The gut microbiota in cardiovascular disease
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CHAPTER 6

Discussion
Introduction
Treatment for cardiovascular disease (CVD) has improved markedly through protection against hyperlipidemia and cardiovascular death by statins and PCSK9-inhibitors. Combining statins and PCSK9-inhibitors successfully reduces the number of cardiovascular events by 50%. This reduction in cardiovascular events is one of the largest success stories in modern medicine (Hansson et al, Circulation, 2017). However, the flip side of the coin is that there is 50% of cardiovascular events remaining, indicating that factors independent of hyperlipidemia also strongly affect the etiology of cardiovascular disease. Over the years an important role for inflammation in the etiology of atherosclerosis has been established. An increase in pro-inflammatory cytokines (e.g IL-1β, IFN-γ) as well as an increase in the number of circulating leukocytes is involved in atherogenesis. Furthermore neutrophils, monocytes and T-cells can infiltrate the atherosclerotic plaque and contribute to atherogenesis by the formation of foam cells and production of reactive oxygen species, increasing the risk of thrombotic events (Libby et al., 2011, Chapter 1).

Until recently our understanding of the importance of inflammation in the etiology of atherosclerosis rested on associative studies in human and mechanistic studies in mice, however, recently the CANTOS-trial showed that administration of Canakinumab, an IL-1β antibody, reduced the number of cardiovascular events by 15%. Importantly, all patients in this study received statin treatment and blood lipids were in the normolipidemic range. This indicates that inflammation is involved in the etiology of atherosclerosis independently of hyperlipidemia. Although directly targeting inflammation by Canakinumab successfully reduced the number of cardiovascular events, there was also an increase in the number of fatal infections and sepsis. This is an intrinsic property of anti-inflammatory drugs, therefore understanding the factors that contribute to systemic inflammation during atherogenesis is of utmost importance.

Gut microbiota-diet-immune interactions in atherogenesis
Systemic inflammation and atherosclerosis have been linked to changes in gut microbiota composition (Jie et al., 2017; Karlsson et al., 2012; Schirmer et al., 2016). The gut microbiota is strongly affected by dietary intake and the intestinal immune barrier plays an important role by controlling the gut microbiota (Brandsma et al., 2015). Consumption of a
high fat cholesterol rich diet (HFC) leads to an expansion of pathobionts and to a reduction in the production of beneficial metabolites such as short-chain fatty acids (SCFA) by the microbiota (Brandsma et al., 2015). This can lead to an increase in intestinal permeability and translocation of bacteria or bacterial components into the circulation, where these can trigger an inflammatory response (discussed in Chapter 2). In addition, the gut microbiota is also influenced by the intestinal immune system (Caricilli et al., 2011; Henao-Mejia et al., 2012; Vijay-Kumar et al., 2010). The intestinal immune system needs to balance between tolerating commensal bacteria, while eliminating pathogens. The first line of defense is formed by a physical-chemical layer formed by the production of mucus and antimicrobial peptides. This layer prevents infiltration of bacteria into the intestinal epithelial barrier and is important to prevent translocation of bacteria from the gut into the systemic circulation (Brandsma et al., 2015; Muniz et al., 2012; Sperandio et al., 2015). Furthermore, pattern recognition receptors (PRRs) can recognize bacterial patterns, promoting signaling to the immune system and promoting clearance of pathogens (Hemmi and Akira, 2005). Dysfunction of several PRRs leads to an altered microbiota composition and this has been linked to decreased immune barrier function, increased translocation of endotoxins into the circulation and promotes the metabolic syndrome (Henao-Mejia et al., 2012, Chapter 2). Thus, the gut microbiota is influenced by dietary intake and the intestinal immune system and the gut microbiota has been linked to systemic inflammation and atherosclerosis. It is however unclear whether alterations in microbiota composition or localization can directly contribute to systemic inflammation and atherogenesis and how these processes are affected by the intestinal immune barrier and diet. Therefore, the aim of this thesis was to understand how the interaction between the diet, gut microbiota and intestinal immune barrier contribute to systemic inflammation and atherosclerosis. To better understand these complex processes, we have manipulated the gut microbiota (Chapter 3) and the intestinal immune barrier (Chapter 4) and studied the effects on atherogenesis. In addition, we assessed the role of dietary cholesterol in Western type diets on gut health in the context of the metabolic syndrome by feeding mice western type diets combined with the gut anti-inflammatory agent 5-ASA (Chapter 5).
Main findings of this thesis

In Chapter 3 we show that transplantation of the pro-inflammatory Casp1Δ/- microbiota into atherosclerosis prone LdlrΔ/- mice promotes atherogenesis and systemic inflammation. These findings are possibly caused by a reduced capacity of the gut microbiome to produce anti-inflammatory SCFAs, hence affecting systemic inflammation and atherosclerosis.

In Chapter 4 we show a protective role for the antimicrobial peptide REG3γ in the development of atherosclerosis. The antimicrobial peptide REG3γ is important to prevent infiltration of the microbiota into the intestinal epithelial barrier. Thus, our study indicates that the intestinal immune barrier plays an important role in the protection against atherogenesis.

In Chapter 5 we show that increased levels of cholesterol in the diet does not affect intestinal barrier function and T2D in C57BL/6 mice.

The microbiota and cardiovascular disease

The gut microbiota has been linked to cardiovascular disease (CVD) by several observational studies (Emoto et al., 2016; Jie et al., 2017; Karlsson et al., 2012). These studies have seen an increased abundance in the taxonomies Collinsella, Ruminococcus Gnavus, Escherichia Coli, Klebsiella and Enterobacter aerogenes in CVD patients. Whereas the SCFA-producing bacteria Eubacterium and Roseburia were decreased (Karlsson et al., 2012). Furthermore, the gut microbiota has been linked to risk factors of CVD, such as blood lipids (TG and HDL) (Fu et al., 2015). However, these observational studies only provide associative evidence that link the gut microbiota to CVD. In addition, the number of studies showing a causal role for the gut microbiota are limited (Li et al., 2016; Wang et al., 2011) and are mostly confined to the understanding of the importance of trimethylamine-oxide (TMAO) in atherogenesis (Gregory et al., 2015; Senthong et al., 2016; Tang et al., 2013; Wang et al., 2011). Choline, phosphatidylcholine, L-Carnitine and other TMA-containing structures are converted by the CutC cluster of the microbiota into TMA (Craciun and Balskus, 2012). TMA is subsequently taken up into the systemic circulation where it is oxidized by Flavin Monoxygenase 3 (FMO3) in the liver leading to formation of TMAO. Increased levels of TMAO augment the development of aortic atherosclerotic plaques (Figure 1a) (Wang et al., 2011). Production of TMA from choline is dependent on the taxonomies; RF39, Erysipelotrichaceae, Coriobacteriaceae,
Allobaculum and Prevotella (Gregory et al., 2015). These studies have further been confirmed in humans, where gut microbiota dependent production of TMA and consequent oxidation to TMAO promotes atherosclerosis (Tang et al., 2013). However, in chapter 3 we provide a novel, alternative mechanism by which the microbiome may contribute to atherogenesis, independent from TMAO and lipids. We show that introduction of the pro-inflammatory gut microbiome of Caspase1⁻/⁻ mice into Ldlr⁻/⁻ mice increased systemic inflammation and accelerated atherosclerosis. We observed an increase in the inflammatory cytokines IL-1β, Il-2 and IFN-γ as well as an increase in circulating monocytes and neutrophils. This indicates that alterations in the gut microbiota promote systemic inflammation and atherogenesis (Figure 1b). This is an intriguing finding since recent studies have indicated that systemic inflammation is causally involved in CVD independent of hyperlipidemia (Hansson, 2017; Ridker et al., 2017). Nevertheless, we cannot conclude from our study whether the increased atherogenesis following dysbiosis is a consequence of the increased systemic inflammation. Future experiments combining microbiota transplantations with anti-inflammatory drugs are needed to confirm the dependence of increased atherogenesis following dysbiosis on increased systemic inflammation.

**SCFA regulators of inflammation**

In chapter 3, we also observed a reduction in the SCFA producing taxonomies Akkermansia, Christensenellaceae and Odoribacter in Ldlr⁻/⁻ mice co-housed with Casp1⁻/⁻ mice. This was accompanied by a reduced cumulative concentration of the anti-inflammatory SCFAs acetate, butyrate and propionate in the cecum of Ldlr⁻/⁻ (Casp1⁻/⁻) mice. In addition to influencing metabolic processes (Chapter 2), SCFAs are involved in the regulation of the immune system and have previously been shown to reduce inflammation (Cox et al., 2009; Tedelind et al., 2007; Vinolo et al., 2011). SCFA affect the immune system locally, by stimulating the production of antimicrobial peptides and IL-18 in intestinal epithelial cells (Corrêa-Oliveira et al., 2016). Production of antimicrobial peptides is important to prevent infiltration of the microbiota into the intestinal epithelial barrier, whereas IL-18 production stimulates the proliferation of intestinal epithelial cells thereby promoting maintenance and repair of the intestinal epithelial barrier (Lewis and Heaton, 1997; Nemoto et
Following uptake of SCFAs into the systemic circulation, SCFAs can also affect the systemic immune system. Administration of SCFAs to macrophages following stimulation by the pro-inflammatory stimulus LPS leads to a reduction in the production of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 (Liu et al., 2012; Singh et al., 2014; Tunaru et al., 2003). Furthermore, butyrate affects antigen presentation by dendritic cells, thereby inhibiting the stimulation of naïve T-cells and hence preventing activation of the adaptive immune response (Millard et al., 2002). Interestingly, butyrate not only inhibits the stimulation of naïve T-cells by dendritic cells, but also skews the differentiation of naïve T-cells from pro-inflammatory IFN-γ producing T-helper 1 cells into more anti-inflammatory regulatory T-cells (Treg) (Gurav et al., 2015). Stimulation of Tregs by butyrate is further supported by in vivo evidence, where administration of butyrate in antibiotic-treated mice increases the number of peripheral Tregs. Acetate has also been reported to have anti-inflammatory properties (Cox et al., 2009; Tedelind et al., 2007) but is not as potent as butyrate in reducing pro-inflammatory cytokines (Vinolo et al., 2011). Acetate promotes production of the anti-inflammatory cytokine IL-10 under steady state conditions and promotes clearance of pathogens during infection by promoting development of effector T-cells. The regulation of T-cell development by acetate are dependent on direct histone deacetylase inhibitor activity. Inhibition of HDAC activity by acetate causes acetylation of p70 S6 kinase and phosphorylation of rS6 which regulate the mTOR pathway and this consequently effects generation of Th1, Th17 and IL-10+ T-cells (Koh et al., 2016; Park et al., 2015). Thus, SCFAs seem to reduce inflammation by affecting pro-inflammatory cytokine production, antigen presentation and skewing of T-cell differentiation to Tregs during steady state and can skew T-helper cell development towards Th1 and Th17 cells during infection (Park et al., 2015). This suggest that the reduced production of SCFAs by the gut microbiota of Casp1−/− mice may augment systemic inflammation and thereby promote atherogenesis (Fig 1B). In line with this, oral administration of butyrate has been shown to slow the progression of atherosclerosis in ApoE−/− mice by attenuating the adhesion and migration of macrophages and decreasing pro-inflammatory cytokines in atherosclerotic plaques (Aguilar et al., 2014). The link between butyrate, inflammation and atherosclerosis is further
supported in humans, where the capacity to produce butyrate by the microbiota is negatively correlated with C-reactive protein concentrations in patients with atherosclerosis (Karlsson et al., 2012). In addition, our study now also reveals a possible role for acetate as protective factor in systemic inflammation and atherogenesis, since the reduced SCFA cecum levels were mainly driven by a significant reduction in acetate. However, future experiments are needed to 1) validate whether reduced SCFA production by the gut microbiota promotes systemic inflammation and atherogenesis and 2) to specify which SCFA or combination of SCFAs are responsible for these effects. A better understanding of SCFA production by bacteria from the gut microbiota in the development of atherosclerosis can possibly reduce the risk for atherosclerosis by administration of specific bacterial strains, dietary fibers or direct administration of SCFA.

Intestinal health and cardiometabolic disease

The intestinal immune barrier plays an important role in controlling the microbiota composition, balancing between tolerance for commensal bacteria and an adequate immune response against pathobionts (Chapter 2). The first line of defense is formed by a physical-chemical layer formed by the production of mucus and antimicrobial peptides (ref). This layer prevents infiltration of bacteria into the intestinal epithelial barrier and is important to prevent translocation of bacteria from the gut into the systemic circulation (Chapter 2). In chapter 4 we investigated whether the mucosal immune system is an additional player in the development of atherogenesis. To do so, we studied the role of the antimicrobial peptide Reg3γ in atherogenesis and found an increased atherosclerotic lesion size in Reg3γ−/− mice fed a HFC diet. This suggests that increased infiltration of the intestinal epithelial barrier by bacteria from the gut contributes to atherogenesis. Thus, in addition to gut microbiota composition we now also show that bacterial localization in the gut is important during atherogenesis giving further insight into the mechanism by which the microbiota can contribute to cardiovascular disease (Figure 1c). Interestingly, Surana et al identified the bacteria Ruminococcus Gnavus and Lactobacillus Reuteri as inducers of Reg3γ expression, administration of these bacteria in mice increased Reg3γ expression (Surana and Kasper, 2017). Therefore, it would be interesting to explore whether administration of these bacteria is protective against atherosclerosis by increasing the
A) Choline, phosphatidylcholine and L-carnitine can be converted by the CutC cluster in bacteria of the microbiota into TMA. TMA is consequently taken up into the circulation where TMA is oxidized in the liver by FMO3 resulting into the formation of TMAO. Increased levels of TMAO promotes atherogenesis. 3,3-dimethyl-1-butanol (DMB) can inhibit the formation of TMA by bacteria in the gut and is protective against atherogenesis.

B) Transplanting the microbiota of Caspase1-/− mice into Ldlr-/− mice promotes systemic inflammation and atherogenesis and leads to a decreased production of the anti-inflammatory SCFA. Future research needs to establish whether enhanced atherogenesis is indeed dependent on 1) a reduction in SCFA, 2) whether this results in increased systemic inflammation and 3) whether an increase in systemic inflammation results into increased atherogenesis.

Figure 1. The involvement of the gut microbiota in atherogenesis.

A) Choline, phosphatidylcholine and L-carnitine can be converted by the CutC cluster in bacteria of the microbiota into TMA. TMA is consequently taken up into the circulation where TMA is oxidized in the liver by FMO3 resulting into the formation of TMAO. Increased levels of TMAO promotes atherogenesis. 3,3-dimethyl-1-butanol (DMB) can inhibit the formation of TMA by bacteria in the gut and is protective against atherogenesis.

B) Transplanting the microbiota of Caspase1-/− mice into Ldlr-/− mice promotes systemic inflammation and atherogenesis and leads to a decreased production of the anti-inflammatory SCFA. Future research needs to establish whether enhanced atherogenesis is indeed dependent on 1) a reduction in SCFA, 2) whether this results in increased systemic inflammation and 3) whether an increase in systemic inflammation results into increased atherogenesis.
levels of REG3γ.
Interestingly, western type diets which are important in the onset of cardiovascular disease affect the intestinal immune barrier, leading to a reduction in antimicrobial peptide production, low-grade intestinal inflammation and increased intestinal permeability facilitating the leakage of pro-inflammatory endotoxins into the circulation (Cani et al., 2008; Luck et al., 2015; Wang et al., 2014). In Chapter 5 we investigated the effects of a western type diet on the immune barrier and how this effects the metabolic syndrome by combining the feeding of a high fat diet (HFD) or HFC with the anti-inflammatory agent 5-ASA in C57BL/6 mice. 5-ASA inhibits intestinal inflammation and prevents increased intestinal permeability following consumption of HFD (Luck et al., 2015). We did not detect differences in intestinal permeability between mice fed a LFD, HFD or HFC indicating that dietary fat or cholesterol does not affect gut barrier function in C57Bl/6 mice. The absence of an effect on intestinal permeability following administration of HFD or HFC is highly surprising and in contrast with our own results (Chapter 3) and many other groups in the field (Cani et al., 2008; Ding et al., 2010; Luck et al., 2015). Previous studies have reported the expansion of pathobionts following feeding of western type diets (David et al., 2014; Devkota et al., 2012; Martinez-Medina et al., 2014). The discrepancy between our study (Chapter 5) and other studies in the field may indicate that effects of western type diets on intestinal inflammation may be dependent on the presence of pathobionts in the gut microbiota of the host. Future experiments should investigate under which circumstances western type diets can disrupt the intestinal epithelial barrier to understand when and how intestinal inflammation could be targeted for therapy in metabolic diseases.

\[ \text{C) Reg3γ-/- mice have increased infiltration of bacteria from the microbiota into the intestinal epithelial barrier and Reg3γ-/- display increased susceptibility for the development of atherosclerosis. Future research needs to establish, whether increase susceptibility for atherosclerosis development is a consequence of translocation of bacteria or endotoxins into the circulation via a yet unknown mechanism.} \]
Understanding the complex interactions of the gut microbiota and the host

The main discovery that has set the stage for microbiota research was done by the group of Jeffrey Gordon in 2006 (Turnbaugh et al., 2006). In this hallmark experiment Gordon’s group discovered that transplanting the gut microbiota of obese mice into lean germfree mice promoted adiposity. This key experiment has sparked the interest of the scientific community and set the stage for a quickly developing research field. Due to the relative short timespan since the “start” of the microbiota field, experimental design and experimental techniques are still under development. The design and choice of model for animal experiments in microbiota research needs careful consideration. There are however some ground rules that apply to all models for the design of animal experiments in general and microbiota studies particular. One of the most important factors influencing microbiota composition is the diet (Brandsma et al). Although macromolecular content is well controlled in most studies, the source of fat, protein and fiber content in commercially available diets is not constant and dependent on price fluctuations in the market (Ericsson and Franklin, 2015). As diets generally contain microbial components, diets used for microbiota studies should be irradiated to provide sterilization of the diet to prevent introduction of new bacteria to the gut microbiota of the host (Ericsson and Franklin, 2015). Thus, it is important to select a vendor for the production of diets that provides irradiated diets with a constant composition to exclude confounding factors and to enable reproducibility of results. However, also the choice of mouse vendor can strongly influence the experimental study outcome. Indeed, several studies have detected differences in gut microbiota composition of mice purchased from different vendors (Denning et al., 2011; Hufeldt et al., 2010; Ivanov et al., 2008). Ivanov et. al. found that mice derived from Taconic farms harbor Segmented Filamentous Bacteria (SFB), whereas these are absent in the same mouse strain from Jackson laboratories. Interestingly SFB stimulate the production of serum amyloid A in the terminal ileum, which stimulates dendritic cells in the lamina propria to promote differentiation of Th17 cells(Ivanov et al., 2009). This induction of Th17-cells by SFB promoted resistance against infection with Citrobacter Rodentium (Ivanov et al., 2009). Therefore, it is important to consider the source of mice carefully. Ideally vendors of mice should keep
track of the microbiota composition of mouse lines over time and publish this data on their websites. In addition, specific mouse lines produced by research groups should be donated to a centralized facility following publication, to prevent variabilities in microbiota composition. This will be important for reproducibility of mouse studies in general and microbiota studies in particular.

Furthermore, housing conditions are important to consider for carefully controlled microbiota studies. Traditionally mice are kept in open cages where they are exposed to the environment. However, the environment in an animal facility is hard to control from the perspective of micro-organisms since researchers, cleaning staff and animal care takers frequently enter the animal rooms where the mice are housed and many different mouse lines are typically kept in the same room. To control for these factors mice should be housed in cages that protects against introduction of micro-organisms from the environment, such as IVC cages or flexible film isolators. Individually filtered cages (IVC) are filter top cages placed in a special rack that filters the air that enters each individual cage and prevents exposure of mice to the environment in the animal facility, thereby limiting the unwanted factors that can affect the experimental outcome. Alternatively, mice can be placed in cages within flexible film isolators, flexible film isolators are a completely sterile environment in which cages with mice can be placed. All material introduced into these isolators is autoclaved, these isolators are typically used for germfree mouse experiments (discussed later).

![Antibiotic Treatment](image)

**Figure 2. Total bacterial content following antibiotic treatment in Ldlr<sup>−/−</sup> mice**

Total bacterial content in feces from Ldlr<sup>−/−</sup> mice treated with broad spectrum antibiotics (Metrodinazole, Ampicillin, Neomycin, Vancomycin) for 10 days. The PCR bands from 5 representative mice are shown.
Mouse models for microbiota research

To investigate the involvement of the microbiota in host physiology and the etiology of disease, several experimental model and designs are available that take into consideration the status of the microbiota of the host. The following mouse models have been used in the microbiota field and have all served their purpose:

Complex microbiota; conventional animals harbor a complex microbiota composition resembling the natural situation

Defined microbiota; animals harboring a microbiota composition of selected bacterial species with a limited complexity, the most well-known example is the altered Schaedler flora containing 8 different bacterial strains.

Mono-associated animals; animals associated with one specific bacterial strains

Germfree animals: animals that are free of all micro-organisms including bacteria, viruses and fungi.

Treating conventional mice with antibiotics as a tool to prove dependence of a phenotype on the gut microbiota is an easy and practical approach to get insight into the involvement of the gut microbiota, an approach that has been successfully applied over time (Elinav et al., 2011; Ichinohe et al., 2011; Rakoff-Nahoum et al., 2004). Administration of broad-spectrum antibiotics can reduce bacterial presence in the gut to 1% of the original (Hill et al., 2010; Rakoff-Nahoum et al., 2004), thereby involvement of the gut microbiota in a phenotype can be investigated. In chapter 3 we also made use of broad-spectrum antibiotics to reduce bacterial presence in the gut. We collected fecal samples before and after the 10-day antibiotic treatment to confirm the effectiveness of the antibiotic treatment. Consistent with previous studies (Hill et al., 2010; Rakoff-Nahoum et al., 2004), total bacterial content was dramatically reduced in $Ldlr^{-/-}$ mice that received antibiotics for 10 days (Fig. 2). It is however noteworthy to realize that this approach relies on the assumption that the registered change in the phenotype is a consequence
of the reduction of the microbiota and not of the antibiotics on the host. Interestingly, Han et al have reported that antibiotics may also directly affect the phenotype of mice independent of the gut microbiota. Disrupting TRAF6 signaling in dendritic cells (TRAF6-DC/-) exacerbates intestinal inflammation, and this effect was ameliorated after administration of antibiotics (Han et al., 2015). However, administration of antibiotics to germfree TRAF6-DC/- mice was also shown to ameliorate intestinal inflammation, indicating that the effects of antibiotics were independent of the gut microbiota (Han et al., 2015). Although this is most likely an exception to the rule, the possibility of direct effects of antibiotics should be taken into account during study design and interpretation of the study results.

In addition to reducing the presence of the gut microbiota via administration of antibiotics, germfree mice have been used extensively in the field to investigate the involvement of the microbiota in a phenotype (Bäckhed et al., 2004; Taurog et al., 1994; Turnbaugh et al., 2006). In this experimental design germfree mice are compared with conventional mice. Differences between the phenotype of germfree and conventional mice indicates the involvement of the microbiota. The advantage of germfree mice with respect to conventional mice treated with broad-spectrum antibiotics is that all bacteria are absent, whereas combining broad-spectrum antibiotics strongly reduces the number of bacteria in the gut but does not remove all bacteria (Figure 2). However, there is a lot of debate about the suitability of germfree mice as a physiological model. Bacteria are essential for the development of the immune system and germfree mice display an underdeveloped immune system (Atarashi et al., 2011; Gaboriau-Routhiau et al., 2009; Helgeland et al., 1996; Ivanov et al., 2009). In addition, intestinal epithelial gene expression is affected (Chowdhury et al., 2007; Hooper et al., 2001), intestinal turnover slowed down (Savage et al., 1981) and germfree mice need specialized diets. Specialized diets are necessary to provide the microbiota dependent Vitamin K and to ensure that the diet is low in fiber content. Due to the absence of bacteria in the gut, fibers cannot be degraded, leading to enlarged ceca and obstipation of germfree mice, to prevent these type of problems specialized diets for germfree mice have been developed. Another important limitation of conducting germfree experiments is the complexity of deriving specific mouse lines to a germfree status.
To transform a specific mouse line into a germfree status, surrogate germfree mice can be purchased and placed in flexible film isolators for maintenance of the germfree status. To rederive a mouse line of interest into a germfree status, mice need to be time mated together with the surrogate germfree mice that will serve as foster mothers. The foster mothers are timed to have pups 1-2 days before the strain of interest, pups from the fosters will be removed and pups from the mouse strain of interest will be removed via hysterectomy and transferred steriley to the germfree foster mothers. During production and maintenance of the new germfree mouse line, the germfree status needs to be monitored regularly to ensure germfree status. A surplus of breeding pairs is needed to achieve sufficient mice for an experiment, due to variability in timed mating and cannibalism of foster mothers. Cannibalism can be reduced by minimizing handling of animals and reducing activity in the room to an absolute minimum (Carter et al., 2002). Altogether, germfree mice are interesting as a model because there is no influence of other microorganisms, but rederiving mice into a germfree status is a tedious process and the germfree status itself has a large effect on physiology of mice.

**Fecal microbiota transplantation**

Next to ablating the microbiota composition, introduction of the microbiota into mice is an important tool to investigate the involvement of the microbiota. A commonly used and probably the simplest approach to investigate causality of the microbiota in disease development is cohousing of diseased mice together with healthy mice. Since mice are coprophagic microbiota transfer occurs naturally via consumption of fecal pellets by mice. This approach has been used to show the involvement of the microbiota in obesity as well as NAFLD (Henao-Mejia et al., 2012; Ridaura et al., 2013). Ridaura et al reported a protective effect against obesity after cohousing obese mice together with lean mice, this protective effect was dependent on the transfer of Christensenellaceae from lean mice to obese mice (Ridaura et al., 2013). On the other hand also exacerbation of phenotypes has been registered after cohousing animals, Henao-Mejia et al have reported exacerbation of NAFLD of WT mice after cohousing with Nlrp3-/- mice (Henao-Mejia et al., 2012). Furthermore, a mixed phenotype has been reported, cohousing the same mouse strain from 2 vendors with low
and high susceptibility for acute liver injury resulted in an intermediate phenotype. (Celaj et al., 2014). In addition, we show increased systemic inflammation and enhanced atherogenesis after cohousing Ldlr-/- mice with Casp1-/- mice harboring a pro-inflammatory microbiota (chapter 3). Thus, cohousing conventional mice is a useful approach to investigate the involvement of the gut microbiota in disease, however the direction and effectiveness of microbiota transfer cannot be controlled and is likely dependent on the stability of the ecosystem in the gut of the mouse strains. To control the direction and effectiveness of fecal microbiota transplantation (FMT) it is important to reduce the microbiota composition of the recipient mice. This can be achieved by making use of mice with altered schaedler flora, germfree mice, treating recipient mice with a mixture of broad-spectrum antibiotics or via cross-fostering. Cross-fostering is achieved via timed matings, where pups that receive an alternate microbiota composition (microbiota recipients) are placed with a foster mother that serves as the microbiota donor. Development of the microbiota start during delivery therefore it is important to transfer the pups with the foster mother directly after birth. Cross-fostering is an effective method (Couturier-Maillard et al., 2013; Fuhrer et al., 2010; Garrett et al., 2007) to transfer the microbiota composition and test the physiological consequences of the altered microbiota composition. As with production of germfree mice, cross-fostering is labour intensive and requires a surplus of breeding pairs. It is however not necessary to have a germfree facility and experiments can be conducted in IVC-cages present in most animal facilities. In addition, cross-fostering is the natural way in which the gut microbiota develops, development of the immune system occurs normally and there is no limitation in types of diets that can be fed to these mice. To maintain the introduced microbiota composition over time, mice should be placed together with donor mice during the entire course of the experiment. Next to cross-fostering, the microbiota can also be transplanted into germfree mice. An important advantage of microbiota transplantations into germfree mice with respect to cross-fostering is the ability to transplant the microbiota composition at different time points, thereby giving insight into the effect of the microbiota during immune development. The microbiota can be transplanted into germfree mice either via oral gavage or by cohousing germfree mice together with the fecal donors. Oral gavage of the gut microbiota is a well-controlled
process, but does increase the stress of animals during experiments due to regular gavages, whereas cohousing leads to natural ingestion of the microbiota but is less well controlled. These approaches for FMT can also be applied to mice in which the microbiota has been reduced with broad-spectrum antibiotics (chapter 3, Figure 2). In case of cohousing, a washout period should be taken into account. Recipients of FMT will have residual antibiotics present in their feces, therefore consumption of fecal pellets of FMT recipients by the FMT donors could affect the microbiota composition and should be prevented. This can be achieved by transferring the bedding of the FMT donors into the cage of the FMT recipients for 1 week following the treatment with antibiotics, after this washout period FMT recipients and FMT donors can be housed together to maintain the transplanted microbiota composition for the rest of the experiment (Chapter 3). Altogether, microbiota experiments should be conducted in a facility in which mice are protected against influences of the environment via IVC-cages or flexible film isolators and food should be irradiated and purchased from a vendor with standardized sources for the macromolecular composition of the food. FMT can be performed by transplanting the microbiota of FMT donors via cross-fostering, treating FMT recipients with broad-spectrum antibiotics or using germfree FMT recipients. Germfree recipients are the most suitable model to investigate single bacteria or to investigate the interaction between a few bacteria, since no other micro-organisms are present in the recipients. To understand the effects of a complex microbiota composition on a physiological process or disease cross-fostering or pretreatment of mice with broad-spectrum antibiotics is more suitable since these models are a better reflection of normal physiology.

**Identification of bacteria causally involved in disease**

Although involvement of the microbiota in disease development has been clearly proven and is widely accepted, identification and validation of specific bacterial strains that contribute to disease development is a major challenge in the microbiota field and a necessity to start targeting the gut microbiota as a therapeutic target. Surana et al recently published a novel method to help address this problem. Specific pathogen free (SPF) mice or gnotobiotic mice harboring either mouse microbiota (MMb) or human microbiota (HMb) (Chung et al., 2012) compositions showed
a different susceptibility to develop colitis following administration of dextran sodium sulfate (DSS) (Surana and Kasper, 2017). SPF mice and HMb mice were protected against development of DSS-induced colitis with respect to MMb mice. To identify which bacteria could contribute to the differences in DSS-induced colitis susceptibility of MMb and HMb mice, MMb and HMb mice were cohoused for 1 or 3 days to produce hybrid microbiota compositions. Cohousing was protective against development of DSS-induced colitis and increasing the length of cohousing lead to a larger survival following DSS-induced colitis. Making use of pairwise comparisons between MMb, HMb, SPF mice and mice with hybrid microbiota compositions led to the discovery that only the taxon Lachnospiraceae was significantly different between all pair-wise comparisons, indicating the relevance of the taxon Lachnospiraceae for development of colitis (Surana and Kasper, 2017). Thereby bacterial candidates with a protective effect were narrowed down to one bacterial family. Feces of the Lachnospiraceae rich HMb microbiota were consequently cultured on a semi-selective medium, to enrich for Lachnospiraceae. Combining 16SrDNA sequencing, MALDI-TOF analysis and biochemical tests identified the novel bacterial species Clostridium Immunis, from the family Lachnospiraceae as the bacterial strain responsible for the protective effect of the HMb microbiota against DSS-induced colitis. Thus, making use of a cohousing approach to generate hybrid microbiotas and applying multiple pair-wise comparisons of microbiota compositions combined with disease phenotype information is an interesting approach to narrow down the number of candidate bacteria. Combining culturing techniques with next-generation sequencing approaches, biochemical assays and MALDI-TOF analysis can consequently be used to identify the individual bacterial species within the candidate taxonomies. Although this is an interesting and promising approach to start understanding which bacteria are involved in disease development, this approach does rely on the ability to culture bacteria from gut microbiota. Until now difficulties to culture strictly anaerobic bacteria has hampered the ability to identify and causally relate bacterial strains to disease development. This limitation has been recognized by multiple groups in the field and efforts to increase the number of culturable bacteria from the gut microbiome are ongoing (Browne et al., 2016; Lau et al., 2016; Rajilić-Stojanović and de Vos, 2014). Comparing classical culturing
techniques with 16S rRNA sequencing of the gut microbiome estimated that 76% of the OTUs observed by culture-independent techniques can be cultured by combining 66 different culturing conditions (Lau et al., 2016). Although this is a promising result, the major limitation of this study and others is the usage of reference-based OTU-picking during 16S sequencing analysis. During reference-based OTU-picking, the OTUs that are not part of the selected reference database are discarded, leading to an underestimation of the bacteria present in the gut microbiome. Since this serves as the culture-independent reference for total bacteria present in the gut microbiome, this will lead to an overestimation of the proportion of bacteria that can be cultured ex vivo. In addition, the resolution of 16S rRNA sequencing is limited and taxonomies are hardly ever identified on the level of bacterial strains. Usage of metagenomics shotgun sequencing has greatly improved the resolution and is able to identify taxonomies from the gut microbiome onto the strain level. More importantly, metagenomics shotgun sequencing also gives insight into function of bacteria by mapping the different genes of all different taxonomies identified. This information can be used to explore which culturing conditions may be successful to capture the full complexity of the gut microbiome. Overall, efforts made until now will help to detect the bacteria that functionally attribute to or protect against disease, it is however necessary to further explore different combinations of culturing conditions. Metagenomic shotgun sequencing can support this process by implementing bacterial gene information to further optimize ex vivo culturing. Furthermore, more accurate estimates of percentages of bacteria from the human gut microbiome that can be cultured ex vivo can be retrieved by comparing the numbers of ex vivo-cultured bacteria to a reference dataset retrieved from the fecal samples of the same individuals by open-reference OTU-picking of metagenomics shotgun sequencing samples. These developments will be a necessary step to move from association to causality and to start understanding the complex mechanisms by which the microbiota interact with each other and their host and how these interactions contribute to physiology and disease development. Ultimately understanding the complex mechanisms in this ecosystem can lead to identification of beneficial or harmful bacteria in a specific microbiota composition for a specific disease, thereby facilitating the ability for development of microbiota targeted therapies in the future.
Concluding remarks
The field of microbiota research in the development of cardiovascular diseases is a fascinating research field with a lot of potency to discover novel mechanisms that are involved in the development of cardiovascular disease. Identifying bacteria or bacterial metabolites that individually or in congregation with other bacteria or metabolites influence the development of cardiovascular disease is however still a major challenge. Understanding the complex interactions between the many bacteria present in the microbiota and human physiology will be instrumental to eventually use the gut microbiota as a target for treatment of cardiovascular disease. Our studies describe a causal role of the gut microbiota in atherogenesis and highlight the importance of gut microbiota composition and localization as well as the intestinal immune barrier in atherogenesis. Furthermore, our studies suggest that the interaction between the intestinal immune barrier, gut microbiota composition and localization influence the development of cardiovascular disease. Our studies warrant follow-up research to validate and specify the suggested mechanisms, to identify which bacteria or bacterial metabolites are important in atherogenesis and to understand whether the role of specific bacteria or bacterial metabolites is dependent on the composition of the gut microbiota.
References


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