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Vostand: Prof. Dr. med. Sebastian Suerbaum

Use of the Oligo-Mouse-Microbiota to generate standardized gnotobiotic mouse lines and to investigate mechanisms underlying *E. coli*-mediated colonization resistance against *Salmonella enterica* serovar Typhimurium

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Zweitgutachter:	Prof. Dr. Till Strowig
Dekan:	Prof. Dr. med. Thomas Gudermann
Tag der mündlichen Prüfung:	29.04.2022

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List of abbreviations

ASF	Altered Schaedler Flora
CR	colonization resistance
OMM^{12}	Oligo-Mouse-Microbiota
SCFA	short chain fatty acids
SIHUMI	simplified human intestinal microbiota
S. Tm	*Salmonella enterica* serovar Typhimurium
T3SS	type III secretion system
T6SS	type VI secretion system

List of publications

Manuscripts published during doctoral studies:

1. **Eberl, C.**, Weiss, A.S., Jochum, L.M., Durai Raj, A.C., Ring, D., Hussain, S., Herp, S., Meng C., Kleigrewe, K., Gigl, M., Basic, M., and Stecher, B. (2021) '*E. coli* enhance colonization resistance against *Salmonella* Typhimurium by competing for galactitol, a context-dependent limiting carbon source', ***Cell Host Microbe***, 29: 1-13.

2. Maier, L., Goemans, C.V., Wirbel, J., Kuhn, M., **Eberl, C.**, Pruteanu, M., Müller, P., Garcia-Santamarina, S., Cacace, E., Zhang, B., Gekeler, C., Banerjee, T., Anderson, E.E., Milanese, A., Löber, U., Forslund, S.K., Patil, K.R., Zimmermann, M., Stecher, B., Zeller, G., Bork, P. and Typas, A. (2021) 'Unravelling the collateral damage of antibiotics on gut bacteria', ***Nature***, 599:120-124.

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8. Herp, S., Brugiroux, S., Garzetti, D., Ring, D., Jochum, L. M., Beutler, M., **Eberl, C.**, Hussain, S., Walter, S., Gerlach, R. G., Ruscheweyh, H. J., Huson, D., Sellin, M. E., Slack, E., Hanson, B., Loy, A., Baines, J. F., Rausch, P., Basic, M., Bleich, A., Berry, D., and Stecher, B. (2019) *'Mucispirillum schaedleri* antagonizes *Salmonella* virulence to protect mice against colitis', ***Cell Host Microbe***, 25: 681-94 e8.

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Münch, P.C., **Eberl, C.**, Ring, D., Franzosa, E.A., Huttenhower, C., Fritz, A., Herp, S., Geffers, R., McHardy, A.C. and Stecher, B. 'Pulsed antibiotic treatments of gnotobiotic mice manifests in complex community dynamics and resilience effects'.

Manuscripts published before doctoral studies:

Eberl, C., Speth, C., Jacobsen, I. D., Hermann, M., Hagleitner, M., Deshmukh, H., Ammann, C. G., Lass-Flörl, C. and Rambach, G. (2019) *'Candida*: Platelet interaction and platelet activity *in vitro*', ***J Innate Immun***, 11: 52-62.

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1. Abstract

The gut microbiota fulfills many beneficial tasks for its host, such as the breakdown of complex carbohydrates, training of the immune system and the protection against enteric pathogens, a phenomenon termed colonization resistance. Due to the high complexity of the gut microbiota and the limitation of appropriate tools, it has been challenging to pin down the causal role of individual bacteria to microbiota functions including colonization resistance and to identify protective species. Bottom-up approaches involving synthetic microbial communities composed of culturable microorganisms provide a means for functional studies.

The Oligo-Mouse-Microbiota (OMM^{12}) is a synthetic bacterial community that consists of 12 bacterial isolates assigned to the 5 most abundant phyla found in the conventional mouse intestine (Brugiroux et al., 2016). This community is nowadays established in many different mouse facilities worldwide and is used by groups working in various areas of functional gut microbiome research. To increase the experimental reproducibility between different research teams using this model, we aimed to establish a standardized inoculation protocol for the colonization of germ-free mice with the OMM^{12} consortium. To compare the reproducibility of the protocol and to find out if varying housing conditions have an impact on the microbial community structure, we included five different European animal facilities in our study. We could show that the bacterial consortium reaches a stable community composition within 2 weeks after the inoculation of germ-free mice in all tested facilities. Furthermore, a second application of the OMM^{12} bacteria after 3 days clearly increases the rate of successful inoculation of specific, oxygen-sensitive members of the consortium in comparison to a single application. Overall, no significant differences in the microbial community composition could be detected between the tested facilities. Thus, we established a protocol, which assures successful implementation of the OMM^{12} model by a wide research community (Eberl et al., 2020).

Using the OMM^{12} mouse model, we further investigated the protective role of individual commensal bacteria during *Salmonella enterica* serovar Typhimurium (*S*. Tm) infections. Mice stably colonized with this synthetic community exhibit intermediate colonization resistance against *S*. Tm in comparison to mice colonized with the Altered Schaedler Flora (ASF) and mice with conventional microbiota. In a previous study it was shown that the addition of three facultative anaerobic bacteria (*Escherichia coli, Staphylococcus xylosus, Streptococcus danieliae)* to the OMM^{12} results in a conventional-like colonization resistance (Brugiroux et al., 2016). Here, we further dissected the role of facultative anaerobic bacteria in colonization resistance and discovered that *E. coli* is solely responsible for the restored colonization resistance against *S*. Tm in this model, while *S. danieliae* and *S. xylosus* are dispensable. Furthermore, we could show that *E. coli*-mediated protection depends on the microbial context, as *E. coli* does not increase colonization resistance against *S*. Tm in ASF mice. Using RNAseq, we found out that *E. coli* utilizes a high number of different carbon sources in ASF mice. In contrast, in OMM^{12} mice only a few genes involved in carbon metabolism are upregulated (e.g. for galactitol utilization). Furthermore, *E. coli* decreases the galactitol levels in the gut of OMM^{12} mice and an *E. coli* mutant deficient in utilizing galactitol is impaired in providing colonization resistance against *S*. Tm infection. Hence, *E. coli* provides colonization resistance against *S*. Tm by depleting galactitol, a limiting

carbon source in those mice. Additionally, we demonstrated that two members of the OMM^{12} consortium, *Blautia coccoides* YL58 and *Enterocloster clostridioformis* YL32 (both *Lachnospiraceae*), that can consume a variety of C5 and C6 sugars available in the gut, contribute to *E. coli*-mediated colonization resistance by creating a carbohydrate restricted environment. In summary, this established the concept that *E. coli* can only provide colonization resistance in a microbial context capable of removing simple sugars from the intestine (Eberl et al., 2021).

2. Zusammenfassung

Die Darmmikrobiota erfüllt viele nützliche Aufgaben für ihren Wirt, wie den Abbau komplexer Nährstoffe, das Training des Immunsystems und den Schutz vor enterischen Krankheitserregern, auch Kolonisierungsresistenz genannt. Aufgrund der hohen Komplexität der Darmmikrobiota und fehlender Untersuchungsmethoden ist es schwierig, den Beitrag einzelner Bakterien spezifischen Funktionen (z.B. Kolonisierungsresistenz) zuzuordnen und schützende Bakterien zu identifizieren. Die verringerte Komplexität von synthetischen Bakterienkonsortien, die aus kultivierbaren Darmisolaten zusammengestellt sind, bietet die Möglichkeit funktionelle Studien durchzuführen.

Die Oligo-Maus-Mikrobiota (OMM^{12}) ist ein synthetisches Bakterienkonsortium, das vor wenigen Jahren in meiner Forschungsgruppe etabliert wurde (Brugiroux et al., 2016). Dieses synthetische Konsortium besteht aus 12 unterschiedlichen bakteriellen Isolaten, die den 5 am häufigsten vorkommenden Phyla im Gastrointestinaltrakt von konventionellen Mäusen angehören. Die OMM^{12} wurde für die funktionelle Mikrobiomforschung im Mausmodell entwickelt und wird heute weltweit von vielen wissenschaftlichen Arbeitsgruppen in unterschiedlichsten Forschungsbereichen verwendet. Zur Erhöhung der experimentellen Reproduzierbarkeit zwischen unterschiedlichen Forschungseinrichtungen, wollten wir ein standardisiertes Inokulationsprotokoll für die Besiedlung keimfreier Mäuse mit dem OMM^{12} Konsortium etablieren. Um die Effizienz des entwickelten Protokolls zu vergleichen und herauszufinden, ob unterschiedliche Haltungsbedingungen einen Einfluss auf die Zusammensetzung der mikrobiellen Gemeinschaft haben, wurden 5 europäische Tiereinrichtungen in die Studie eingeschlossen. Es konnte gezeigt werden, dass die Bakterien innerhalb von 2 Wochen nach der Inokulation von keimfreien Mäusen in allen getesteten Einrichtungen eine stabile Gemeinschaft bilden. Des Weiteren kann die Rate der erfolgreichen Kolonisierung spezifischer, Sauerstoff-sensitiver Bakterien deutlich durch eine zweite Inokulation der Mäuse mit den OMM^{12} Bakterien nach 3 Tagen erhöht werden. Insgesamt konnten keine signifikanten Unterschiede in der Zusammensetzung der mikrobiellen Gemeinschaft zwischen den getesteten Einrichtungen festgestellt werden. Somit haben wir ein Protokoll etabliert, dass eine erfolgreiche Implementierung des OMM^{12} Modells in verschiedenen Forschungseinrichtungen ermöglicht (Eberl et al., 2020).

Des Weiteren haben wir das OMM^{12} Mausmodell dazu verwendet, um die schützenden Funktionen einzelner kommensaler Bakterien während *Salmonella enterica* serovar Typhimurium (*S.* Tm) Infektionen zu untersuchen. Mäuse, die stabil mit diesem Konsortium besiedelt sind, weisen eine intermediäre Kolonisierungsresistenz gegen *S.* Tm auf, verglichen mit Mäusen, die mit der Altered Schaedler Flora besiedelt wurden und Mäusen mit konventioneller Mikrobiota. In einer früheren Studie konnte gezeigt werden, dass die Zugabe von drei fakultativ anaeroben Bakterien (*Escherichia coli, Staphylococcus xylosus, Streptococcus danieliae)* zum OMM^{12} Konsortium zu einer Kolonisierungsresistenz führt, die der von Mäusen mit einer komplexen Mikrobiota ähnelt (Brugiroux et al., 2016). Hier wurde die Rolle der fakultativ anaeroben Bakterien weiter untersucht und herausgefunden, dass in diesem Modell *E. coli* allein für die Kolonisierungsresistenz gegen *S.* Tm verantwortlich ist, während *S. danieliae* und *S. xylosus* entbehrlich sind. Außerdem konnte gezeigt werden, dass der durch *E. coli* vermittelte Schutz vom mikrobiellen Kontext abhängt, da *E. coli* in ASF

Mäusen die Kolonisationsresistenz gegen *S.* Tm nicht erhöhen kann. Mittels RNAseq wurde festgestellt, dass *E. coli* in ASF Mäusen viele verschiedene Kohlenstoffquellen nutzen kann. Im Gegensatz dazu, wurden in OMM^{12} Mäusen nur wenige Gene hochreguliert, die am Kohlenstoffstoffwechsel beteiligt sind (z. B. für die Galactitol-Metabolisierung). Wir konnten zeigen, dass *E. coli* den Galactitolspiegel im Darm von OMM^{12} Mäusen senkt und dass die Besiedlung von OMM^{12} Mäusen mit einer *E. coli* Mutante, die Galactitol nicht verstoffwechseln kann, zu signifikant erhöhten *S.* Tm Zahlen führt. Daraus kann man schließen, dass *E. coli* durch das Depletieren der limitierenden Kohlenstoffquelle Galactitol in OMM^{12} Mäusen die Kolonisierungsresistenz gegen *S*. Tm vermittelt. Zwei Mitglieder des OMM^{12} Konsortiums, *Blautia coccoides* YL58 und *Enterocloster clostridioformis* YL32 (beide *Lachnospiraceae*), sind in der Lage viele im Darm frei verfügbare C5 und C6 Zucker zu konsumieren und zur *E. coli* vermittelten Kolonisierungsresistenz beizutragen, indem sie eine kohlenhydratarme Umgebung schaffen. Daraus kann man schließen, dass *E. coli* Kolonisierungsresistenz gegen *S.* Tm nur in einem mikrobiellen Kontext vermitteln kann, indem leicht verwertbare Zucker von der restlichen Mikrobiota depletiert werden (Eberl et al., 2021).

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3. Introduction

3.1 Gnotobiotic mouse models

3.1.1 Intestinal microbiota

The mammalian gastrointestinal tract is associated with a highly diverse microbial community that is composed of several hundred different bacteria, viruses, bacteriophages and some archaea, fungi, protozoa and worms (Vemuri et al., 2020). There is an increasing microbial density along the gastrointestinal tract, with only 10^1 microbial cells per gram content in the stomach to numbers up to 10^{12} cells per gram in the colon (Miller et al., 2021; Sommer and Bäckhed, 2013). The colonic microbiota is dominated by strictly anaerobic bacteria belonging to the phyla Bacteroidetes and Firmicutes (Clavel et al., 2016). Of note, the microbiota composition varies between healthy individuals and is influenced by diet, lifestyle and diseases (Fan and Pedersen, 2021).

The microorganisms in the gut fulfill crucial functions for their human host in several aspects. For example, they facilitate the breakdown of otherwise indigestible polysaccharides (e.g. fiber) and synthesize essential vitamins (LeBlanc et al., 2013; Oliphant and Allen-Vercoe, 2019). The microbiota contributes to developmental processes including a fully functional immune system by educating and priming host defenses (Clavel et al., 2017; Hooper et al., 2012). Another central function of the microbiota is to provide protection against enteric pathogens. This phenomenon is also termed colonization resistance (Buffie and Pamer, 2013; Stecher, 2015) and is conserved among humans, higher as well as lower animals and plants (Engel and Moran, 2013; McLaren and Callahan, 2020).

Recent advancements in the ‘omics’-technologies including metagenomics, metatranscriptomics, metaproteomics and metabolomics approaches as well as bioinformatic analysis pipelines (Bharti and Grimm, 2021) made it possible to get a more comprehensive view on the compositional variability of the microbiota, functional potential and alterations in patients (Miller et al., 2021). These studies revealed that characteristic microbiota signatures are associated with various diseases, such as inflammatory bowel disease (Khan et al., 2019), cardiovascular disease (Kazemian et al., 2020), colorectal cancer (Wong and Yu, 2019), depression (Barandouzi et al., 2020) and diabetes (Gurung et al., 2020) when compared to healthy controls. However, there is a gap in knowledge to translate microbiome signatures to functionality. Specifically, microbiome analyses do not allow us to predict a healthy microbiota by its composition or metabolic output. This is due to tremendous inter-individual differences of the microbiota on the taxonomic level, which are often explained by demography, ethnicity, age, health status, drug intake and dietary habits (Fan and Pedersen, 2021). Furthermore, microbiota functionalities are for the most part not conferred by individual microorganisms, but complex microbial networks engaged in metabolic and signaling interactions.

To unravel these functions, a combination of ‘top-down’ and ‘bottom-up’ studies has been implemented in the past years (**Figure 1**). Metagenomics-based approaches to study the microbiota have facilitated a ‘top-down’ view on the intestinal microbial community (correlations and associations of community

patterns), but also ‘bottom-up’ studies that focus on individual organisms and pathways yield important insights into mechanisms of microbial community assembly and stability as well as interactions between different members of the community and with their host. For these kind of studies, synthetic bacterial gut communities are valuable tools because they allow us to study model microorganisms and their interactions *in vitro* and *in vivo*, using gnotobiotic (Greek ‘gnotos’ known, ‘bios’ life) mouse models. Eventually, the aim is to generate computational models to predict and manipulate microbiome functionalities *in silico* (Shreiner et al., 2015).

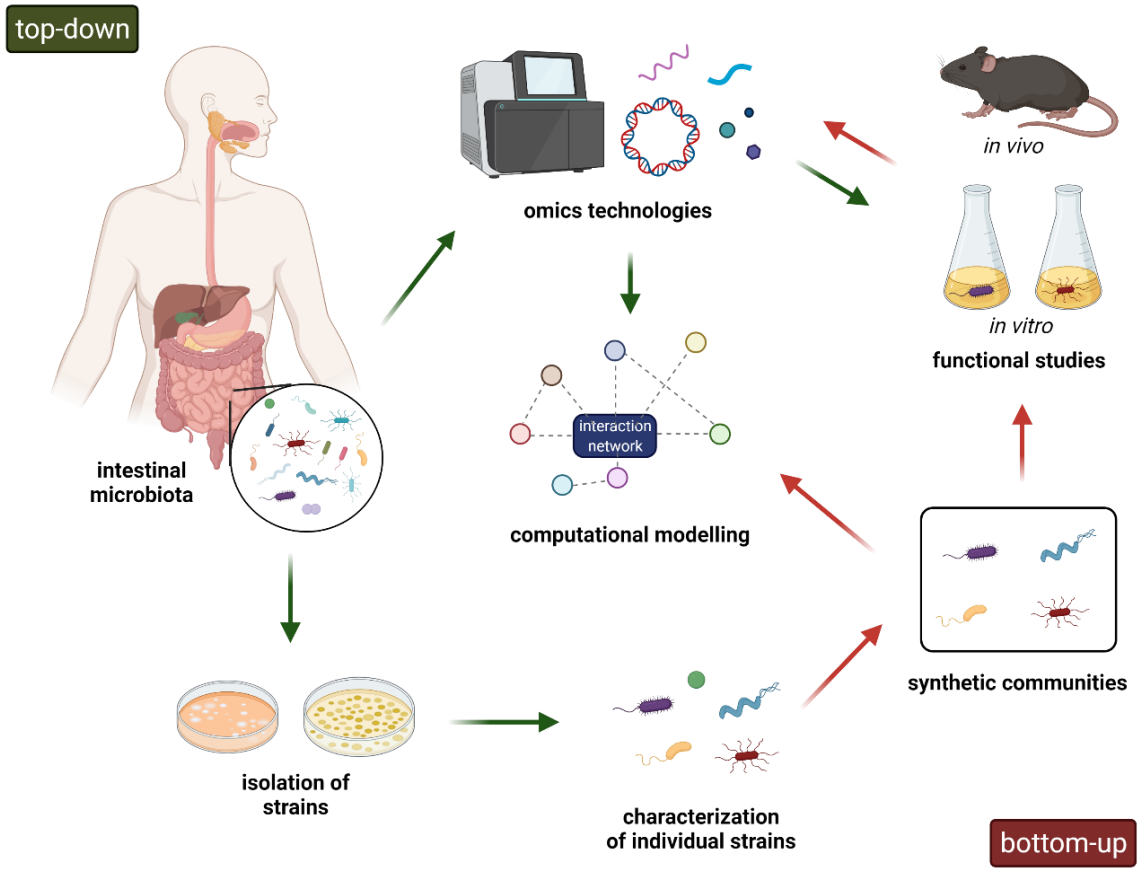

Figure 1: Strategies in microbiome research. The top-down approach relies on data generated by omics technologies (metagenomics, metatranscriptomics, metaproteomics, metabolomics) and comparing healthy and diseased individuals using complex microbial communities. In bottom-up studies, the interactions of individual gut isolates and different pathways are investigated using cultured isolates or synthetic communities for functional studies and predictive computational modelling. (Figure created with BioRender.com)

3.1.2 Synthetic bacterial gut communities

Due to the high complexity of the intestinal microbiota, it is difficult to investigate the molecular mechanisms underlying host-microbe or microbe-microbe interactions in health and disease. Synthetic bacterial communities offer a way to study microbial ecology and functionality *in vitro* (Fischbach, 2018). The main advantages of these models are that the strains of interest can be first extensively characterized *in vitro*, then the community can be assembled according to the research question, the

experimental conditions can be tightly controlled, and the results can be used to model bacterial interactions *in silico* (Clavel et al., 2016). For example, the Papin group identified interspecies metabolic interactions between 6 different bacterial strains using pairwise cultures and metabolome profiling (Medlock et al., 2018). Venturelli and colleagues developed a model to decipher interactions in a 12-member bacterial community. They show that pairwise interactions are important to understand community dynamics in a consortium with multiple bacterial strains (Venturelli et al., 2018).

Synthetic communities can be used to investigate functions of individual bacteria *in vivo* in germ-free mouse models. Mice can be re-derived germ-free by embryo transfer or caesarian section and then housed under germ-free conditions in isolators or gnoto-cages (Macpherson and McCoy, 2015) (**Figure 2**). From a germ-free state, the microbiota can be experimentally modified and colonized with individual bacteria of interest, with strain combinations, complex human-derived or defined synthetic communities (Elzinga et al., 2019). Most studies have used bacteria originating from the gastrointestinal tract of human or mice. To increase the clinical relevance, many studies use human isolates, although there are some problems associated with that. Strains of human origin are not always able to establish itself in the mouse gut and not necessarily fulfill the same functions as in their original host (Chung et al., 2012; Clavel et al., 2016) as the host and its resident microbiota have in part co-evolved over a long period of time. It was shown that a murine microbiota can simply outcompete an already established human-derived microbiota in the mouse gut, highlighting the host-specificity of the intestinal microbiota (Seedorf et al., 2014).

In comparison to mice with a conventional microbiota, germ-free mice have several abnormalities, such as an underdeveloped immune system, an altered metabolite and bile acid composition, a drastically enlarged cecum, decreased numbers of goblet cells, an altered mucus layer, and an increased susceptibility to bacterial infections (Fiebiger et al., 2016). One of the earliest attempts of synthetic community research was to normalize these parameters with defined bacterial mixtures (Schaedler et al., 1965). Unfortunately, bacterial communities developed in these early studies have not been preserved in public strain collections. In the recent years, a number of synthetic communities for the use in gnotobiotic mouse models have been established (Clavel et al., 2016; Elzinga et al., 2019), which I will summarize below.

In 1965 Russel W. Schaedler established a mouse colony with a defined microbial community (Schaedler et al., 1965), which was later modified bei P. Orcutt and then named Altered Schaedler Flora (ASF) (Orcutt, 1987). The ASF consists of 8 mouse-derived strains that are assigned to the phyla Firmicutes (*Anaerotignum* species ASF 356, *Lactobacillus intestinalis* ASF 360, *Ligilactobacillus murinus* ASF 361, *Eubacterium plexicaudatum* ASF 492, *Pseudoflavonifactor* species ASF 500, *Schaedlerella arabinosiphila* ASF 502) Bacteroidetes (*Parabacteroidetes goldsteinii* ASF 519) and Deferribacteres (*Mucispirillum schaedleri* ASF 457). This consortium is often used as reference or defined minimal microbiota (Brand et al., 2015), but the bacterial strains are currently not available in public strain collections. Other problems are that the community lacks some of the dominant intestinal bacteria found in the mouse gut and that ASF mice do not differ from germ-free mice in many microbiota-associated characteristics like the degradation of mucin or the induction of mucosal immune responses (Elzinga et al., 2019; Norin and Midtvedt, 2010).

The simplified human intestinal microbiota (SIHUMI) was introduced in 2011 and is composed of four Firmicutes (*Anaerostipes caccae*, *Lactiplantibacillus plantarum*, *Blautia producta*, and *Clostridium ramosum*), one Bacteroidetes species (*Bacteroides thetaiotaomicron*), one member of Actinobacteria (*Bifidobacterium longum*), and one of the Proteobacteria (*Escherichia coli*). These human-derived strains were selected according to their prevalence in humans, the availability of the genome sequences, their metabolic capabilities, and their ability to stably colonize the gut of rodents (Becker et al., 2021). The SIHUMI is normalizing gut parameters much better and is phylogenetically more diverse in comparison to the ASF, but is also lacking Verrucomicrobia, which is one of the five most abundant phyla found in the murine and human gut (Elzinga et al., 2019).

Another synthetic community with increasing popularity is the Oligo-Mouse-Microbiota (OMM^{12}) (Brugiroux et al., 2016).

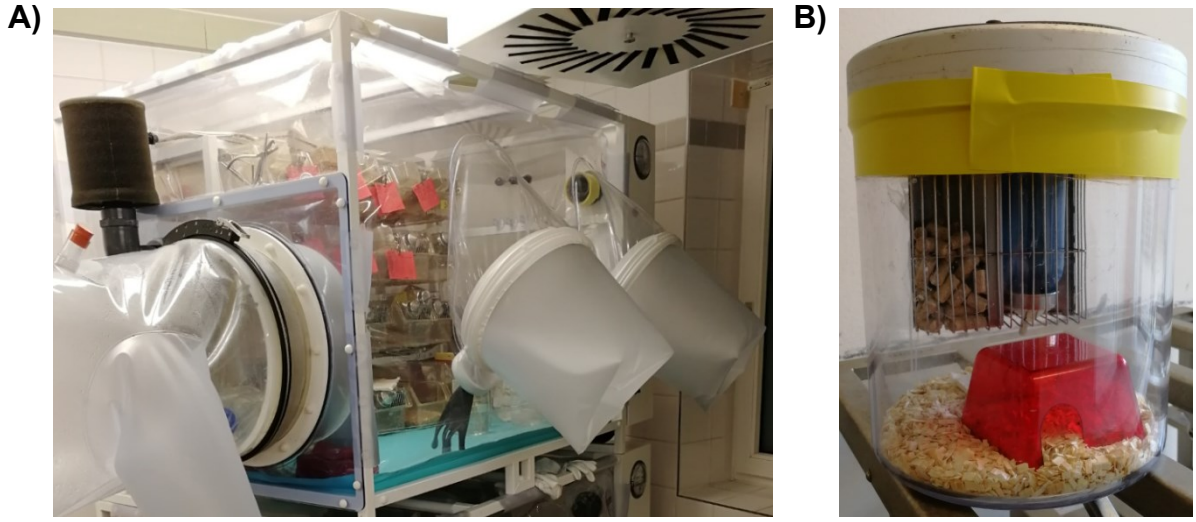

Figure 2: Housing conditions for gnotobiotic mice. (A) Flexible film isolators and **(B)** gnoto-cages ensure a germ-free environment during breeding of mice and experiments. The mice only receive autoclaved diet as well as water and are handled under sterile conditions.

3.1.3 Oligo-Mouse-Microbiota (OMM^{12})

The OMM^{12} is a synthetic minimal consortium that was recently established in the Stecher laboratory (Brugiroux et al., 2016). The OMM^{12} consists of 12 bacterial mouse isolates (*Enterococcus faecalis* KB1, *Limosilactobacillus reuteri* I49, *Bifidobacterium animalis* YL2, *Clostridium innocuum* I46, *Blautia coccoides* YL58, *Enterocloster clostridioformis* YL32, *Flavonifractor plautii* YL31, *Acutalibacter muris* KB18, *Bacteroides caecimuris* I48, *Muribaculum intestinale* YL27, *Akkermansia muciniphila* YL44 and *Turicimonas muris* YL45) representing five abundant bacterial phyla (Firmicutes, Bacteroidetes, Verrucomicrobia, Actinobacteria and Proteobacteria) of the human and mouse microbiota (**Figure 3A**). Besides based on their phylum-level diversity, the strains were selected according to their ability to stably colonize the mouse intestine over several generations. All strains are fully sequenced (Garzetti et al., 2017; Lamy-Besnier et al., 2021) and publicly available for non-commercial use within the mouse intestinal bacterial collection (miBC) (Lagkouvardos et al., 2016).

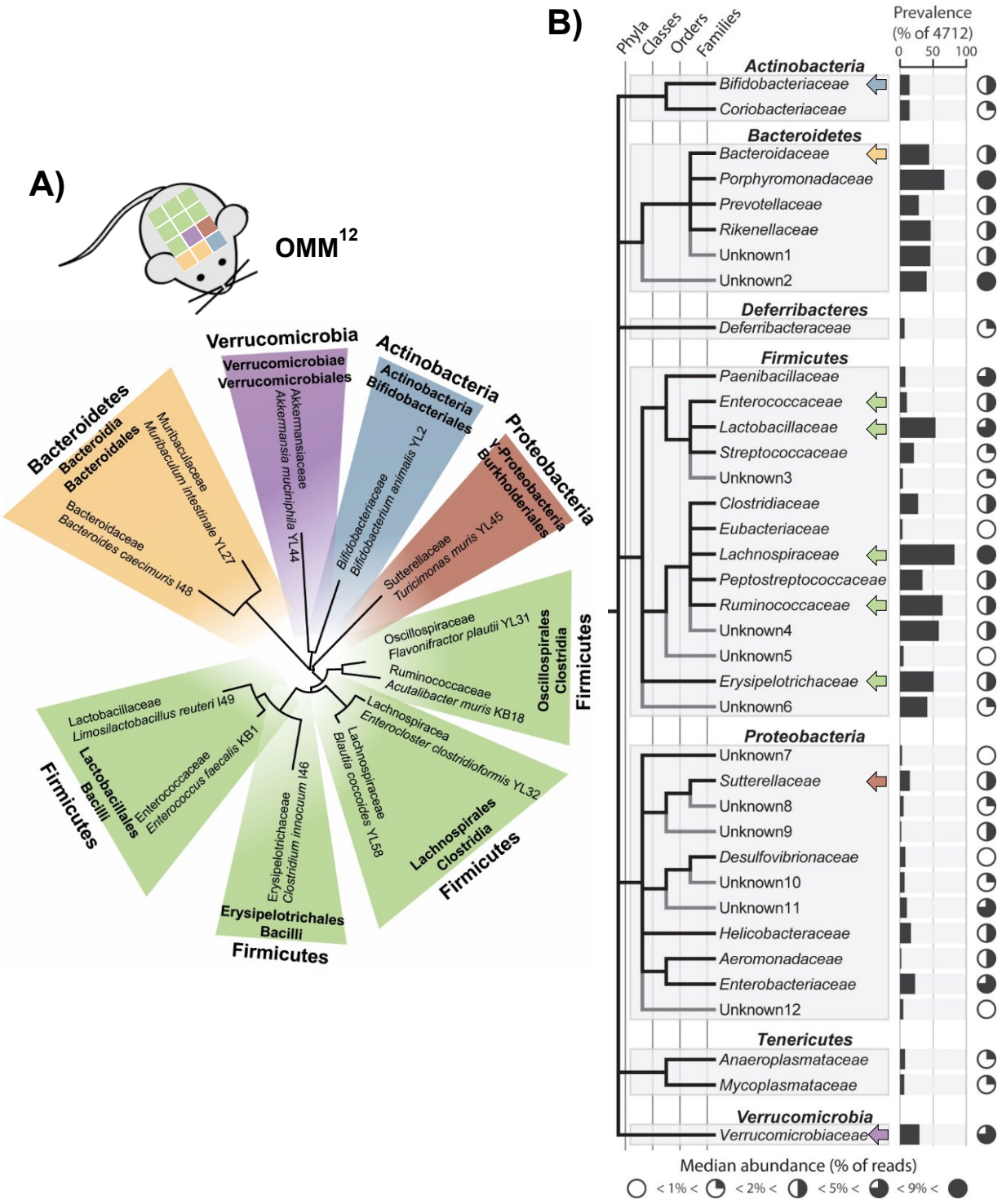

Figure 3: Phylogenetic membership of the OMM[12] bacteria. A) Phylogenetic tree of the OMM[12] community based on bacterial 16S rRNA gene sequences. Reproduced from (Weiss et al., 2021) with permission from corresponding author. **B)** Common bacterial families found in conventional mice. Prevalence and abundance of the families are indicated with histograms or pie charts, respectively. Families represented in the OMM[12] community are marked with arrows. Due to ongoing changes in taxonomic classification and nomenclature, *Muribaculaceae* (Bacteroidetes) and *Oscillospiraceae* (Firmicutes) are not listed, although they are frequent members of the murine gut microbiota. Reproduced from (Clavel et al., 2016) with permission from publisher.

To date, the OMM^{12} model is used in over 60 different laboratories worldwide for addressing various research questions (e.g. infection biology, microbe-host interactions, metabolism, immunology, and microbial ecology). Publications in which the OMM^{12} consortium was used are summarized in **Table 1**. In some studies, the OMM^{12} consortium is also designated as stable defined moderately diverse mouse microbiota 2 (sDMDMm2).

In a recent study, the metabolic potential and the interactions between the individual members of the OMM^{12} community were characterized *in vitro* using a bottom-up approach and metabolic models were generated as a reference for functional studies. It was demonstrated that the community interaction network is shaped by exploitative as well as interference competition and that *E. faecalis* KB1 and *B. coccoides* YL58 are important drivers of the community structure *in vitro*. Moreover, the main producers of short chain fatty acids, which are functionally important microbiota-derived metabolites in the gut, were identified. For example, both Bacteroidetes strains and the *Lachnospiraceae* strains are able to generate high amounts of acetic acid, while butyric acid is only produced by *F. plautii* YL31 and *C. innocuum* I46 (Weiss et al., 2021).

In contrast to the SIHUMI, the OMM^{12} contains one bacterium belonging to the phylum Verrucomicrobia: *Akkermansia muciniphila* YL44. *A muciniphila* is a specialized mucus-degrader (Ottman et al., 2017) and has been associated with a healthy intestine, as the numbers in the gut inversely correlate for example with obesity and diabetes (Dao et al., 2016; Plovier et al., 2017; Zhang et al., 2013). However, the OMM^{12} community is lacking representatives of the phyla Deferribacteres and Tenericutes, which are often found in the gut of conventional mice. This may explain phenotypic differences of OMM^{12} and conventional mice. Some important bacterial families like the facultative anaerobic *Enterobacteriaceae* and the sulfate-reducing *Desulfobacteriaceae* are also missing (**Figure 3B**). The absence of normally abundant bacterial species can have an impact on functionality and metabolic capacity of the microbial community (Brugiroux et al., 2016; Herp et al., 2019). Therefore, it will be important to add bacteria fulfilling these missing functions in studies aiming to better reflect the capabilities of a conventional microbiota. On the other hand, the reduced complexity of the OMM^{12} model offers the opportunity to complement the community with representative strains of the missing phyla/families and then investigate their impact on the microbiota as well as on the host.

Eventually, one future goal is to establish a synthetic community with as little bacterial strains as possible that can recapitulate phenotypes of a conventional microbiota when transplanted in germ-free mice. Recently, a new gnotobiotic mouse model consisting of 15 murine isolates has been introduced (three Bacteroidetes strains: *Bacteroides acidifaciens* MD185, *Bacteroides caecimuris* MD237, *Parabacteroides goldsteinii* MD072; eleven Firmicutes strains: *Clostridium cocleatum* I50, *Enterocloster clostridioformis* YL32, *Clostridium* sp. MD294, *Clostridium* sp. MD300, *Subtilibacillum caecimuris* MD335, *Longibacillum caecimuris* MD329, *Irregularicoccus caecimuris* MD308, *Lactobacillus johnsonii* MD006, *Ligilactobacillus murinus* MD040, *Limosilactobacillus reuteri* MD207, *Anaerotruncus colihominis* JM4-15; one Proteobacterium: *Escherichia coli* Mt1B1). This synthetic community recapitulates more phenotypical features of mice with a conventional microbiota and also covers more prevalent intestinal bacterial families in comparison to the OMM^{12}, although the strains only belong to 3 different phyla (Darnaud et al., 2021).

Table 1: Publications using the OMM[12] model.

Article	Research area	Title
(Li et al., 2015)	Microbial ecology	The outer mucus layer hosts a distinct intestinal microbial niche
(Brugiroux et al., 2016)	Model establishment/ Infection biology	Genome-guided design of a defined mouse microbiota that confers colonization resistance against *Salmonella enterica* serovar Typhimurium
(Lagkouvardos et al., 2016)	Strain collection/ Public resource	The Mouse Intestinal Bacterial Collection (miBC) provides host-specific insight into cultured diversity and functional potential of the gut microbiota
(Uchimura et al., 2016)	Genomics	Complete genome sequences of 12 species of stable defined moderately diverse mouse microbiota 2
(Studer et al., 2016)	Infection biology	Functional intestinal bile acid 7α-Dehydroxylation by *Clostridium scindens* associated with protection from *Clostridium difficile* infection in a Gnotobiotic Mouse Model
(Garzetti et al., 2017)	Genomics	High-quality whole-genome sequences of the Oligo-Mouse-Microbiota bacterial community
(Steinert et al., 2017)	Immunology	The stimulation of macrophages with TLR ligands supports increased IL-19 expression in inflammatory bowel disease patients and in colitis models
(Kindt et al., 2018)	Metabolism	The gut microbiota promotes hepatic fatty acid desaturation and elongation in mice
(Herp et al., 2019)	Infection biology	*Mucispirillum schaedleri* antagonizes *Salmonella* virulence to protect mice against colitis
(Wotzka et al., 2019)	Infection biology	*Escherichia coli* limits *Salmonella Typhimurium* infections after diet shifts and fat-mediated microbiota perturbation in mice
(Bolsega et al., 2019)	Infection biology	Composition of the Intestinal Microbiota Determines the Outcome of Virus-Triggered Colitis in Mice
(Rolhion et al., 2019)	Infection biology	A *Listeria monocytogenes* bacteriocin can target the commensal *Prevotella copri* and modulate intestinal infection
(Marion et al., 2019)	Metabolism	In vitro and in vivo characterization of *Clostridium scindens* bile acid transformations
(Eberl et al., 2020)	Model establishment	Reproducible colonization of germ-free mice with the Oligo-Mouse-Microbiota in different animal facilities
(Fischer et al., 2020)	Metabolism, IBD	Dietary cellulose induces anti-inflammatory immunity and transcriptional programs via maturation of the intestinal microbiota
(Wyss et al., 2020)	Immunology	Using precisely defined in vivo microbiotas to understand microbial regulation of IgE
(Nowosad et al., 2020)	Immunology	Tunable dynamics of B cell selection in gut germinal centres
(Lehmann et al., 2020)	Immunology	Microbiota-induced tissue signals regulate ILC3-mediated antigen presentation
(Schaupp et al., 2020)	Immunology	Microbiota-Induced Type I Interferons Instruct a Poised Basal State of Dendritic Cells
(Lourenco et al., 2020)	Phages/ Microbial ecology	The spatial heterogeneity of the gut limits predation and fosters coexistence of bacteria and bacteriophages
(Marion et al., 2020)	Metabolism	Biogeography of microbial bile acid transformations along the murine gut
(Kuczma et al., 2020)	Immunology	Commensal epitopes drive differentiation of colonic T regs
(Feuerstein et al., 2020)	Immunology	Resident macrophages acquire innate immune memory in staphylococcal skin infection
(Mager et al., 2020)	Immunology	Microbiome-derived inosine modulates response to checkpoint inhibitor immunotherapy

(van Tilburg Bernardes et al., 2020)	Microbial ecology	Intestinal fungi are causally implicated in microbiome assembly and immune development in mice
(Lamy-Besnier et al., 2021)	Genomics	Closed and high-quality bacterial genome sequences of the Oligo-Mouse-Microbiota community
(Zünd et al., 2021)	Genomics/ Phages	High throughput sequencing provides exact genomic locations of inducible prophages and accurate phage-to-host ratios in gut microbial strains
(Streidl et al., 2021)	Metabolism	The gut bacterium *Extibacter muris* produces secondary bile acids and influences liver physiology in gnotobiotic mice
(Yilmaz et al., 2021)	Microbial ecology	Long-term evolution and short-term adaptation of microbiota strains and sub-strains in mice
(Weiss et al., 2021)	Microbial ecology	Exploring the interaction network of a synthetic gut bacterial community
(Maier et al., 2021)	Microbial ecology	Unravelling the collateral damage of antibiotics on gut bacteria
(Eberl et al., 2021)	Infection biology	*E. coli* enhance colonization resistance against *Salmonella* Typhimurium by competing for galactitol, a context-dependent limiting carbon source

3.1.4 Generation of standards of use for the OMM^{12} model

An increasing number of research groups worldwide are currently using the OMM^{12} mouse model to address a variety of research questions (**Table 1**). This offers a huge potential as it allows to compare results between different laboratories and to generate a database collecting phenotypes and functionalities conferred by this community. To this end, it is important to generate certain standards in using the community. Therefore, the aim of the first part of my PhD thesis was to establish a protocol for the reproducible colonization of germ-free mice with the OMM^{12} consortium and to determine the time that is required by the OMM^{12} bacteria to form a stable community in the mouse gut. We further wanted to compare the efficiency of the established protocol in different European germ-free rodent facilities and investigate if the varying housing conditions (housing type, standard chow, water etc.) of the mice in the different facilities have a major impact on the microbial community composition.

3.2 *Salmonella* enterica serovar Typhimurium (*S*. Tm) infection

3.2.1 General information

S. Tm is a Gram-negative, facultative anaerobic, rod-shaped bacterium belonging to class Gamma-Proteobacteria, family *Enterobacteriaceae*. The serovar Typhimurium is one of the most important *Salmonella enterica* serotypes causing infections worldwide. It is often acquired through the consumption of contaminated food or water (Eng et al., 2015). The most common symptoms are diarrhea, abdominal cramps, fever, and nausea. *Salmonella*-induced gastroenteritis is often self-limiting and does not need specific medical treatment, but disease can be severe in young children, elderly and immunocompromised patients (Coburn et al., 2006). In the European Union, Salmonellosis is the second most common cause of bacterial gastrointestinal infections and is responsible for many foodborne outbreaks. In 2017, 92649 cases were reported of which 156 ended fatal (ECDC, 2020).

Interestingly, only a minority of the people (approximately 1-5%) exposed to *Salmonella* develop clinical symptomatic infections (Simonsen et al., 2009). Thus, in most individuals the infection seems to be efficiently blocked. This could be due to the absence of genetic predisposition or immunosuppression but also to varying degrees of colonization resistance mediated by the resident gut microbiota (Stecher, 2021). Hence, strengthening microbiota-mediated colonization resistance in people at risk to contract an infection might be a promising approach to improve public health.

3.2.2 Phases of *S*. Tm infection

After ingestion, *S*. Tm has to pass through the stomach, where the acidic pH eliminates up to 99% of the pathogen population (Giannella et al., 1972). The ability of the few surviving bacteria to grow in the gastrointestinal tract depends on the resident microbial community. The proposed mechanisms of colonization resistance are described in detail in section 3.2.3. The capacity of the microbiota to prevent *S*. Tm expansion can be impaired for example by antibiotics (Bohnhoff et al., 1954), high-fat diet (Wotzka et al., 2019) or pre-existing inflammation (Stecher et al., 2007).

Another important host barrier against pathogen invasion is the intestinal mucus layer (Zarepour et al., 2013). *S*. Tm is able to cross the inner mucus layer by using flagella and chemotaxis (Stecher et al., 2004). Upon reaching the epithelial tissue, *S*. Tm uses the adhesin SiiE to adhere at the epithelial cells and invades into the cells using the type III secretion system (T3SS) (Zhang et al., 2018). *S*. Tm is also able to invade host cells via T3SS-independent mechanisms (Boumart et al., 2014).

The invasion of *S.* Tm stimulates a complex cascade of immune responses, involving the activation of the NAIP/NLRC4 inflammasome, interleukin-18 secretion and finally interferon-gamma production (Wotzka et al., 2017). These immune defenses efficiently reduce *S*. Tm tissue loads (Sellin et al., 2014), but the resulting inflammation also drastically alters the intestinal environment which leads to dysbiosis and blooming of *Enterobacteriaceae* (Stecher, 2015; Zeng et al., 2017). *S*. Tm as well as other *Enterobacteriaceae* are more resistant against antimicrobial host defenses in comparison to other microbiota members (Gill et al., 2012; Raffatellu et al., 2009) and can profit from the generation of

anaerobic electron acceptors like tetrathionate (Winter et al., 2010) and nitrate (Lopez et al., 2012), host-derived metabolites like lactate (Gillis et al., 2018), and increased luminal oxygenation near the epithelium (Rivera-Chávez et al., 2016) in the inflamed gut. Furthermore, respiratory growth enables *S.* Tm to metabolize otherwise unfavored carbon sources like ethanolamine, propanediol and succinate (Faber et al., 2017; Spiga et al., 2017; Thiennimitr et al., 2011).

The recovery from *S.* Tm infection is mediated by innate and adaptive immune responses. For example, *S.* Tm-specific IgA is secreted into the intestinal lumen and so promotes the clearance of the pathogen (Moor et al., 2017). In this phase, inflammation decreases, the microbiota recovers, colonization resistance is reestablished, and the pathogen is displaced (**Figure 4**).

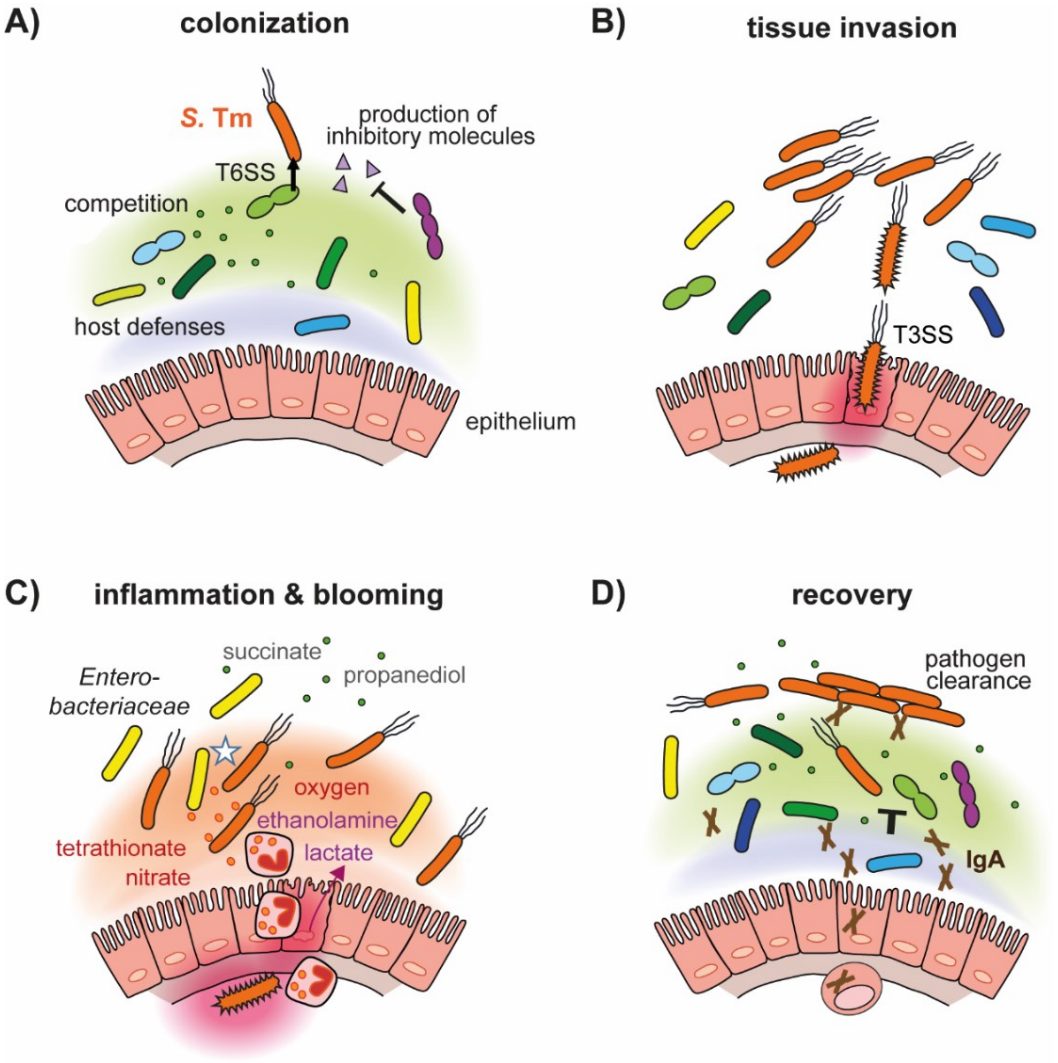

Figure 4: Phases of *S.* Tm infection. A) Intestinal colonization. The resident microbiota can prevent the establishment of the pathogen by producing inhibitory molecules (e.g. bacteriocins, SCFA), competing for limiting resources, direct killing using T6SS or stimulation of host immune defenses. **B)** Tissue invasion is mediated by the T3SS. **C)** Inflammation leads to a drastically altered gut environment resulting in a bloom of *Enterobacteriaceae*. **D)** Recovery and clearance of the pathogen is facilitated by innate and adaptive immune responses. Adopted from (Stecher, 2021).

3.2.3 Colonization resistance

The importance of the resident microbiota in the defense against enteric pathogens was first described in the 1950s by Bonhoff and colleagues. They showed that mice in which the microbiota was disturbed by antibiotics require a 10^5-fold lower dose of *S*. Tm to get infected (Bohnhoff et al., 1954). Since then, many studies aimed to identify members of the microbiota involved in colonization resistance. Due to the high diversity of the intestinal microbiota, it is still challenging to causally relate specific species in protective microbiome signatures to colonization resistance. Therefore, germ-free mice and gnotobiotic mouse models with reduced complexity are very useful for deciphering the interactions among the microbiota, their host and the incoming pathogens (Stecher et al., 2013). Some key mechanisms of colonization resistance are summarized in the next section.

3.2.3.1 Resource competition

Specific members of the microbiota can compete with *S*. Tm for similar nutrient niches. In 1983 Rolf Freter formulated the nutrient-niche hypothesis, stating that if all available nutrient niches are blocked by the autochthonous microbiota, pathogens are not able to invade the ecosystem (Freter et al., 1983). Most likely, *S*. Tm is competing with closely related bacterial species from the family *Enterobacteriaceae* (e.g. *E. coli*) due to their high metabolic similarity (Rogers et al., 2020). It was already shown that commensal *Enterobacteriaceae* are able to increase the colonization resistance against different *Salmonella* serovars in conventional (Velazquez et al., 2019) as well as in gnotobiotic mice (Brugiroux et al., 2016) and in gnotobiotic piglets (Splichalova et al., 2011). Litvak and colleagues demonstrated that *Enterobacteriaceae* contribute to colonization resistance against *Salmonella* Enteritidis by competing for oxygen in the gut of neonatal chicks (Litvak et al., 2019). It was also shown that closely related *Enterobacteriaceae* compete for microbiota-liberated mucosal sugars (Maltby et al., 2013) and that *S*. Tm can benefit from the altered mucosal carbohydrate availability in the gut of antibiotic-treated mice (Ng et al., 2013). Oliveira and colleagues recently reported that *Klebsiella michiganensis* mediates colonization resistance against *Salmonella* via substrate competition (Oliveira et al., 2020). Furthermore, *E. coli* can also confer colonization resistance by competing for hydrogen or C4-dicarboxylates (Nguyen et al., 2020) and the probiotic *E. coli* Nissle competes with *S*. Tm for the trace element iron in the inflamed gut (Deriu et al., 2013). Overall, these data suggest that closely related commensal *Enterobacteriaceae*, in particular *E. coli* and *Klebsiella* spp., play an important role in mediating pathogen exclusion by substrate competition. So far, however, the importance of different substrates as well as the contribution of other members of the microbiota remain unclear.

3.2.3.2 Direct killing or inhibition

Besides competing for substrates with the pathogen, the microbiota can produce inhibitory compounds (e.g. bacteriocins) that negatively affect the growth of the invading pathogen. For example, *E. coli* Nissle produces microcins in the inflamed gut that target *S.* Tm (Sassone-Corsi et al., 2016). Various anaerobic bacteria produce short chain fatty acids (SCFA) like acetate, propionate, and butyrate as fermentation by-products. These SCFA induce a local pH reduction and this can inhibit the proliferation of *Enterobacteriaceae* (Sorbara et al., 2019). *Bacteroides* species mediate colonization resistance against *S*. Tm through the production of propionate. Propionate negatively influences *S*. Tm proliferation by

disrupting the intracellular pH hemostasis (Jacobson et al., 2018). Notably, SCFA also interfere with *S.* Tm virulence gene expression and can so modulate the infectivity of the pathogen in later phases of infection (Durant et al., 2000; Gantois et al., 2006; Hung et al., 2013).

Another strategy is the direct injection of toxins into the invading pathogen using a contact-dependent type VI secretion system (T6SS) (Chen et al., 2019), but to date no cases of T6SS-mediated killing of *S.* Tm by the microbiota have been reported. Overall, the microbiota can kill the pathogen or attenuate its growth by a variety of different mechanisms.

3.2.3.3 Stimulation of host immune responses

The microbiota can also indirectly prevent the outgrowth of pathogens in the gut by stimulating antimicrobial host defenses (Caballero and Pamer, 2015). For example, the gut microbiota triggers the expression of lectins that have a bactericidal effect on a wide range of bacteria (Cash et al., 2006). Thiemann and colleagues showed that distinct members of the microbiota prevent *S.* Tm infection by stimulating antibacterial interferon-gamma responses (Thiemann et al., 2017). This mechanism limits pathogen invasion, leads to a delayed onset of *S.* Tm induced inflammation, and increases survival of the host.

3.2.3.4 Community-dependent colonization resistance

There is increasing evidence that colonization resistance is not mediated by a single bacterial species but rather by the concerted activity of a complex community. Protective bacteria may require a specific microbial context to counteract the invading pathogen. A study of the Stecher group showed that a mixture of 3 facultative anaerobes only confers protection to mice colonized with the OMM^{12} but not to ASF mice (Brugiroux et al., 2016). Similarly, *Blautia producta* requires the presence of 3 other commensal bacteria (*Bacteroides sartorii*, *Parabacteroides distasonis Clostridium bolteae*) to protect against vancomycin-resistant *Enterococci* (VRE) (Caballero et al., 2017). There are several explanations for this context-dependency (**Figure 5**). First, the bacterial community cooperatively restricts the range of substrates in the gut and this way mediates pathogen exclusion. Second, the other bacteria create a specific environment that is required for the successful colonization of the protective strain, and third, the presence of the protective strains triggers functions in other members of the community that limit pathogen colonization - or the other way around (Stecher, 2021).

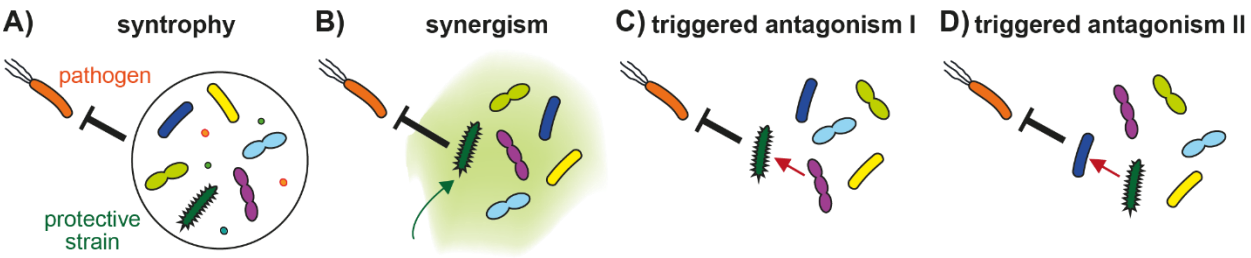

Figure 5: Microbial context-dependent colonization resistance. A) The community cooperatively mediates pathogen exclusion by limiting available substrates. **B)** The microbiota facilitates the colonization of the protective strain by creating a specific environment. **C)** Other bacteria trigger protective functions in the protective strain. **D)** The protective strain triggers protective functions in other members of the microbiota.

3.2.4 *S*. Tm infection studies in OMM^{12} mice

In the past, the OMM^{12} mouse model has been used to investigate different stages of *S*. Tm infection.

Two studies investigated mechanisms underlying colonization resistance against *S*. Tm in the OMM^{12} model. Brugiroux and colleagues showed that mice stably colonized with the OMM^{12} consortium exibit an intermediate degree of colonization resistance against *S*. Tm compared to ASF mice and mice with a conventional laboratory microbiota. The addition of 3 facultative anaerobic bacteria (*Escherichia coli, Staphylococcus xylosus, Streptococcus danieliae)* to the OMM^{12} consortium leads to a significant increase in colonization resistance that is comparable to that of conventional mice. Interestingly, the addition of the 3 facultative anaerobic bacteria to mice colonized with ASF does not increase the colonization resistance against *S*. Tm. Hence, the protective effect of the facultative anaerobic bacteria depends on the microbial context (Brugiroux et al., 2016). It has remained unclear, which of the facultative anaerobic bacteria confers the resistance and what are the mechanisms of context-dependent protection in this case.

Wotzka and colleagues demonstrated that a shift to high-fat diet drastically decreases the colonization resistance against *S*. Tm in OMM^{12} mice. An increased fat intake results in elevated bile acid levels in the gut. They showed that *S*. Tm and *E.coli* have a higher intrinsic resistance to bile salts in comparison to other bacteria of the microbiota and that this explains the outgrowth of *S*. Tm in the gut of mice lacking *E. coli*. In their model, the presence of competitive *E.coli* are protective in the fat-challenged gut by limiting *S*. Tm infection, but the mechanism of this protective effect is still unclear (Wotzka et al., 2019).

Herp and colleagues described that a member of the Deferribacteres is able to interfere with *S*. Tm virulence. While *Mucispirillum schaedleri* does not confer colonization resistance against *S*. Tm to OMM^{12} mice, it protects from *S.* Tm tissue invasion and colitis. The underlying mechanism is that *S*. Tm downregulates its invasion machinery (T3SS) in presence of *M. schaedleri*, which is presumably mediated by nitrate competition between the two bacteria (Herp et al., 2019). This is an example where a commensal bacterium influences the second infection stage (tissue invasion), but does not interfere with *S.* Tm colonization.

3.2.5 Investigation of OMM^{12}-mediated colonization resistance

It is challenging to determine the contribution of individual bacterial species in providing colonization resistance against invading pathogens. The reduced complexity of the OMM^{12} model enables us to pin down the role of single bacterial species and to get mechanistic insights into the interplay of different bacterial species. In a previous study (Brugiroux et al., 2016), work in the Stecher group showed that the addition of facultative anaerobic bacteria leads to a significantly increased colonization resistance against *S*. Tm in OMM^{12} mice, but not in mice colonized with ASF. In the second part of my thesis, I aimed to elucidate the mechanism of colonization resistance mediated by the facultative anaerobic bacteria and further investigated the microbial context dependency of this protective effect (**Figure 5**).

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4. Publications

4.1 Paper I

Reproducible colonization of germ-free mice with the Oligo-Mouse-Microbiota in different animal facilities

Claudia Eberl, Diana Ring, Philipp C. Münch, Markus Beutler, Marijana Basic, Emma C. Slack, Martin Schwarzer, Dagmar Srutkova, Anna Lange, Julia S. Frick, André Bleich, Bärbel Stecher

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4.1.1 My contributions I

I am the sole first author of this study. Together with my supervisor Bärbel Stecher, I conceived and designed the experiments. I performed colonization experiments with germfree mice, coordinated the sampling in the other tested facilities with the co-authors and processed all samples. With the help of my co-authors, I analyzed the data. Besides editing the original draft written by Bärbel Stecher, I created all figures and tables included in this manuscript and wrote the ‘Materials and Methods’ section and the figure legends.

4.2 Paper II

E. coli enhance colonization resistance against Salmonella Typhimurium by competing for galactitol, a context-dependent limiting carbon

Claudia Eberl, Anna S. Weiss, Lara M. Jochum, Abilash Chakravarthy Durai Raj, Diana Ring, Saib Hussain, Simone Herp, Chen Meng, Karin Kleigrewe, Michael Gigl, Marijana Basic, Bärbel Stecher

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4.2.1 My contributions II

I am the sole first author of this study. Together with my supervisor Bärbel Stecher, I conceived and designed the experiments. With exception of the spent media experiments and phenotypic microarrays (Figure 4), I performed all experiments and created all genetically modified bacterial strains described in this manuscript. Together with my co-authors, I analyzed the data. Besides editing to original draft written by Bärbel Stecher, I created all figures and tables included in this manuscript and wrote the 'Materials and Methods' section and the figure legends.

Danksagung

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