Figure 3.8: NM-MDS based on Bray-Curtis distance measure showing the results from the DGGE gel of mice gut microbiota 0 weeks after the antibiotics treatment. Both the single-housed mice and the co-housed mice are plotted. Lcn 2 KO-mice are marked in red, Ht-mice in green and Wt-mice in blue and in addition the outer-points for each group are marked with a line. The points on the graphs are marked with the mice litter number (Table 2.1).

Table 3.8: Two-way ANOSIM of gut microbiota of both the single housed mice and the co-housed mice, 0 weeks after antibiotics treatment, with sequential Benferroni significance.

Factor	**ANOSIM R-value**	**p-value**
Genetics	0.21083	0.0316
Habitation	0.28052	0.027

The NM-MDS analysis of the co-housed mice produced a stress value of 0.1153 (< 0.2) and the results are therefore reliable to be interpreted (figure 3.9). The plot show that the mice in cage 7 that were siblings except for one mouse and was dominated by lcn2 KO-mice, are clustered together, and the mice in cage 8 that were siblings and was dominated by Wt-mice are clustered together. These results indicate that the mice in cage 7 had developed a similar microbial profile and the mice in cage 8 had developed a similar microbial profile.

The NM-MDS plots also show a clear tendency of the Lcn2 KO-mice to be clustered to the left side of the coordinate system, while the Wt/Ht-mice are clustered to the right. Cage number 9 was a special case as two of the mice from litter 4, mice number 27 (Ht) and 56 (Wt), had to be placed in separate cages due to fighting amongst the males. This meant that only mice number 26 (Ht) and 28 (Lcn 2 KO) lived in the same cage together throughout the whole experiment of part 2. Despite the fact that the two former lived alone throughout the whole experiment, they are still found on the right hand side in the coordinate system where the rest of the Wt/Ht-mice are found. Results from one-way ANOSIM however, showed that the factor of genotype was not significant for the gut microbiota of the co-housed mice (Table 3.9).

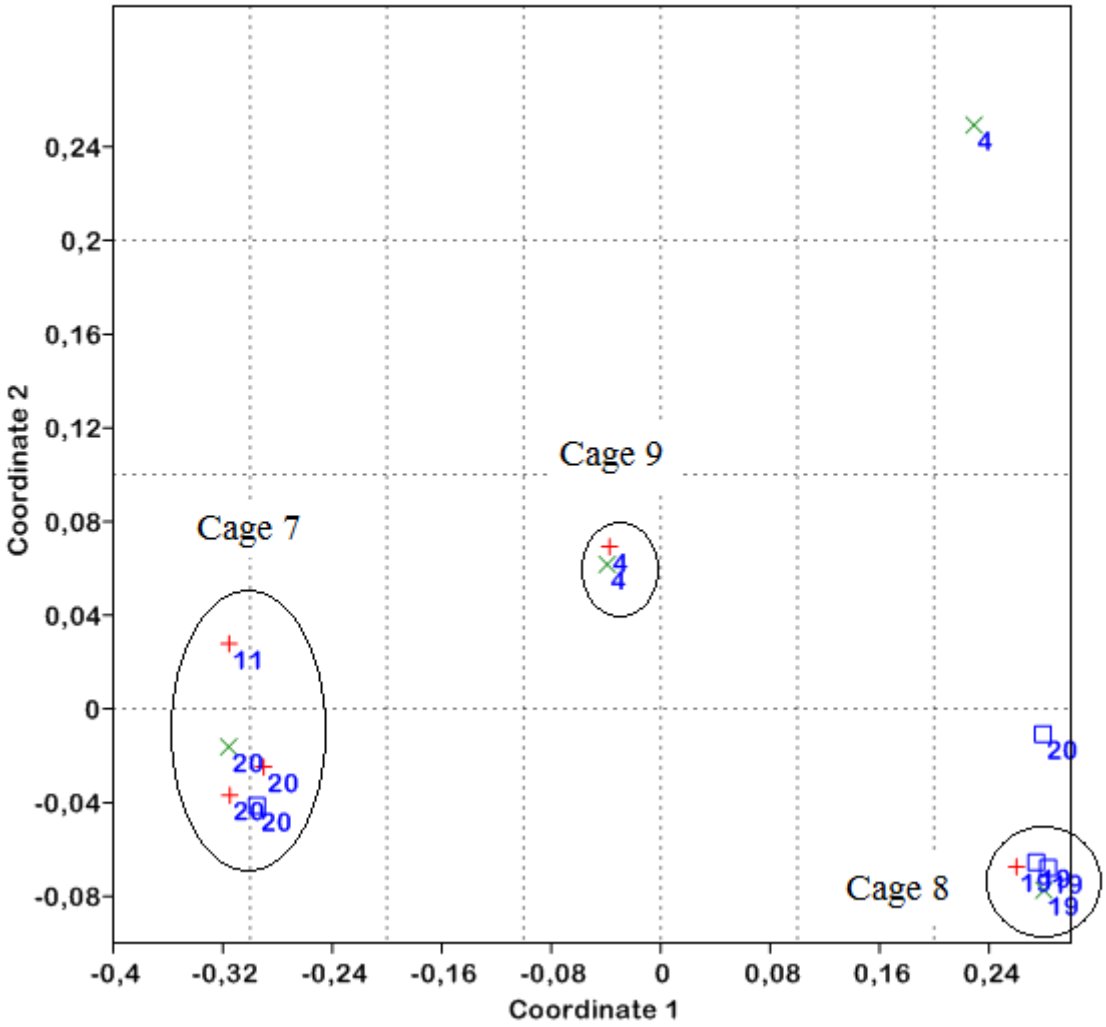

Figure 3.9: NM-MDS based on Bray-Curtis distance measure from the DGGE gel, 0 weeks after the antibiotics treatment. Only results from the co-housed mice are shown. The points on the graphs are marked with the mice litter number. Lcn 2 KO-mice are marked in red, Ht-mice in green and Wt-mice in blue. The circles indicate the mice that had shared the same cage together.

Table 3.9: One-way ANOSIM of gut microbiota, 0 weeks after antibiotics treatment. The analysis was only performed on the co-housed mice with sequential Benferroni significance.

	Groups	**ANOSIM R-value**	**p-value**
Total	-	0.02597	0.3406
Between groups	KO and Wt	0.1375	0.1964
	KO and Ht	-0.0375	0.5807
	Wt and Ht	-0.1042	0.5666

The produced coordinate system obtained from the NM-MDS analysis of the single-housed mice clearly separates the three groups with no overlaps (figure 3.10). The stress value was 0.1697 and thereby < 0.2. Results from the one-way ANOSIM analysis indicate that there is a difference between the Lcn 2 KO-mice and the Ht-mice (R= 0.5947), and this difference is significant ($p<0.05$). Despite that the comparison between Lcn 2 KO-mice and Wt-mice produced an R-value of 0.4359, which indicate that there is a difference between the two groups of genotypes, the difference however was not significant (Table 3.10).

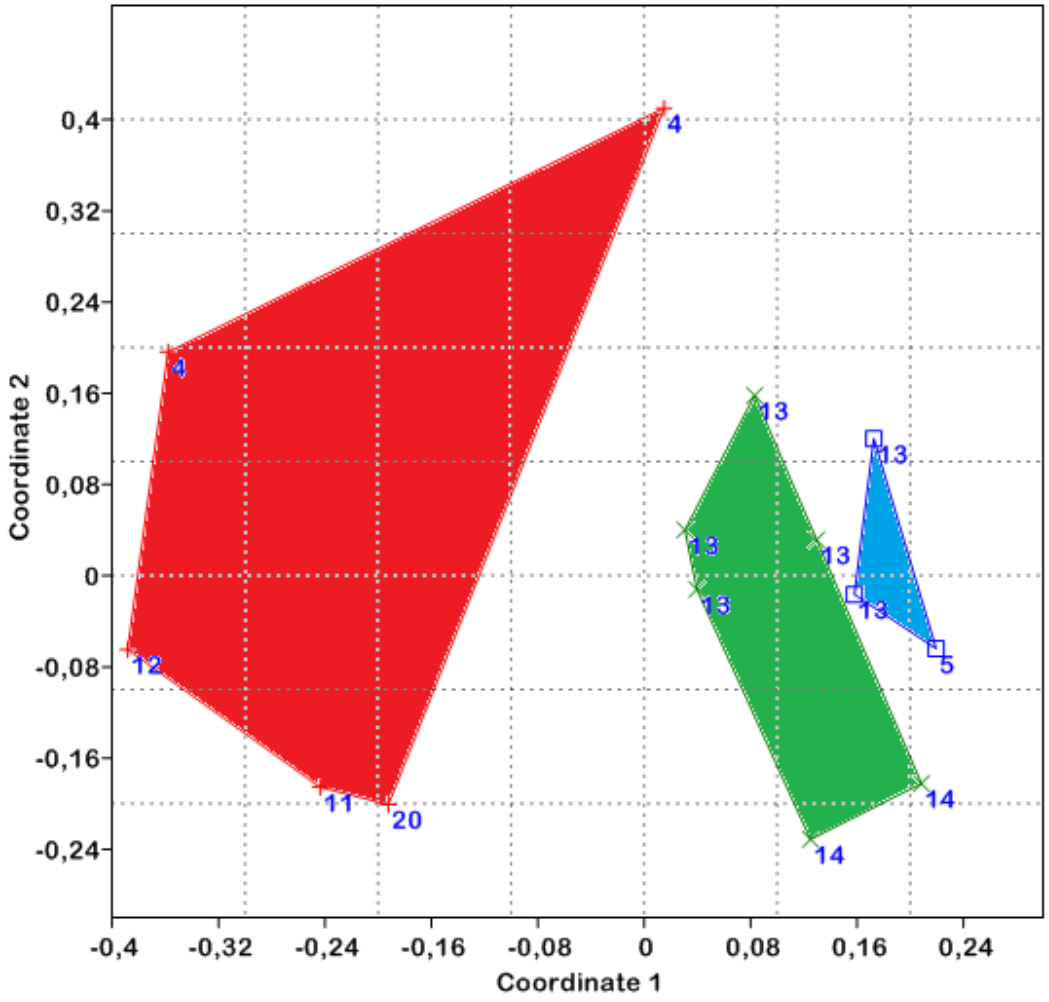

Figure 3.10: NM-MDS based on Bray-Curtis distance measure showing the results from the DGGE gel of mice gut microbiota 0 weeks after antibiotics treatment. Only the results from the single housed mice have been plotted. Lcn 2 KO-mice are marked in red, Ht-mice in green and Wt-mice in blue and in addition the outer-points for each group are marked with a line and filled in with the respective colour. The points on the graphs are marked with the mice litter number

Table 3.10: One-way ANOSIM of gut microbiota, 0 weeks after antibiotics treatment. The analysis was only performed on the single housed mice with sequential Benferroni significance.

	Groups	ANOSIM R-value	p-value
Total	-	0,3957	0,0039
Between groups	KO and Wt	0,4359	0,0738
	KO and Ht	0,5947	0,0028
	Wt and Ht	-0,07407	0,605

3.4.2 MOUSE MICROBIOTA 2 WEEKS AFTER ANTIBIOTICS TREATMENT

Figure 3.11 shows the DGGE gel from the GI microbiota 2 weeks after the antibiotics treatment. The mice were at this stage 23 weeks old. The gel contains 60 bands.

For the single housed mice, the average band richness (K), Shannon diversity index (H') and Evenness index (J') were fairly similar between the KO-mice, Ht-mice and Ht-mice (Table 3.10). Results from one-way ANOVA showed no significant difference of neither of the diversity indexes.

For the co-housed mice the diversity indices were also observed to be similar between the Lcn 2 KO-mice, Wt-mice and Ht-mice and one-way ANOVA confirmed that there were no significant differences.

When comparing the single housed mice with the co-housed mice in respect to band richness, the single-housed mice were observed to have a higher average band richness compared to the mice that had been living co-housed. Results from the two-way ANOVA showed that the habitat of mice is a significant factor on band richness ($p<0.05$), but not the factor of genotype alone, nor the interaction of genotype and habitat. This means that the single housed mice had a significantly higher band richness compared to the co-housed mice. The single-housed mice generally also had a higher average diversity index (H') compared to the mice that had been living co-housed. Results from two-way ANOVA showed that habitation is a significant factor ($p<0.05$) for the Shannon diversity index, but neither the factor of genotype alone, nor the interaction between genotype and habitat. The average evenness indices for the different groups were fairly similar and showed no significant differences in respect to genotype and habitat.

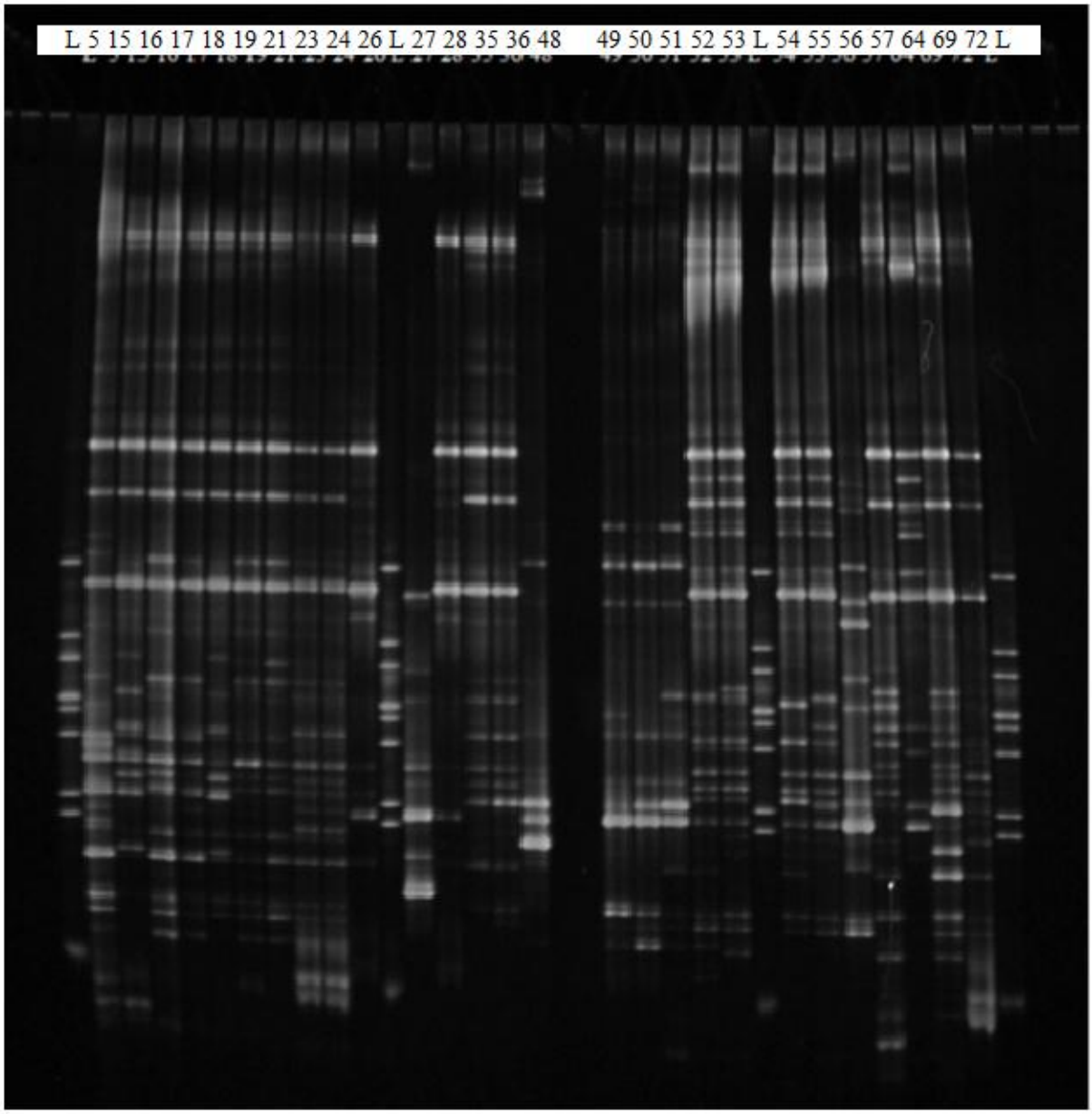

Figure 3.11: DGGE gel (35-55 % denaturing gradient) obtained from stool samples taken 2 weeks after the mice had ended the antibiotics treatment. The various lanes are marked with the mice identification numbers and lanes marked with an L are the standard.

Table 3.11: Diversity indices obtained from DGGE gel of mice gut microbiota 2 weeks after antibiotics treatment. The sample names refer to the mice identification number. The average and standard deviation was calculated for the co-housed (CH), the single-housed (SH) and both of them together (all). Lcn 2 KO-mice are shown in red, Wt-mice in blue and Ht-mice in green.

	Mouse id	Band richness (K)	Shannon index (H')	Evenness index (J')
KO-mice	23	23.0	2,77	0,882
	24	19.0	2,56	0,869
	72	16.0	2,27	0,820
	57	24.0	2,67	0,841
	69	24.0	2,73	0,858
	28	9.0	1,74	0,792
	51	11.0	1,97	0,820
	52	24.0	2,80	0,881
	55	25.0	2,83	0,880
	64	16.0	2,62	0,945
	Average all	19.1±5.9	2.50±0.38	0.859±0.043
	Average CH	17.0±7.3	2.39±0.50	0.864±0.060
	Average SH	21.2±3.6	2.60±0.20	0.854±0.024
Wt-mice	5	24.0	2,77	0,873
	15	20.0	2,74	0,915
	18	23.0	2,65	0,846
	48	9.0	1,91	0,867
	50	13.0	2,14	0,835
	54	25.0	2,78	0,864
	56	15.0	2,53	0,935
	Average all	18.4±6.2	2.50±0.35	0.876±0.036
	Average CH	15.5±6.8	2.34±0.39	0.875±0.042
	Average SH	22.3±2.1	2.72±0.06	0.878±0.035
Ht-mice	16	28.0	3,04	0,913
	17	21.0	2,59	0,851
	19	22.0	2,47	0,798
	21	23.0	2,62	0,835
	35	21.0	2,51	0,824
	36	21.0	2,42	0,794
	26	11.0	1,96	0,819
	27	11.0	1,97	0,820
	49	11.0	2,02	0,844
	53	28.0	3,01	0,903
	Average all	19.7±6.5	2.46±0.39	0.840±0.040
	Average CH	15.3±2.7	2.24±0.51	0.846±0.040
	Average SH	22.7±2.7	2.61±0.23	0.836±0.044

The NM-MDS analysis of both the single-housed mice and the co-housed mice produced a stress value of 0.1846 (< 0.2) and thus the results are reliable to be interpreted. The coordinate system is not shown, as the plot resembles the one in figure 3.8 and thus, it is difficult to visualize particular patterns. The hypothesis testing with two-way ANOSIM however, showed that both genetics and habitation were significant factors ($p<0.05$) (Table 3.12). This result indicates that there is a significant difference in microbiota between the three different genotypes, and also that there is a significant difference in microbiota between the single housed mice and the co-housed mice.

Table 3.12: Two-way ANOSIM of gut microbiota of both the single housed mice and the co-housed mice, 2 weeks after antibiotics treatment, with sequential Benferroni significance.

Factor	**ANOSIM R-value**	**p-value**
Genetics	0.21087	0.0253
Habitation	0.46872	0.0001

The NM-MDS analysis from the co-housed mice produced a stress value of 0.0778 (< 0.2) and the results are therefore reliable to be interpreted (figure 3.12). Results from the one-way ANOSIM (Table 3.13), showed that genetics was not a significant factor of differences in gut microbiota between the Lcn 2 KO-mice, Wt-mice and Ht-mice that had been living co-housed ($p>0.05$).

Two weeks after the antibiotics treatment, the plot shows the same pattern as figure 3.8. The mice in cage 7 that were siblings except for one mouse and that was dominated by Lcn2 KO-mice, are clustered together, and the mice in cage 8 that were siblings and was dominated by Wt-mice are clustered together. These results indicate that the mice in cage 7 had developed a similar microbial profile and the mice in cage 8 had developed a similar microbial profile.

The NM-MDS plots show a clear tendency of the Lcn2 KO-mice to be clustered to the right side of the coordinate system, while the Wt/Ht-mice are clustered to the left. The two mice that lived alone in cage 9 throughout the whole experiment are found on the left hand side in the coordinate system, where the rest of the Wt/Ht-mice are found.

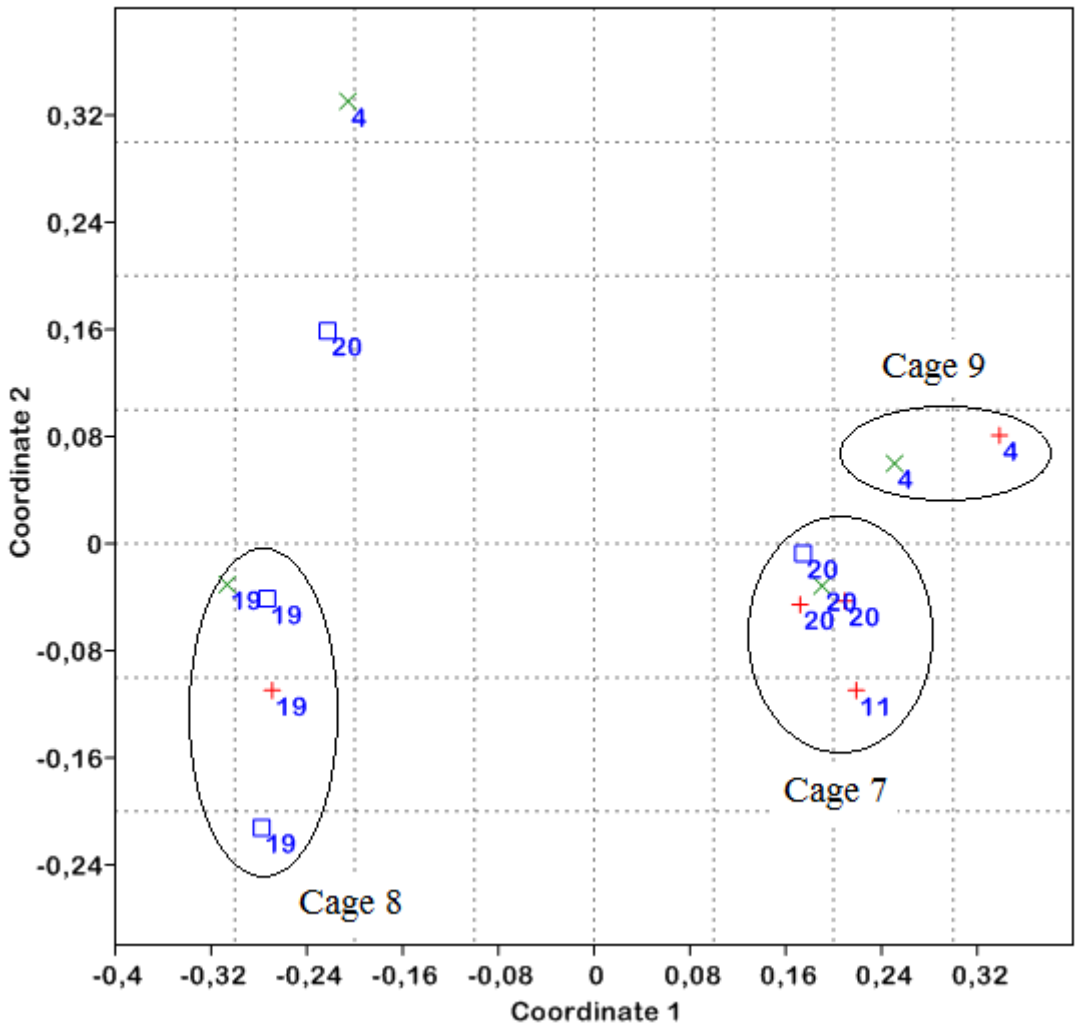

Figure 3.12: NM-MDS 3D plot based on Bray-Curtis distance measure showing the results from the DGGE gel of mice gut microbiota, 2 weeks after antibiotics treatment. Only the results from the co-housed mice are shown. Lcn 2 KO-mice are marked in red, Ht-mice in green and Wt-mice. The circles indicate the mice that had shared the same cage together and the points on the graphs are marked with the mice litter numbers (Table 2.1).

Table 3.13: One-way ANOSIM of gut microbiota, 2 weeks after antibiotics treatment. The analysis was only performed on the co-housed mice with sequential Benferroni significance.

	Groups	ANOSIM R-value	p-value
Total	-	0.06169	0.2579
Between groups	KO and Wt	0.2313	0.116
	KO and Ht	0.01875	0.3399
	Wt and Ht	-0.08333	0.5474

The stress value obtained from the NM-MDS analysis of the single-housed mice was 0.2141 and thus > 0.2 gives an uncertain result (figure 3.13). The coordinate system clearly separates the three groups, with a small overlap between the Wt-mice and the Ht-mice. From one-way ANOSIM the results from the comparison between Lcn 2 KO-mice and Ht-mice (R = 0.632) shows a strong significant difference in the composition of gut microbiota (Table 3.13). The comparison between Lcn 2 KO-mice and Wt-mice gave an R-value = 0.4974, thereby indicating a smaller difference, and the p-value is on the border line of not being significant. There is no significant difference between the gut microbiota of Wt-mice and Ht-mice (Table 3.14).

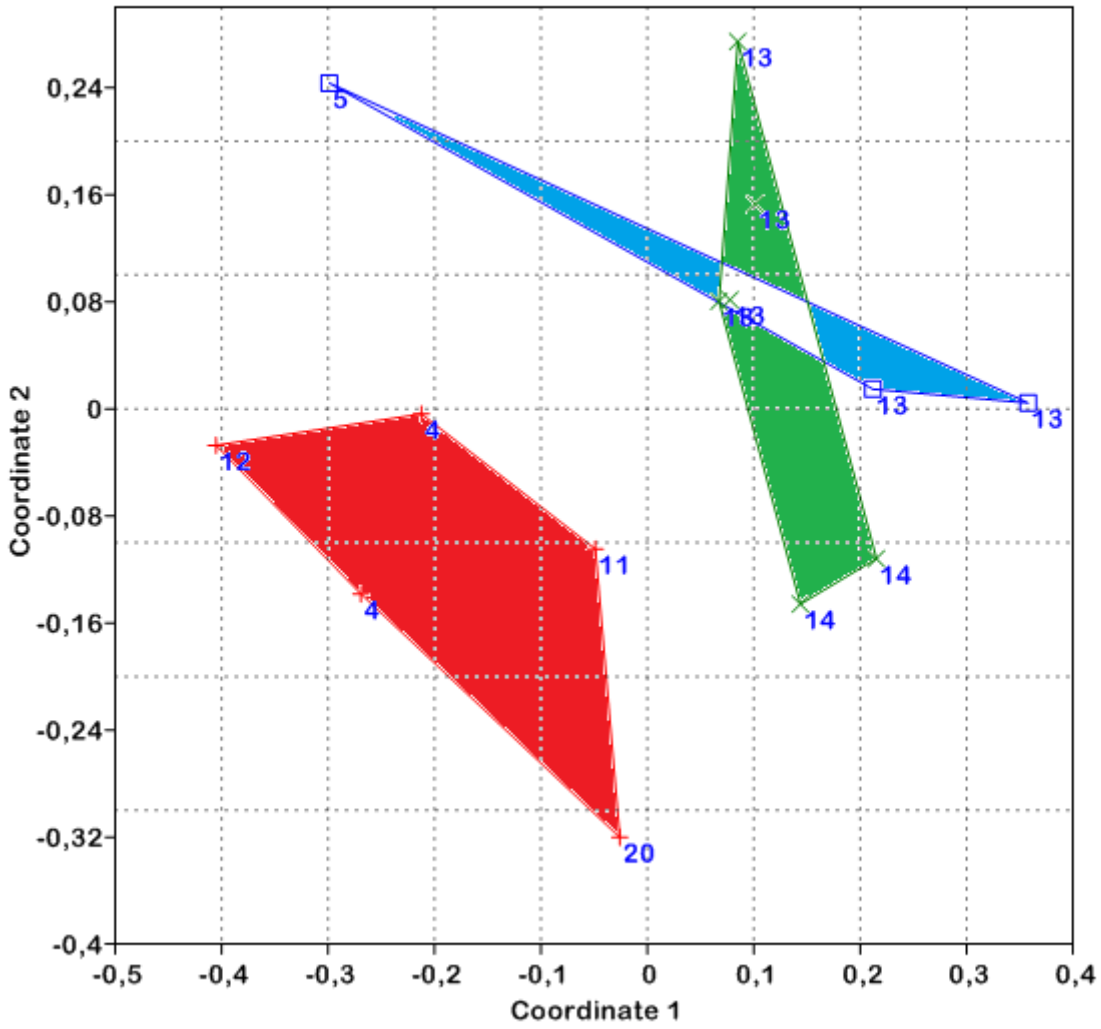

Figure 3.13: NM-MDS based on Bray-Curtis distance measure showing the results from the DGGE gel of the gut microbiota 2 weeks after the antibiotics treatment. Only the result from the single housed mice have been plotted. Lcn 2 KO-mice are marked in red, Ht-mice in green and Wt-mice in blue and in addition the outer-points for each group are marked with a line. The points on the graphs are marked with the mice litter number (Table 2.1).

Table 3.14: One-way ANOSIM analysis of the gut microbiota, 2 weeks after antibiotics treatment. The analysis is performed only on the single-housed mice with sequential Benferroni significance.

	Groups	ANOSIM R-value	p-value
Total	-	0,4603	0,0019
Between groups	KO and Wt	0,4974	0,0576
	KO and Ht	0,632	0,0016
	Wt and Ht	0,284	0,1335

3.4.3 MOUSE MICROBIOTA 6 WEEKS AFTER ANTIBIOTICS TREATMENT

Figure 3.14 shows the DGGE gel from the GI microbiota 6 weeks after the antibiotics treatment, when the mice were 23 weeks old. The gel contains 68 bands.

For the single housed mice, the average band richness (K), Shannon diversity index (H') and evenness index (J') were observed to be similar between the Lcn 2 KO-mice, Ht-mice and Ht-mice (Table 3.14). These observations were confirmed by one-way ANOVA, which showed no significant differences between the diversity indices with regards to genotype.

For the co-housed mice the diversity indices were also observed to be similar between the three different genotypes. One-way ANOVA showed no significant differences of neither K'-, H'- or J'-values with regards to genotype.

When comparing the single housed mice with the co-housed mice, the average band richness was observed to be quite similar for the two groups. Results from two-way ANOVA confirmed these observations, that the effects of genotype and habitation were not significant factors of band richness. The average H'-values were generally higher for the single-housed mice compared to the co-housed mice. Results from two-way ANOVA however, showed no significant differences of diversity with respect to the factors of genotype and habitat. There was also no significant difference between the groups with regards to evenness.

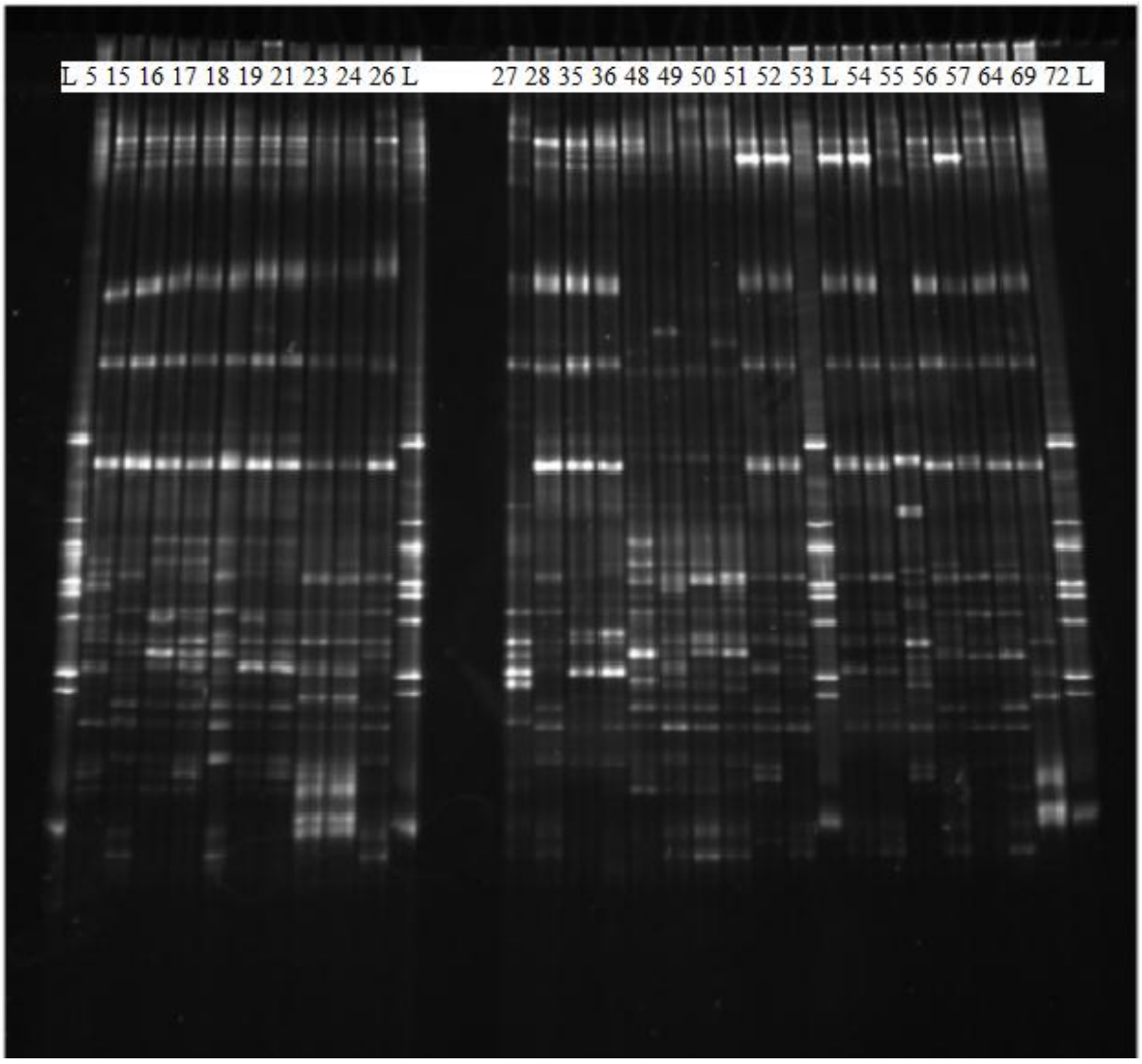

Figure 3.14: DGGE gel (35-55 % denaturing gradient) obtained from stool samples taken 6 weeks after the mice had ended the antibiotics treatment. The various lanes are marked with the mice identification numbers and lanes marked with an L are the standard.

Table 3.15: Diversity indices obtained from the DGGE-gel 6 weeks after antibiotics treatment. The average and standard deviation was calculated for the co-housed (CH), the single-housed (SH) and both of them together (all). Lcn 2 KO-mice are shown in red, Wt-mice in blue and Ht-mice in green.

	Mouse id	Band richness (K)	Shannon index (H')	Evenness index (J')
KO-mice	23	24.0	2.82	0.886
	24	24.0	2.77	0.871
	72	23.0	2.83	0.904
	57	29.0	2.77	0.821
	69	27.0	2.98	0.904
	28	30.0	2.77	0.814
	51	35.0	3.14	0.884
	52	24.0	2.57	0.809
	55	23,0	2.49	0.795
	64	19,0	2.27	0.770
	Average all	25.8±4.5	2.74±0.25	0.846±0.049
	Average CH	26.2±6.3	2.65±0.33	0.814±0.042
	Average SH	25.4±2.5	2.83±0.09	0.877±0.034
Wt-mice	5	29.0	2.95	0.877
	15	29.0	2.84	0.844
	18	31.0	3.19	0.928
	48	28.0	2.76	0.827
	50	25.0	2.86	0.887
	54	17.0	2.38	0.841
	56	30.0	2.95	0.867
	Average all	27.0±4.8	2.85±0.25	0.867±0.034
	Average CH	25.0±5.7	2.74±0.25	0.855±0.027
	Average SH	29.7±1.2	2.99±0.18	0.883±0.042
Ht-mice	16	27,0	2.83	0.859
	17	27,0	3.00	0.910
	19	25,0	2.79	0.865
	21	34,0	3.11	0.881
	35	17,0	2.52	0.889
	36	29,0	2.75	0.815
	26	28,0	2.81	0.844
	27	24,0	2.58	0.812
	49	31,0	3.21	0.936
	53	23,0	2.48	0.790
	Average all	26.5±4.7	2.81±0.25	0.860±0.046
	Average CH	26.5±3.7	2.77±0.33	0.846±0.064
	Average SH	26.5±5.6	2.83±0.21	0.870±0.032

The NM-MDS analysis of both the single-housed mice and the co-housed mice produced a stress value of 0.2114 (> 0.2) and thus gives an unclear result (figure 3.15). The coordinate system shows that the three different genotypes overlap each other, although seven samples from the Lcn 2 KO-mice, three samples from the Wt-mice and three samples from the Ht-mice stand out and are not part of the overlapping area.

The hypothesis testing with two-way ANOSIM showed that habitation was a significant factor for the profiles of microbiota, and the factor of genetics is just on the borderline of being significant (Table 3.16). This result indicates that there is a significant difference in microbiota between the three different genotypes, and also that there is a significant difference in microbiota between the single housed mice and the co-housed mice. The R-values were all > 0 although quite low, reflecting a weak difference between the groups.

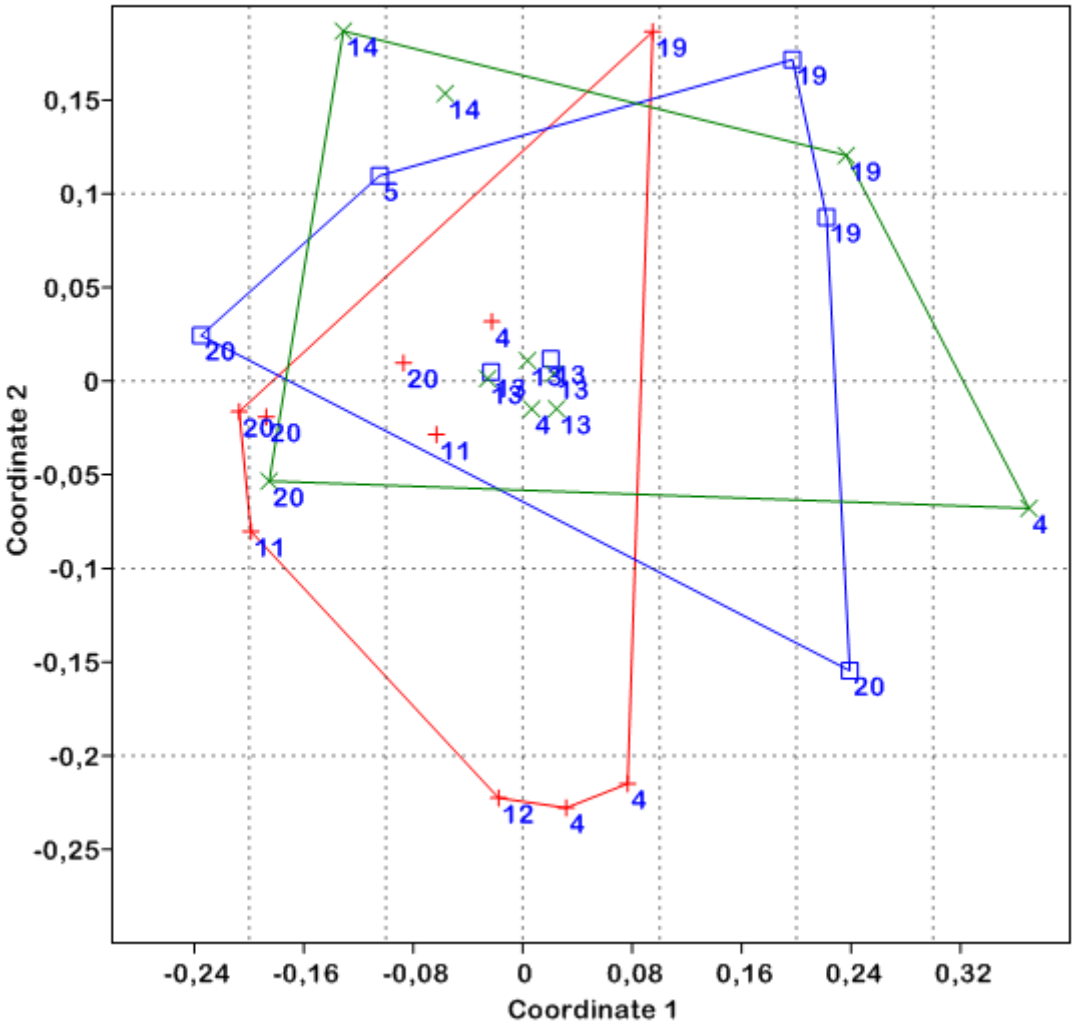

Figure 3.15: NM-MDS based on Bray-Curtis distance measure showing the results from the DGGE gel of mice microbiota 6 weeks after the antibiotics treatment. Both the single-housed mice and the co-housed mice are plotted. Lcn 2 KO-mice are marked in red, Ht-mice in green and Wt-mice in blue and in addition the outer-points for each group are marked with a line. The points on the graphs are marked with the mice litter number (Table 2.1).

Table 3.16: Two-way ANOSIM of gut microbiota of the single housed mice and the co-housed mice, 6 weeks after antibiotics treatment, with sequential Benferroni significance.

Factor	**ANOSIM R-value**	**p-value**
Genetics	0.1677	0.0528
Habitation	0.28314	0.0147

The NM-MDS analysis of the co-housed mice produced a stress value of 0.1496 (<0.2) and the results are therefore reliable to be interpreted (figure 3.16). The tendency for the mice in cages with mixed genotypes to have developed a microbiota in favour of the genotype in majority follows the same pattern as shown in figure 3.8. Results from the one-way ANOSIM (Table 3.17), showed that there was no significant differences in gut microbiota between the Lcn 2 KO-mice, Wt-mice and Ht-mice that had been living co-housed (p>0.05).

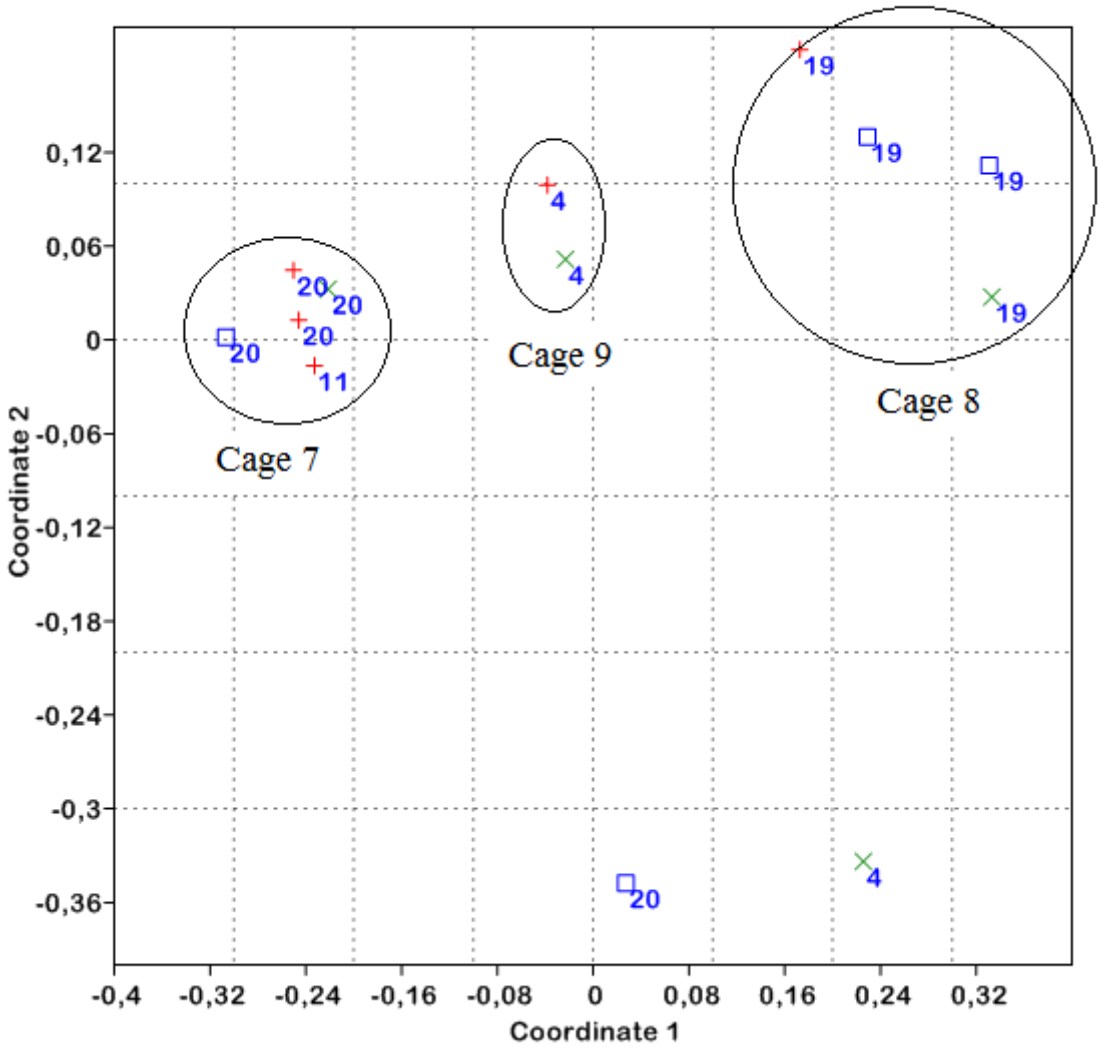

Figure 3.16: NM-MDS based on Bray-Curtis distance measure showing the results from the DGGE gel 6 weeks after antibiotics treatment, where only the co-housed mice are shown. Lcn 2 KO-mice are marked in red, Ht-mice in green and Wt-mice in blue and in addition the outer-points for each group are marked with a line. The points on the graphs are marked with the mice litter number (Table 2.1).

Table 3.17: One-way ANOSIM analysis of the mice gut microbiota, 6 weeks after antibiotics treatment. The analysis was only performed on the co-housed mice.

	Groups	ANOSIM R-value	p-value
Total	-	0.05195	0.2918
Between groups	KO and Wt	0.175	0.1604
	KO and Ht	0.1	0.2031
	Wt and Ht	-0.2396	1

The stress value obtained from the NM-MDS analysis of the single-housed mice was 0.1159 (< 0.2) and the results is therefore reliable to be interpreted. The coordinate system clearly separates the three groups with no overlaps (figure 3.17). Results from one-way ANOSIM showed that the comparison between Lcn KO-mice and Ht-mice (R = 0.4053) gave a significant difference in the composition of their microbiota. The comparison between Lcn 2 KO-mice and Wt-mice and between Wt-mice and Ht-mice all show R-values > 0, but the differences are weak and showed no significant difference (Table 3.18).

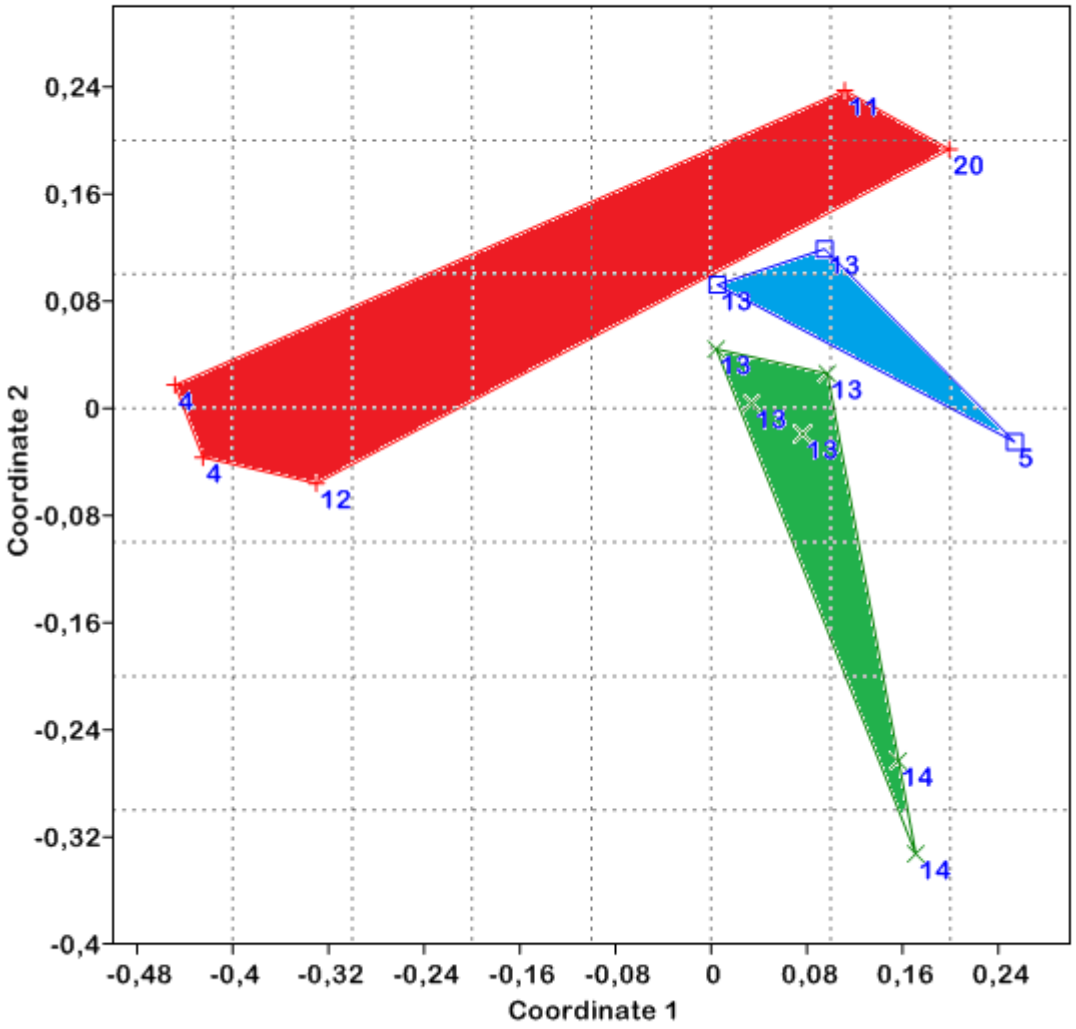

Figure 3.17: NM-MDS based on Bray-Curtis distance measure showing the results from the DGGE gel where only the results from the single-housed mice have been plotted. Lcn 2 KO-mice are marked in red, Ht-mice in green and Wt-mice in blue and in addition the outer-points for each group are marked with a line. The points on the graphs are marked with the mice litter number (Table 2.1).

Table 3.18: One-way ANOSIM analysis of the gut microbiota, 6 weeks after antibiotics treatment. The analysis was performed only on the single-housed mice.

	Groups	**ANOSIM R-value**	**p-value**
Total	-	0.2834	0.0183
Between groups	KO and Wt	0.08718	0.3202
	KO and Ht	0.4053	0.0097
	Wt and Ht	0.1235	0.2049

3.4.4 SUMMARY OF RESULTS

Lipocalin 2 prevents growth of bacteria that rely on catechol type siderophores for iron acquisition (Goetz, *et al.*, 2003; Flo, *et al.*, 2004). It hypothesized that Lcn2 can impart a selection pressure on establishment of the gut microbiota and thus influence the commensal diversity.

Summing up the results, Lcn 2 seems to effect the natural colonization of the mice gut, as well as the re-establishment of the microbiota after a perturbation with antibiotics treatment. However, the factors of environment (habitation) and maternity were also important factors for the observed differences in microbiota between Wt/Ht-mice and Lcn2 KO-mice.

4. DISCUSSION

4.1 DESCRIPTIVE STUDIES

The aim of this project was to find out whether the antimicrobial protein, Lipocalin 2 influences the colonization of the gut microbiota in mice. To study the composition of the microbiota in mice over time, the most convenient method was to collect stool samples from each mouse. In this way the mice would not suffer any pain or discomfort. Although the bacterial composition is not the same in all parts of the gastrointestinal tract, the majority of the bacteria leave the faecal route and thus, the faecal composition may reflect the gastrointestinal tract (Zoetendal, *et al*., 2001). The hypotheses for this study were:

1) *Lipocalin 2 deficient mice (Lcn 2 KO-mice) establish a different gut microbial profile than Wt/Ht-mice.*
2) *The dominant genotype in cages of mice with mixed genotypes influences the minority in respect to the composition of the microbiota.*

To test the hypotheses, the experiment was designed as a two factor experiment in which the factors of genotype and habitation were investigated. The cage set-up was designed to have some cages with only Lcn2 KO-mice, wild type mice and heterozygous mice (single housing), and a few cages with mice of mixed genotypes (co-housing). In this way it would be possible to differentiate between the impact of genetics and habitation. The results from the single-housed mice are discussed firstly and secondly the co-housed mice.

By using an inbred strain of chimaeric C57BL/6 BL mice, it provided a way to control the genetics by using mice that were as similar as possible. The mice were reared in cages adjacent to each other, where exposure to microbes from sources other than littermates, parents and animal caretakers were limited. If differences in gut microbial profiles were observed, then the genotype of the mice would be one of the main factors determining the result. Based on one-way ANOSIM, the samples from the single housed mice in part 1 and part 2 of the research, all showed a significant difference ($p<0.05$) of gut microbiota between

Lcn2 KO-mice and Wt/Ht-mice. These differences are visualized in the NM-MDS plots were the Lcn2 KO-mice are all clustered together and are clearly separated from the Wt/Ht-mice (figure 3.6, 3.10 and 3.13). These results support the first hypothesis that Lipocalin 2 deficient mice establish a different gut microbial profile than Wt/Ht-mice.

The diversity indices were in general fairly similar for the single housed mice between the three different genotypes, both before and after the antibiotics treatment. What then could explain these differences observed in gut microbiota between Lcn2 KO-mice and Wt-mice? In the simplest scenario, specific host alleles would result in a different microbiota that may either be harmful or beneficial to host health (Spor *et al*., 2011). As was mentioned in chapter 1.2, the study by Flo *et al.*, (2004), demonstrated that Lcn2 KO-mice were more sensitive to certain Gram-negative bacteria and more readily died of sepsis than normal mice. One possible explanation for this result could be that Lcn2 KO mice are colonized and develop a different microbial composition compared to Wt-mice. By lacking the antimicrobial protein, Lcn 2, it may be speculated that harmful bacterial strains more easily get access to, and establish themselves in the gut of Lcn2 KO-mice, and thus this group of mice would be more susceptible towards bacterial infections. Studies have suggested that a specific combination of microorganisms in the gut can affect host health, thus, host control over the gut microbiota is an important factor for regulation (Spor *et al*., 2011). A study by Zhang *et al*., (2010) investigated the contribution of host genetics and diet in shaping the gut microbiota. The DGGE analysis indicated that the microbiota of APOA1 deficient mice (the main protein component of plasma high-density lipoprotein) had a different community structure from that of wild-type mice. The Wt-mice were healthy and had normal weight and glucose tolerance, whereas the APOA1 deficient mice were obese and had impaired glucose tolerance. Variations in those host genes that contribute to host properties of the gut habitat thus have a strong potential to affect the community structure in the gut microbiota (Spor, *et al*., 2011).

To evaluate the influence of habitation versus genotype on the gut microbiota, mice of mixed genotypes were cohabited at 6 weeks of age. As was explained in chapter 1.6, mice tend to pursue coprophagy, therefore mice living together often develop similar composition of their gut microbiota (Bugle & Rubin, 1993). If mice of mixed genotypes were placed in the same cage together, how would this affect the microbial profiles of the mice? It was hypothesized that the dominant genotype would influence the minority in respect to the composition of the microbiota. To test this hypothesis, one of the cages of mixed genotypes contained more Lcn 2 KO-mice than normal mice, the majority of the second cage consisted of Wt-mice and the

last cage was dominated by heterozygote mice. If the prediction was correct, one would expect cage number 7, which was dominated by Lcn 2 KO-mice, to be all clustered together in the NM-MDS plot. This result would indicate that all the mice had developed a similar composition of gut microbiota, as the distance between the samples would be proportional to the similarity between them (Holland, 2008). Same scenario would apply for cage number 8, which was dominated by Wt-mice. If genetics was one of the main factors influencing the colonization of the microbiota, then the mice in cage 7 would be clustered on one side of the coordinate system, while the mice in cage 8 would be clustered on the other side.

Results from the NM-MDS plots where only the co-housed mice were shown (figure 3.8 and 3.11), both showed that the mice in cage 7 (dominated by lcn2 KO-mice), were clustered together and the mice in cage 8 (dominated by Wt-mice) were clustered together. These results indicate that the mice in cage 7 had diverged to develop similar microbial profiles and the mice in cage 8 had diverged to develop a similar microbial profile. These observations are likely achieved through coprophagy. What was interesting, however, was that the NM-MDS plots also showed a clear tendency of the Lcn2 KO-mice to be clustered to the left side of the coordinate system, while the Wt/Ht-mice were clustered to the right. These observations of lcn2 KO-mice and Wt-mice being placed differently in the plots support the hypothesis that lipocalin 2 deficient mice establish a different gut microbial profile than Wt/Ht-mice, and that the dominant genotype influenced the minority in respect to the composition of the microbiota, thereby supporting the second hypothesis.

The question of why the majority of one mice genotype would influence the minority in the same mice cage to develop a gut microbiota that resembled the dominant genotype still remained unclear. If it is assumed that Lcn 2 KO-mice have a different composition of bacteria in their GM compared to Wt-mice, and that it's also assumed that Lcn KO-mice habit more harmful bacteria in their GM compared to wild-type mice, then a lcn 2 KO-mice placed in a cage with only Wt-mice might establish a healthy micobiota, whereas a Wt-mice placed in a cage with only Lcn 2 KO-mice might develop a more harmful microbiota. Research has shown that microbial transplantation experiments, where the microbiota of a diseased mouse is transplanted to a germ-free healthy mouse resulted in the diseased phenotype being transferred with the microbiota and vice versa. Such studies have not only been demonstrated in mice, but also on humans. Borody *et al*., (2003) showed that colonic infusion of donor human intestinal microbiota reversed ulcerative colitis in the selected patients, and You *et al*.,

(2008) reported to have successfully treated a patient with *C.difficile* infection by donor stool. These results support the theory that an imbalance of the microbiota is the cause and not a consequence of the diseases.

The experimental set-up was designed with the intention of comparing littermates in order to minimize other effects than the knockout of Lcn2. Unfortunately this design meant that the effect of genotype and maternity could not be differentiated. All the mice in cage 7, except for one were from the same litter, and all the mice in cage 8 were from the same litter, thus it may seem that mouse faecal microbiota clustered according to litter, regardless of Lcn2 genotype. By investigating only the two factors: genotype and habitation, it is difficult to determine if the clustering of Lcn 2 KO-mice to one side and Wt-mice to the other side, was influenced by the maternal environment or if it was due to host genetics. Although the mice used in this experiment were an inbred strain of C57BL/6, thus genetically highly similar, and all the mice were exposed to the same environment, the composition of the microbiota between the different mothers is not identical. As was explained previously (chapter 1.1), one of the earliest factors that influence the colonization of the microbiota is the maternal environment. This maternal effect occurs when the mice are born vaginally and therefore the microbiota of the mother is their primary inoculum (Spor *et al*., 2011). Siblings from one litter may thus be exposed to a microbial community that differs slightly from siblings of another litter, which may explain the clustering of the two litters on each side of the plot. Several approaches have been used to try and limit the maternal effect, e.g. cross fostering (swapping offspring between two mothers after birth) and inoculation of microbiota into germ-free mice in order to standardize the microbiota. In general however, genetic polymorphism, rearing and housing conditions as well as their interactions, need to be incorporated into models when studying the impact of genetics on the microbiota (Spor *et al*., 2011).

If the observed pattern, with the majority of Lcn 2 KO-mice found on one side of the coordinate system and the majority of Wt-mice found on the other side was in fact due to differences in gut microbiota and not the maternity factor, then one would expect the single-housed Lcn 2 KO-mice to be found amongst the mice in cage 7 (dominated by Lcn 2 KO-mice) and the single-housed Wt-mice to be found amongst the mice in cage 8 (dominated by Wt-mice). By observing figure 3.4 (before the antibiotics treatment) it seems that the single-housed Lcn 2 KO-mice are found amongst the other co-housed Lcn 2 KO-mice, while the single-housed Wt-mice are found amongst the other co-housed Wt-mice. This result indicates

that the observed differences in microbiota between co-housed Lcn2 KO-mice and co-housed Wt-mice are influenced by genotype and to a less extent by the maternity factor. However, by observing figure 3.8 (after the antibiotics treatment) the single-housed Wt-mice are clustered amongst the co-housed Lcn2 KO-mice, thus in this case it seems that the maternity factor is the superior factor for the co-housed mice.

Ideally all the mice used in the experiment should have had the same mother. If that was the case and the same pattern occurred (with Lcn 2 KO-mice clustered to the left in the plot while Wt-mice were clustered to the right), then it would be safer to say that genotype would be one of the main factors contributing to the difference in microbiota, as the mice were siblings and thus genetically highly similar. However, as this was not possible, the mice should have been more randomized in respect to maternity when planning the cage set-up in order to minimize the maternity effect. Another drawback with the experiment was the limited number of mice in each cage. According to Mandelian statistics it was expected to get approximately 25 % normal mice (Wt), 50 % heterozygote mice (Ht) and 25 % lipocalin 2 deficient mice (Lcn2 KO). Unfortunately the number of Lcn2 KO-mice and Wt- mice were at minimum, with only 10 and 9 mice respectively. To get even numbers of each group and by not using more animals than was absolutely necessary, 10 Ht-mice were chosen to be a part of the research, along with 10 Lcn2 KO mice and 9 Wt-mice. By having a limited number of mice meant that only a few mice could be distributed to each cage. This had consequences for the statistical tests such as ANOVA, which assumes that the observations are normally distributed, which is not the case for this experiment.

Summing up, the results show that the co-housed mice from the same litters had little variation in their microbial profiles. By contrast, the microbiota of the single-housed mice where the litters were split among different cages diverged in composition. This divergence seemed to be dependent on the genotype of the mouse. A similar result was found in a study by Alexander *et al*., (2006) who analyzed the abundance of the eight members of the altered Schaedler flora in mice. The results showed that the mice that had been living co-housed at weaning, whether from the same or different litters, showed a similar microbiota. However, the microbiota of litters that were split among different cages at weaning diverged in composition and the degree of divergence was dependent on the genotype of the mice. What can thus be concluded is that although the microbiota of the mother may be the primary

inoculum, stochastic differences in the colonization process between mice and small differences of the environment may interact with the genotype to determine the composition of the microbiota (Spor *et al*., 2011).

4.2 EVALUATION OF METHODS

To find out if the antimicrobial protein, lipocalin 2, influences the colonization of the gut microbiota in mice, the gut microbiota of wild type mice (Wt), heterozygote mice (Ht) and Lipocalin 2 deficient mice (Lcn2 KO) were studied by collecting stool samples. As microbial ecology is the study of interactions amongst microorganisms and between microorganisms and their environment, microbial ecosystems have to be studied over longer time periods (Muyzer & Smalla, 1998). For this purpose the cloning approach was found to be less useful, because it is time consuming and labor intensive, and hence impractical for multiple sample analysis. In order to monitor changes in microbial community structure in mouse gut microbiota over time, PCR/DGGE was used to make microbial profiles. The DGGE method was preferred as it's a fast procedure, produces a good overview over the bacterial community present in a sample and has the advantage that many samples can be analysed on the same gel.

The DGGE method generally produced gels with high quality and showed a good separation of the bacteria present in the stool samples. A denaturing gradient of 30-60 % was first tested, but it seemed that the bands were slightly jammed together (figure 3.3). A drawback with DGGE is that different conditions might result in different resolutions of separation making gels difficult to reproduce (Muyzer, 1993). To obtain higher resolution profiles, a narrower gradient (35-55%) was tried out. The result showed that this denaturing gradient seemed to show a better separation of bands (figure 3.1, 3.7 and 3.14).

As with any PCR based technique, the DGGE method suffers from biases from the DNA extraction and amplification steps. The three different DNA isolation kits that were tested demonstrated this by showing varying results of purity and the amount of DNA extracted. As for the amplification of the 16S rRNA genes, some bacterial phylotypes can be amplified more than others with PCR because the primer pair used makes a better fit. Thus, by

comparing the amount of fluorescence on a DGGE gel from two separate bands belonging to the same stool sample, one cannot postulate with certainty that one stool sample contains more of one bacterium than the other. The reason may simply be that more DNA of that specific bacterium was amplified during PCR. Thus, DGGE is criticized for being only semi quantitative. What is possible with DGGE, however, is to compare the same bands from different stool samples. This may give an indication of bacterium abundance in the various stool samples.

An obstacle with the amplification step was the use of universal primers, as the presence of bacterial DNA in the Taq DNA polymerase and master mixture is a known problem. The contaminating DNA is amplified, giving rise to false positives (Tseng *et al.,* 2003). The contamination could be seen in the control sample as it showed the same band as the rest of the samples on the agarose gel and often showed the same amount of product. This made it sometimes necessary to increase the number of PCR cycles. Taq DNA polymerase and PCR master mix from both VWR and QIAGEN were tested out in order to see which had the least amount of contamination, although with varying results.

Despite some difficulties with the amplification step, the DGGE method was a good method to detect differences in microbial profiles between Wt-, Ht- and Lcn2 KO-mice. The bands on the DGGE gel were not characterized as the objective of this thesis was to find out if the microbial profiles of Lcn 2 KO-mice were different from that of Wt-mice.

5. CONCLUSION

5.1 CONCLUSION

The study of mice gut microbiota revealed differences in the microbial profiles between Wt-, Ht- and Lcn 2 KO-mice. The result showed that both the factor of genotype and habitation were significant factors for the observed differences.

For the single-housed mice (mice of same genotype), a significant difference of gut microbiota was found between Wt/Ht-mice and Lcn 2 KO-mice, indicating that the genotype was the main factor for the observed differences. Lcn 2 thus seems to influence the natural colonization of the mice gut, as well as the re-establishment of the microbiota after a perturbation with antibiotics treatment.

For the co-housed mice (mice of mixed genotypes) both the effect of genotype and maternity seemed to influence the composition of the microbiota, although the factor of maternity was not taken into account in the analysis. The experimental set-up was designed with the intention of comparing littermates in order to minimize other effects than the knockout of Lcn2. Unfortunately this design meant that the effect of genotype and maternity could not be differentiated.

5.2 FUTURE PROSPECTS

This study focused on analysing and comparing microbial profiles of Wild-type mice (Wt), Heterozygote mice (Ht) and Lcn2 KO-mice (Lcn2 KO) in order to find out if the microbial profiles of Lcn 2 KO-mice were different from that of Wt-mice. As this study showed that there were significant differences, the same experiment could be repeated, but this time the number of mice could be scaled up in order to get more statistical reliable data. The experimental design could be planned in a different way by randomizing the mice from different litters in order to minimize the maternity effect, and the bacteria present in the gut microbiota could be sequenced in order to compare and thus find out what these differences are. When the influence of the host genome on the microbiota is clearly documented, multi-level models can be developed that take into account the environment, genetics and the microbiome to better predict the outcome of perturbations in the gut, such as diet change, the onset of disease or the administration of antibiotics (Spor, et al., 2011).

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APPENDIX

Appendix A: DNA isolation protocol

Appendix B: Primers used for PCR

Appendix C: DGGE solutions

Protocol

Please wear gloves at all times

1. To the 2 ml **Dry Bead Tubes** provided, add 0.25 g of fecal sample. Add 550 µl of **Bead Solution** (For larger sample sizes up to 10 grams, try our UltraClean® Mega Soil DNA Isolation Kit, Catalog# 12900-10).
2. Gently vortex to mix.
3. Check **Solution S1.** If **Solution S1** is precipitated, heat solution to dissolve before use.
4. Add 60 µl of **Solution S1** and invert once to mix.
5. Add 200 µl of **Solution IRS** (Inhibitor Removal Solution).
6. Secure bead tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes. (See alternative lysis method for less DNA shearing).
7. Make sure the 2 ml tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
8. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).
   Note: With 0.25 g of fecal sample and depending upon fecal type, expect 400 to 500 µl of supernatant. Supernatant may still contain some fecal particles.
9. Add 250 µl of **Solution S2**, Vortex 5 seconds. Incubate at 4°C for 5 minutes.
10. Centrifuge the tubes for 1 minute at 10,000 x *g*.
11. Avoiding the pellet, transfer 450 µl of supernatant to a clean **2 ml Collection Tube** (provided).
12. Shake Solution S3 to mix. Add 900 µl of **Solution S3** to the supernatant and vortex 5 seconds.
13. Load approximately 650 µl onto **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute. Discard the flow through and add the remaining supernatant to the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute.
    Note: Depending on sample type, a total of up to three loads for each sample processed may be required.
14. Add 300 µl of **Solution S4** and centrifuge for 30 seconds at 10,000 x *g*.
15. Discard the flow through.
16. Centrifuge again for 1 minute.
17. Carefully place **Spin Filter** in a new clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution S4** onto the **Spin Filter.**
18. Add 50 µl of **Solution S5** to the center of the white filter membrane.
19. Centrifuge for 30 seconds.
20. Discard the **Spin Filter**. DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20°C to -80°C). **Solution S5** contains no EDTA.

Thank you for choosing the UltraClean® Fecal DNA Isolation Kit.

APPENDIX B: PCR PRIMERS

The different primer sequences for the primers used in the amplification of the V3-region of the 16S rRNA and the primers used for amplifying the Lipocalin 2 gene. The primer Lcn2 Wt was used together with Lcn2-extra for amplifying the wild type Lcn2 allele, and Lcn2-neo1500 was used together with Lcn2-extra to amplify the Lcn2 knockout allele.

Table B1: Primer names with their respective sequences.

Primer name	**Primer sequence 5'-3'**
338F-GC	5'- ACT CCT ACG GGA GGC AGC AG-3'
518R	5'- ATT ACC GCG GCT GCT GG-3'
Lcn2-wt	5'- GTC CTT CTC ACT TTG ACA GAA GTC AGG -3'
Lcn2-extra	5'-CAC ATC TCA TGC TGC TCA GAT AGC CAC -3'
Lcn2-neo1500 (knock-out)	5'- ATC GCC TTC TAT CGC CTT CTT GAC GAG - 3'

APPENDIX C: SOLUTIONS FOR CASTING GEL FOR DGGE

For making 8 % acrylamide in 0, 5 * TAE (per 250 ml):

Acrylamid solution (0 % denaturing):
40 % acrylamide solution, 50 ml
50 * TAE, 2, 5 ml
H_2O, 197, 5 ml

For making 8 % acrylamide, 5, 6 M urea, 32 % formamide in 0, 5 * TAE (per 250 ml):

Deionized formamide:
Formamide, 200 ml
DOWEX RESIN AG 501X8, 7, 5 g

Stirred for 1 hour at room temperature

Denatured acrylamide solution (80 % denaturing):
40 % acrylamide solution, 50 ml
50 * TAE, 2, 5 ml
Urea, 84 g
Deionized formamide, 80 ml

The bottle was stored at 4 º C and covered with aluminium foil in order to protect the solution from light. This solution must be sterile filtered before pouring the gel.

Denaturing %	**0 %**	**80 %**	**TMED + 10 % APS**	**Total volume**
30	15 ml	9 ml	16 µl + 87µl	24 ml
35	13.5 ml	10.5 ml	16 µl + 87µl	24 ml
55	7.5 ml	16.5 ml	16 µl + 87µl	24 ml
60	6 ml	18 ml	16 µl + 87µl	24 ml

0 % stacking gel:
0 % acrylamide solution, 8 ml
10 % APS, 40 µl
TMED, 10 µl