



## Synthetic Biology Strategies for Engineering of Human Microbiome Related Species for Therapeutic Applications

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**Engineering Advanced Microbiome Therapeutics Using  
*E. coli* Nissle 1917 for Bile Acid and Neurodegenerative  
Related Disorders**

Ph.D. Thesis  
August 2020

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The Novo Nordisk Foundation Center for Biosustainability  
Technical University of Denmark

**Engineering Advanced Microbiome Therapeutics Using *E. coli* Nissle 1917 for Bile Acid and Neurodegenerative Related Disorders**

**Felipe Gonzalo Tueros Farfan**

## Preface

The present Ph. D. thesis serves as a partial fulfillment of the requirements to obtain a Ph. D. degree from the Technical University of Denmark (DTU). The work presented, was performed at the Bacterial Synthetic Biology Section at the Novo Nordisk Foundation Center for Biosustainability (NNF-CFB) at DTU, from 1<sup>st</sup> of September 2016 to the 31<sup>st</sup> of August 2020. The projects were supervised by Professor Morten O. A. Sommer from the (NNF-CFB), DTU. The Ph. D. was funded by the Novo Nordisk Foundation, Ph. D fellowship grant no. NNF16CC0020908.



Felipe Gonzalo Tueros Farfan, 31<sup>st</sup> of August 2020  
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## Abstract

Advances in DNA synthesis, sequencing and a deeper understanding of biological and biochemical mechanisms have enabled the development of engineered microbes, and has expedited the speed at which they are constructed, tested and optimized. This has led to the revolution of biotechnology and its further application through different industrial fields. Even though this advancements have had significant repercussions regarding the discovery of new drugs and an increased efficiency in production capabilities derived from microbial fermentations, the medical sector has not benefited fully from the immense potential biotechnology has to offer.

With the advent of microbiome research, diseases are being understood from a different perspective, taking into account the role that bacteria inhabiting our bodies have towards these events and how they modulate positively or negatively diseases. In this view, engineered microorganisms have the prospective to be much more than simple cell factories. Engineered probiotic strains, for example, could be targeted to address diseases on site, responding to the patient's needs and to the environmental cues where they have established. This could prove to be a novel approach for treating chronic diseases, which often impair a normal life style, affecting deeply the life quality of patients.

On the same line of thought, supporting amount of evidence is tilting the balance towards a more personalized treatment, as more insights into how drugs are absorbed, metabolized, regulated and degraded, are seen to depend greatly on the characteristics of each individual. Advanced microbiome therapeutics (AMT)s are envisioned to address the hurdles that modern medicine has come a crossed. These engineered microbes could potentially colonize the environment of choice, within the human body to deliver therapeutic action, by the production of specific drugs, degradation of toxic molecules or by modulating harmful microbial communities that pose a risk for normal wellbeing. Additionally, the development of such strains contributes dramatically to the transition into a more sustainable society, making us less dependent on natural resources for production capabilities, as these therapeutic microorganisms will produce the needed drugs by utilizing normal occurring resources in their given environments.

This technology, however, is in its early stages and needs further research to properly assess its potential and fully understand its capabilities. In the present thesis, bile acid and neurodegenerative disorders are taken as examples for the engineering and development of advanced microbiome therapeutics. Envisioning them as possible approaches to address pathologies in these areas, enabling a more efficacious treatment approach. *E. coli* Nissle 1917, a well-documented probiotic, was employed as the bacterial chassis for such intended strains.

## Dansk Sammenfatning

Fremskridt inden for DNA-syntese, sekventering og en dybere forståelse af biologiske og biokemiske mekanismer har muliggjort udviklingen af konstruerede mikrober og har fremskyndet den hastighed, hvormed de konstrueres, testes og optimeres. Dette har ført til revolutionen inden for bioteknologi og dens yderligere anvendelse gennem forskellige industrielle områder. Selvom disse fremskridt har haft betydelige konsekvenser med hensyn til opdagelsen af nye lægemidler og en øget effektivitet i produktionskapaciteten, der stammer fra mikrobielle gæringer, har den medicinske sektor ikke haft fuldt udbytte af det enorme potentiale, som bioteknologi har at tilbyde.

Med fremkomsten af mikrobiomeforskning forstås sygdomme fra et andet perspektiv under hensyntagen til den rolle, som bakterier, der lever i vores kroppe, har over for disse begivenheder, og hvordan de modulerer positive eller negative sygdomme. I denne opfattelse har konstruerede mikroorganismer muligheden for at være meget mere end enkle cellefabrikker. Konstruerede probiotiske stammer kunne f.eks. Målrettes mod sygdomme på stedet, svarende på patientens behov og de miljømæssige signaler, hvor de er etableret. Dette kan vise sig at være en ny tilgang til behandling af kroniske sygdomme, som ofte forringer en normal livsstil, der påvirker patienternes livskvalitet dybt.

På samme tankegang vipper understøttende mængde bevis balancen mod en mere personlig behandling, da mere indsigt i, hvordan stoffer absorberes, metaboliseres, reguleres og nedbrydes, afhænger meget af de enkelte personers egenskaber. Advanced Microbiome Therapeutics (AMT)s er planlagt til at tackle de forhindringer, som moderne medicin er kommet på tværs af. Disse konstruerede mikrober kan potentielt kolonisere det valgte miljø inden for den menneskelige krop for at levere terapeutisk virkning ved produktion af specifikke lægemidler, nedbrydning af toksiske molekyler eller ved at modulere skadelige mikrobielle samfund, der udgør en risiko for normal velvære. Derudover bidrager udviklingen af sådanne stammer dramatisk til overgangen til et mere bæredygtigt samfund, hvilket gør os mindre afhængige af naturlige ressourcer for produktionsmuligheder, da disse terapeutiske mikroorganismer vil producere de nødvendige lægemidler ved at udnytte normale forekommende ressourcer i deres givne miljøer.

Denne teknologi er imidlertid i sine tidlige faser og har brug for yderligere forskning for korrekt at kunne vurdere dens potentiale og fuldt ud forstå dens evner. I den foreliggende afhandling tages galdesyre og neurodegenerative lidelser som eksempler på konstruktion og udvikling af avanceret mikrobiom-terapi. At forestille sig dem som mulige tilgange til behandling af patologier i disse områder, hvilket muliggør en mere effektiv behandlingsmetode. *E. coli* Nissle 1917, et veldokumenteret probiotikum, blev anvendt som bakteriekabinet til sådanne tilsigtede stammer.

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Thanks to all present and former members of the Sommer lab, you have made my time here incredible, I have learned so much and had so much fun at the same time. Sarunas, Simone, Sang-woo, Felipe, Alex (Caaarl), Max, William, Anne, Carina, Frederick, Jeremy, Andreas, Mari, Sara, Vanessa, Carmen, Dennis and Hitesh, never a boring lunch with you guys. Special thanks to the people I have worked and collaborated with, Stefano, Mostafa, Hans, Leonie, Carola, Peter, Kira, Scott, Michael, Anne, Mareike (also for reviewing part of the thesis), Anette, Ruben, Marcus, Vibeke, Hongbin, Lei, Hao, Christian and Alex. To the people of the analytical core at CfB, Mette, Charlotte, Tune, Lars and Lars, thank you so much for helping me out in these 4 years. To Rebeca for always keeping an eye on the PhD students at CfB. Also, to all the members of the PhD club, I was lucky to be part of.

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Finally, I would like to thank the Novo Nordisk Foundation, for allowing me to be part of the Copenhagen Bioscience PhD Program and granting me a PhD fellowship to undertake my studies.

Thank you all!

## List of Publications

The work included in this thesis has contributed to the following manuscripts:

- 1. Bile Acids Are Not a Native Product of Microbial Marine Species.**  
Felipe G. Tueros, Mostafa M. H. Ellabaan, Marcus Ståhlman, Ruben V. Uribe, Fredrik Bäckhed, Morten O. A. Sommer. *Manuscript under preparation*
- 2. Exploring strategies for secondary bile acid degradation and neutralization**  
Felipe G. Tueros, Marcus Ståhlman, Mostafa M. H. Ellabaan, Scott Quainoo, Christian B. Jendresen, Alex T. Nielsen, Fredrik Bäckhed, Morten O. A. Sommer. *Manuscript under preparation*
- 3. Engineering Advanced Microbiome Therapeutics for Production and Delivery of L-DOPA and Dopamine**  
Felipe G. Tueros, Mareike Bongers, Lei Yang, Hao Lau, Hongbin Zhang, Vibeke H. K. Kruse, Line V. Morgensen Anette W. Bruun, Susanne Kammler, Anders S. Andersen, Morten O. A. Sommer. *Manuscript under preparation*

Additionally, contributions were made to the following publication (not included).

1. S. Cardinale, F. G. Tueros, M. O. A. Sommer. (2017) **Genetic-metabolic coupling for targeted metabolic engineering**. Cell Reports. 20, 1029-1037.  
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## Synopsis

The purpose of this thesis was to construct, test and explore the applicability of advanced microbiome therapeutics (AMT)s, aiming to treat diseases related to bile acid and neurodegenerative disorders. In view of this, we first attempted to identify metabolic capabilities of bacterial species for producing bile acids, which could enable further biotechnological applications. Strategies to identify microbial cells or mechanisms for secondary bile acid degradation and/or neutralization are explored as well, with the aim of removing accumulation of toxic bile acids *in vivo*. Identified neutralizing capabilities are further engineered into a probiotic chassis that could serve as a mean to remove these molecules from the gut and possible metabolic engineering targets are investigated. Along the same lines, capabilities to produce L-DOPA, a common drug used for treating patients of Parkinson's disease, were transferred to *E. coli* Nissle 1917 and its preliminary *in vivo* delivery efficacy is assessed. Metabolic optimizations for the production of L-DOPA are implemented, as well as, possible strategy for modulating bacterial species that commonly affect L-DOPA uptake in the gut. Lastly, this thesis elaborates on how these AMTs could be used to treat diseases, aspects needed to optimize and possible challenges for their application.

## Thesis Structure

### Chapter I – Introduction

Gives a general Introduction to the field of biotechnology and its perspectives in healthcare. It also includes an overview of the role of bile acids and catecholamines in health and disease, an Introduction to Parkinson's disease and finalizes by describing the objective behind advanced microbiome therapeutics, their advantages over conventional treatments and their applicability in real scenarios.

### Chapter II – Bile Acids Are Not a Native Product of Microbial Marine Species

Includes a brief manuscript, in which microbial production of bile acids is investigated for its high biotechnological value. Nonetheless, no evidence of these metabolic capabilities were identified and further characterization of these bacteria suggested that bile acids came from the media fraction and not as a result of microbial production. Using bioinformatics tools putative bile salt hydrolases were identified in three marine species, as well as a cholate catabolic pathway in *Rhodococcus marinonascens*. These results support and give further evidence for a possible misinterpretation in previous published reports.

### Chapter III – Exploring Strategies for Secondary Bile Acid Degradation and Neutralization

Contains a manuscript, where different strategies were explored for identifying suitable routes for bacterial neutralization and/or degradation of secondary bile acids, which have been shown to affect negatively health and promote certain diseases. The approaches explored consist on: 1) A functional metagenomic screening for identifying genes capable of degrading, neutralizing or giving resistance towards deoxycholic acid 2) Isolating intestinal bacterial strains capable of utilizing deoxycholic acid as the sole carbon source, and 3) Transferring secondary bile acid sulfonation capabilities to *E. coli* Nissle 1917, a probiotic strain.

#### Chapter IV – Engineering Advanced Microbiome Therapeutics for Production and Delivery of L-DOPA and Dopamine

Includes a manuscript, outlining the construction, metabolic optimization and proof of concept of L-DOPA delivery *in vivo* using an engineered *E. coli* Nissle 1917 strain, as an advanced microbiome therapeutic for Parkinson’s disease. Bacteriocin co-expression was also explored as a mean to modulate *E. faecalis*, a known to bacterium to decrease treatment efficacy of L-DOPA in PD patients. Lastly, initial steps towards the development of a dopamine AMT are reported.

#### Chapter V – Concluding Remarks and Perspectives

Contains the concluding remarks and future perspectives of the present thesis

## I. Introduction

### A. Biotechnology and its perspective in healthcare

Biotechnology is a broad area within biology, which uses whole organisms or biological parts for the generation of goods or services for the well-being of society. There are countless examples that could be mentioned, however, probably the one that most people might be familiarized with is the production of alcoholic beverages. In this process, there is a fundamental step, in which living microorganisms are used to produce ethanol. These organisms, when added, consume the given carbohydrate, ferment it and as a metabolic product they produce ethanol. Nowadays, this process is denoted as bioconversion and several examples can be outlined since the beginning of human societies until now<sup>[1]</sup>.

For decades, biotechnology has risen to be an important backbone of today's modern industry, allowing certain processes to be faster, cleaner and more efficient. Besides this, Biotechnology has allowed a transition into a more sustainable industrial era, where industrial capabilities are not limited by the availability of specific compounds, contrary to the case of the chemical industry, but rather depend on renewable compounds<sup>[2]</sup>. Perhaps, one of the best examples are the ethanol biorefineries which use yeast to produce ethanol from biomass. This characteristic allows biorefineries to be versatile and adaptable to future challenges, that might limit the accessibility of certain resources<sup>[3]</sup>.

Several drugs used in the healthcare are produced by genetically modified organisms (GMO)s, including bacterial and yeast strains, animal and vegetal cells<sup>[4]</sup>. Frequently, genes encoding necessary enzymes to produce the target drugs or compounds, are introduced into these organisms or cells, via plasmids or genomic integrations. In bacteria, these genes are normally expressed using plasmids. A fine example of the first plasmid based therapeutic molecule produced in *E. coli* is insulin<sup>[5]</sup>. However, since plasmids can be silenced, genomic integrations of these genes are preferred in some cases. In animal cells, for example, chromosomal integrations are routinely performed<sup>[6]</sup>. Large scale fermentations are then carried out with the aim of mass producing these compounds by growing these organisms extensively<sup>[7]</sup>. Additional steps involving separation and purification of the products (downstream processing) are carried out, once the fermentations are completed<sup>[8]</sup>. Improvements in fermentation technologies has been instrumental for generating drugs at reduced costs and making biotechnology a more appealing industry.

A clear example is the generation of antibodies, these molecules have a complex structure, which depends on native cellular mechanisms to be folded and assembled correctly in order to be functional<sup>[9]</sup>. Routinely, large animal-derived polyclonal antibody production is achieved by injecting antigens into large farm mammals, such as horses. After a short period of time, these animals would become immunized and produce antibodies. The blood would then be collected and the antibodies purified<sup>[10]</sup>. As a result of the time and effort invested, this technology is considered rather expensive, in addition, there is more and more emphasis with regards to animal welfare and using alternatives technologies to generate these antibodies<sup>[11]</sup>. Fermentations and cell culture technologies allow a more efficient production of a variety of antibody forms, hybridoma technology<sup>[12]</sup> (Nobel prize in 1975) and Chinese Hamster Ovary<sup>[13]</sup> (CHO) cells are among the most used mammalian platforms for this purpose and offer a more robust, reliable and cost-efficient alternative.

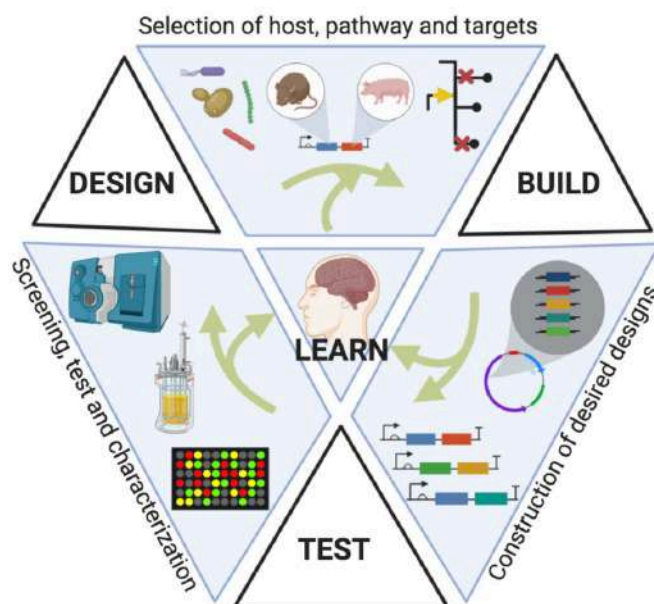
**Table 1.** Top 10 Biological drugs sold in the U.S in 2017 (based on reported sales 2018 - USD)<sup>[190]</sup>.

Drug	Generic Name	Molecule	Sales (Bn)	Produced in	Launch date
Humira	Adalimumab	Recombinant human monoclonal antibody	18.4	CHO	2002
Rituxan	Rituximab	Recombinant humanized monoclonal antibody	9.2	CHO	1997
Enbrel	Etanercept	Recombinant soluble dimeric fusion protein	7.9	CHO	1998
Herceptin	Trastuzumab	Recombinant humanized monoclonal antibody	7.4	CHO	1998
Avastin	Bevacizumab	Recombinant humanized antibody	7.1	CHO	2004
Remicade	Infliximab	Recombinant chimeric, humanized tumor necrosis factor alpha (TNF) monoclonal antibody	7.1	Hybridoma cell line	1998
Lantus	Insulin glargine	Insulin receptor agonist	5.7	<i>E. coli</i>	2000
Neulasta	Pegfilgrastim	Recombinant human granulocyte colony-stimulating factor	4.7	<i>E. coli</i>	2002
Avonex	Interferon beta-1a	Interferon beta-1a	2.1	CHO	1996
Lucentis	Ranibizumab	Recombinant humanized monoclonal antibody fragment	1.5	CHO	2006

It is important to note that microorganisms used in the biotechnological industry have not been designed by nature to perform on large scale bioreactors, on the contrary, they have spent millions or billions<sup>[191,192]</sup> of years evolving to be the fittest in their given environment or niche, competing with other species and developing robust mechanisms to withstand adverse conditions<sup>[14]</sup>. When these organisms are subjected to artificial environments to massively produce non-native compounds, their metabolic and physiological traits are greatly compromised, resulting in toxicity, poor performance and productivity. System metabolic engineering, which integrates systems biology, synthetic biology, evolutionary engineering and metabolic engineering has improved the rate at which cell factories are developed and optimized<sup>[15]</sup>.

Traditional metabolic engineering focuses in genetically optimizing the metabolism of organisms in order to overproduce a desired compound in an industrial context<sup>[16]</sup>. To accomplish this, the pathway of interest is studied in detail, identifying enzymatic bottlenecks, appropriate gene expression levels, stress responses, and deciding which approach to take in order to efficiently redirect the metabolic flux for optimal production of the compound of interest<sup>[17]</sup>. This high level of optimization towards a heavy producer phenotype, comes at a heavy cost, affecting growth and other native physiological features at the expense of higher titers<sup>[18]</sup>. Studies are now trying to grasp a deeper understanding of changes and adaptations that take place in the organisms hosting these heterologous pathways by using powerful omics techniques such as genomics<sup>[19]</sup>, transcriptomics<sup>[20]</sup>, proteomics<sup>[21]</sup>, metabolomics<sup>[22]</sup> and fluxomics<sup>[23]</sup> with the goal of rationally improving production capabilities. These technologies give an extremely detailed panorama of the events happening within the cell, enabling researchers to integrate and model complex physiological events, identifying further targets to engineer and unveiling previously unknown cellular features<sup>[24, 25]</sup>.

Synthetic biology has been the corner stone with regards to the rate of improvement and innovation taking place in Biotechnology. This field has enabled a rapid iterative cycle for cell factory development (figure 1), by applying technologies that ease the design, construction, test and optimization of cell factories, and thus the learning outcomes<sup>[26]</sup>. Among these technologies, DNA assembly techniques such as BioBrick assembly<sup>[27]</sup>, Gibson assembly<sup>[28]</sup>, Uracil specific excision reagent (USER) cloning<sup>[29]</sup>, between others<sup>[30,31]</sup>, have been instrumental for enabling rapid construction of genetic parts. In the same manner, chromosome engineering technologies such as site-specific integration<sup>[32]</sup>, recombineering<sup>[33]</sup>, multiplex automated genome engineering (MAGE)<sup>[34]</sup> and CRISPR/Cas technologies<sup>[35]</sup> have allowed rapid and more efficient introduction of point mutations, gene knock outs (KO) and knock ins (KI), as well as integration of heterologous pathways, improving production stability<sup>[36]</sup>. Other technologies, which modulate<sup>[37]</sup> and stabilize gene expression<sup>[38]</sup>, channel specific substrates<sup>[39]</sup>, have biosensing capabilities<sup>[40]</sup>, enable synthetic circuitry<sup>[41]</sup> and enzyme engineer<sup>[42]</sup> are also part of the vast repertoire of tools in synthetic biology. In addition, Synthetic Biology also gives us vast data sets, by which we can comprehend the flaws and virtues of each design, and most importantly, deepen our understanding of complex biological mechanisms.



**Figure 1.** Overview of the design, build, test and learn cycle in biotechnology.

In all, Biotechnology gives us the immense potential to harness the power of biology for the benefit of human kind. Its benefits have already started to revolutionize the pharma and chemical industry. Further innovation and discoveries will certainly improve other aspects of our society, hopefully redirecting it to be more sustainable and environmentally aware.

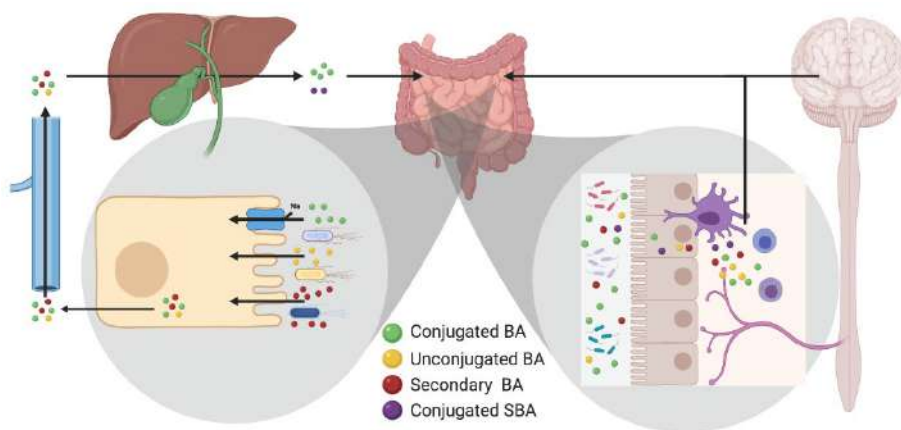
## B. The Role of Bile Acids in Health and Disease

Bile acids (BA)s are among nature's most interesting designs. These molecules not only exhibit extraordinary properties but, in addition, a plethora of physiological functionalities. BAs were first reported in studies dating back to the 19<sup>th</sup> century<sup>[43]</sup>, however, it was not until the studies of Heinrich Otto Wieland, in which the structure of three isolated BAs were confirmed to be remarkably similar to cholesterol<sup>[44]</sup>. Due to the importance of his findings, he was later awarded the Nobel prize in 1928. Over time, extensive knowledge has been accumulated regarding the functionality of bile acids as part of the digestive process<sup>[45]</sup>. Nevertheless, recent findings propel the role of BAs to be largely complex<sup>[46]</sup>, having different phases of interplay within the host's physiology and affecting deeply health and disease states.

BAs are produced in the hepatocytes from cholesterol, being the sterol rings their main structural component<sup>[47]</sup>. The intriguing spatial arrangements of their hydroxyl groups makes them amphipathic molecules, having a hydrophilic face due to the hydroxyl groups, and a hydrophobic face comprised of the sterol body. The biosynthetic pathway requires of at least 14 different enzymatic steps for two main purposes. 1) Shorten the sterol tail by several oxygenation steps, mainly involving P450 cytochrome oxidases; 2) Modify the cyclopentanoperhydrophenanthrene ring structure or nucleus<sup>[48]</sup>. In humans, the two most prominent bile acids are chenodeoxycholic acid (CDCA) and cholic acid (CA), they differ from each other by one hydroxyl group in position 12. These bile acids can be either be conjugated with taurine or glycine, as a consequence, their critical micelle concentration (CMC) and passive reabsorption in the intestine are greatly reduced<sup>[49]</sup>. All of these features make BAs virtuous surfactants, which greatly aid the digestion and absorption process of lipids and liposoluble vitamins<sup>[50]</sup>. After their main digestive task is finished, up to 95% of all bile acids are actively transported back into the blood, through the enterocytes, where they are taken back to the liver, as part of the enterohepatic circulation (figure 2). These BAs will then be re-conjugated and secreted into the intestine as part of the digestive process of the next meal<sup>[51]</sup>. Nevertheless, in this transit time, BAs are able to interact with receptor molecules such as the farnesoid X receptor (FXR) and the G protein-coupled membrane receptor 5 (TGR5), working as signaling molecules. As a result, these receptors trigger signaling cascades, modulating the expression of genes involved in the metabolism of bile acids, carbohydrates, energy expenditure and inflammation, both in enterohepatic and peripheral tissues<sup>[52]</sup>. These findings have brought major attention to the relevance that BAs play in health and disease, motivating research that could make use of these molecules or their receptors as potential therapeutic targets<sup>[53]</sup>.

From the moment bile acids are secreted into the intestine, they come in close contact with microbial species. As a matter of evolution and survival to their environment, bacterial species have adapted to coexist and use BAs in their favor. The first microbial modification that bile acids undergo, is the deconjugation of the amino acid moiety (taurine or glycine), which is performed by bile salt hydrolases (BSH)<sup>[54]</sup>. This step is regarded as the gatekeeper for further microbial modifications, since no other modification can take place without deconjugating the amino acids<sup>[55]</sup>. There is a great diversity of identified BSHs in nature, belonging to species of bacteria and archaea inhabiting animals, marine environments and to a less extent soil. The reason why this enzyme is widely spread among different environments remains unclear, however, BSHs have been demonstrated to aid colonization by reducing the toxicity of conjugated bile acids<sup>[56]</sup>. Further down, in the large intestine primarily, other species have specialized in de-hydroxylating, hydroxylating and epimerizing unconjugated primary bile acids. The term "secondary bile acid" corresponds to primary bile acids that have been further modified by these mechanisms<sup>[57]</sup>.

In the last decade, more than 20 variants of secondary bile acids (SBAs) have been reported in the literature. From these, deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA) account for the most prominent fraction. Depending on the specific modification of the hydroxyl groups, these compounds will have increased (DCA, LCA) or decreased (UDCA, ISO-BAs) hydrophobicity<sup>[58]</sup>. Besides their chemical properties, SBAs are also able to cross the intestinal layer by passive diffusion, of course to a much lower extent, compared to the active transport of primary BAs in the small intestine.



**Figure 2.** Schematic representation of the enterohepatic circulation (left) and the influence of bile acids towards the immune and nervous system (right).

The presence of bile acids and their metabolites trigger several physiological responses in the host. These molecules are able to bind numerous receptors in several tissues and are thought to influence lipogenesis, bile acid metabolism, glucose metabolism<sup>[196]</sup>, immunity, energy regulation among others<sup>[59]</sup>. This suggests that BAs could be even more intricately involved in regulating physiological events than previously thought. Several studies have demonstrated the different effects that SBAs have in health and disease<sup>[60,61]</sup>. For instance, UDCA has been extensively used for treating several cholestatic liver diseases such as cholesterol gallstones, primary biliary cholangitis (PBC) and cholestasis of pregnancy<sup>[193-195]</sup>. As it contributes to lower the cytotoxic potential of the BA pool due to its less hydrophobic structure<sup>[62]</sup>. Yet, recent studies suggest that the therapeutic effect of UDCA could involve stimulation of hepatocellular and ductular secretions, cytoprotection against bile acids and cytokine-induced injury, and immunomodulation<sup>[63]</sup>.

On the other hand, increased concentrations of SBAs such as DCA and LCA, have been correlated to the presence of gallstones<sup>[64]</sup>, inflammatory diseases and the onset of hepatic and colon cancer<sup>[65]</sup>, as a consequence of their poor solubility, cytotoxicity and negative immunomodulatory traits<sup>[66,67]</sup>. Most studies suggest that this could be a result of an unbalanced microbiome or dysbiosis<sup>[70]</sup>, however, it still remains unclear under which specific circumstances these bile acids accumulate and trigger these diseases. It is worth mentioning that there are not only negative properties linked to SBAs. Deoxycholic acid and lithocholic acid have also been reported to have strong binding affinity to FXR, TGR5, PXR, VDR and CAR receptors<sup>[196,197]</sup>, which positively



regulate metabolism and several other physiological features. Thus, could be of importance for treating diseases such as cholestatic liver disease, non-alcoholic steatohepatitis (NASH), Inflammatory bowel disease (IBD), osteoporosis, among others<sup>[68]</sup>. DCA has also been reported to inhibit proliferation of vegetative *C. difficile* cells in the intestine, protecting against possible outgrowth and future infections<sup>[69]</sup>. In addition, SBAs are thought to relieve dysbiosis induced inflammation in mouse models<sup>[70]</sup>.

Intriguingly, it seems that the nervous system does not escape the ring of influence that bile acids have. Recent studies have reported the presence of bile acids and associated receptors in rodent and human brains<sup>[71-73]</sup>. Furthermore, BA biosynthesis could also take place in the brain as a metabolic route to eliminate 27-hydroxylated sterols<sup>[74]</sup>, important signaling molecules that modulate neuronal activity and cholesterol biosynthesis in the brain<sup>[75]</sup>. Even though circulating bile acids could also have access to the brain by crossing through the blood brain barrier (BBB), both by passive diffusion and active transport, the concentrations may not be sufficient to trigger any effect. On the other hand, an indirect pathway mediated by TGR5-GLP-1 could signal the CNS by the innervations of the vagal nerve in the intestine, likely influencing glucose and energy homeostasis, among other things<sup>[76]</sup>. Lastly, studies have also revealed neuroprotective effects of bile acids in models of Huntington's disease<sup>[77]</sup>, Alzheimer's disease<sup>[78]</sup>, Parkinson's disease<sup>[79]</sup>, among other diseases<sup>[80]</sup>.

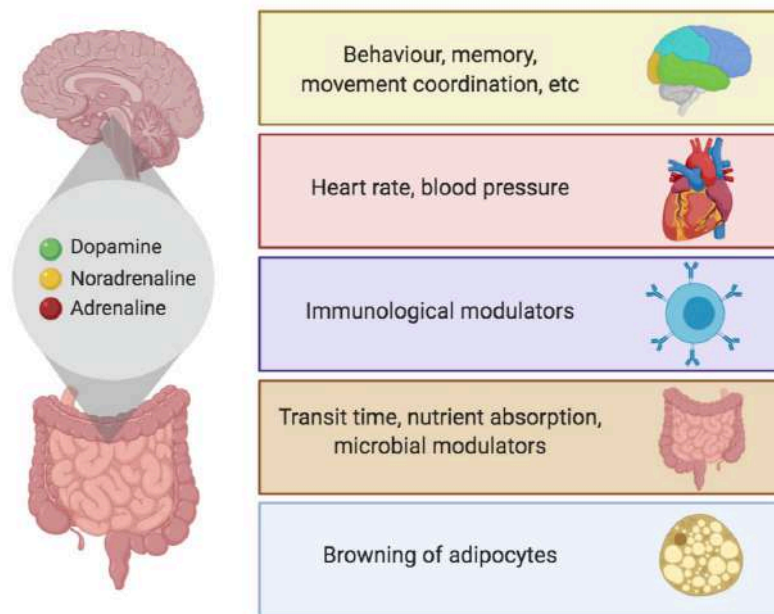
At first glance, it appears that the implications of bile acids in health and disease might be an area excessively complex to understand, especially when taken into consideration the numerous interactions between them and the microbiome, and how both of these factors affect the physiology of the host. Nevertheless, more and more efforts are being focused towards revisiting the field of bile acids in order to deepen the understanding of their role as signaling molecules in health and disease, and hopefully enable better and more efficacious treatments.

### C. The importance of catecholamines, a systemic perspective

The nervous system is comprised of an intricate and complex network of neurons that coordinate and execute functions with outstanding precision. Its role to synchronize biological functions and give us the experience of life through consciousness puzzles scientist until this day<sup>[198]</sup>. Neurotransmitters play a fundamental role communicating neurons within the nervous system and with other tissues. These molecules are routinely secreted into the synaptic cleft and bind receptors in post-synaptic neurons to transmit signals or information<sup>[81]</sup>. However, several other cell types have also been reported to express receptors for these molecules, which reinforces the theory that neurotransmitters are able to cross talk with other tissue types. There are 2 main classifications of these molecules: 1) classic or amino acid derived neurotransmitters and 2) neuropeptides<sup>[82]</sup>; It is in the scope of this thesis to mainly discuss classic neurotransmitters. Furthermore, amino acid derived neurotransmitters such as dopamine, noradrenaline, adrenaline and serotonin are produced in vast amounts in order organs such as the intestine and kidneys, enabling communication from these organs towards the CNS and other tissues<sup>[83]</sup>.

It is a common misconception that neurotransmitters are solely located in the nervous system and therefore are only involved in the transduction of signals among neurons. On the contrary, catecholamines, such as dopamine (DA), norepinephrine (NEP) and epinephrine (EP) are involved in complex communications between different tissues and organs (figure 3). There are 2 types of catecholamine receptors  $\alpha$  and  $\beta$ .  $\alpha$ -receptors have mainly excitatory effects: vasoconstriction, splenoconstriction, bronchoconstriction, contraction of the arrectores pilorum (horripilation),

contraction of the uterus, mydriasis, contraction of the sphincters and relaxation of the smooth intestinal musculature, and activation of glycogenolysis<sup>[84]</sup>.  $\beta$ -receptors, on the other hand, produce bronchodilation, vasodilation, relaxation of the uterus, positive cardiac inotropism with increased heart rate<sup>[84]</sup>. These receptors are expressed in numerous tissues such as in the intestine<sup>[85]</sup>, muscles<sup>[86]</sup>, kidneys<sup>[87]</sup>, immune cells<sup>[88]</sup>, adipocytes<sup>[89]</sup>, neurons<sup>[90]</sup> among others.



**Figure 3.** Overview of known physiological features modulated by catecholamines.

Catecholamines are metabolic derivatives of tyrosine, an aromatic amino acid. The first enzymatic step for catecholamine biosynthesis involves the hydroxylation of tyrosine into 1-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH; EC 1.14.16.2), requiring both tetrahydrobiopterin (BH4) and molecular oxygen for the reaction<sup>[91]</sup>. Due to the great physiological importance of catecholamines, the activity of this enzyme is tightly controlled by complex phosphorylation of the enzyme. In addition, TH is the rate limiting enzyme for catecholamine biosynthesis, as it is feedback inhibited by the of catecholamines (DA, NEP, EP) with the active site<sup>[92]</sup>, resulting in a decrease enzyme activity primarily by reducing  $V_{max}$ <sup>[93]</sup>. Moreover, the structure of TH is comprised of three domains, a regulatory domain (R) in the amino terminal, followed by a catalytic domain (C) and a coiled-coil domain at the carboxyl-terminus<sup>[94]</sup>. The regulatory domain has been target of several studies, as phosphorylation of specific serine residues modulate the enzymatic activity drastically<sup>[95]</sup>. Among these residues, phosphorylation of serine in position 40 has shown to make the enzyme resistant to feedback inhibition, by altering the conformational structure of the enzyme leading to a variant of TH that is still inhibited but the dissociation of the catechol but in a reversible manner<sup>[96]</sup>.

Further down in the catecholamine biosynthetic pathway, L-DOPA is decarboxylated into dopamine. This is accomplished by the DOPA decarboxylase (DDC; EC 4.1.1.28). This enzyme is pyridoxal 5'phosphate dependent and catalyzes the irreversible decarboxylation of both L-DOPA and L-5-hydroxytryptophan (5-HTP), thus producing dopamine and serotonin respectively<sup>[97]</sup>. It has also been documented that it can decarboxylate other aromatic amino acids, therefore also called aromatic amino acid decarboxylase (AAAC)<sup>[98]</sup>. An additional hydroxylation step, performed by dopamine  $\beta$ -hydroxylase (DBH; EC 1.14.17.1), takes place to convert dopamine into norepinephrine, using ascorbate and oxygen<sup>[99]</sup>. Finally, norepinephrine is methylated by phenylethanolamine N-methyltransferase (PNMT; EC:2.1.1.28), using S-adenosyl-L-methionine (SAM), as the methyl donor<sup>[100]</sup>.

Diseases affecting dopamine biosynthesis lead to serious voluntary movement impairment and neurological syndromes. Tyrosine hydroxylase deficiency, a rare autosomal recessive disease, takes place when two copies of a mutated TH are inherited, this leads to low levels of tyrosine hydroxylase enzyme and therefore low levels of catecholamine biosynthesis<sup>[101]</sup>. Moderate signs of this disease involve abnormal gait patterns, clumsy movements, muscle stiffness and involuntary repetitive movements. Severe signs include poor control of voluntary symptoms, abnormal rigidity in arms and legs, decrease motor functions and involuntary muscle spasms<sup>[102]</sup>. Another disease which is characterized by loss of catecholamine, specifically dopamine production, is Parkinson's Disease (PD). In contrast to TH deficiency, PD is a neurodegenerative disease, in which dopaminergic neurons, responsible for producing and secreting dopamine in the brain are lost. There is not a clear consensus regarding the relation that  $\alpha$ -synuclein, a protein majorly located in the brain forming presynaptic terminals, plays in the onset of this disease, however, it has been successfully identified as a potential biomarker for PD<sup>[103]</sup>. Several studies have pointed out that accumulation of  $\alpha$ -synuclein into aggregations called Lewy bodies may interfere with normal processes and as a consequence cause oxidative stress responses, which eventually lead to the neuron's death<sup>[104]</sup>. Parkinson's disease will be discussed in a greater detail in the following section of the introduction.

Systemic circulation of catecholamines play an important role as well. Approximately 50% of all dopamine is produced in the gut by enteric neurons and intestinal epithelial cells, resulting in increased dopamine concentrations in the hepatic portal vein<sup>[105]</sup>. Catecholamines circulate systemically, and are also secreted into the lumen of the intestine. These molecules are known to have implications modulating immunity, muscular contractions and neuronal activity, affecting transit time<sup>[106]</sup>, nutrient absorption, blood flow, exocrine secretion, motility, cytoprotection<sup>[107]</sup>, immune response<sup>[108]</sup>, and possibly microbial dynamics<sup>[109]</sup>. Intestinal secreted catecholamines are later taken up actively and transported via the portal vein to the liver, where they are thought to modulate immunity, regulating for example, iNKT immune cells in cell-mediated hepatitis<sup>[110]</sup>. Another example of immune modulation is the role of dopamine and its D2 receptor in inflammatory bowel disease, it has been shown that dopamine agonist can decrease the severity of ulcerative colitis (UC) in animal models, attenuating vascular permeability and preventing excessive vascular leakage<sup>[111]</sup>. Systemic levels of catecholamine have also been described to induce browning of white adipocytes for energy generation, which could have great implications to treat obesity<sup>[112]</sup>. Blood pressure can also be affected due to catecholamines interacting with specialized receptors in the heart and causing higher contraction rate, thus increasing blood pressure<sup>[113]</sup>.

In all, catecholamines exhibit a great potential to be used as therapeutics, as these molecules are highly interconnected with different physiological aspects of health and disease. Nevertheless, it is this highly complex interconnection that must be understood in order to fully exploit their

applicability. Deciphering these specific mechanisms poses a great challenge but promises superior approaches to address pathologies.

#### **D. Parkinson's disease, a silent pandemic**

Parkinson's disease is the fastest growing neurological disorder that leads to disability, according to the Global Burden of Disease study, having increased 118% from 1990 to 2015 globally<sup>[114]</sup>. The disease was first described by the British physician, Dr. James Parkinson in 1817, affecting six individuals with symptoms not yet classified in any specific nosology<sup>[115]</sup>. Even though PD is not infectious, it shows many features of a pandemic<sup>[116]</sup>. First, it extends over large geographical areas, increasing in most parts of the planet, due to increased life expectancy and exposure to chemicals or pollutants, that may influence the onset of the disease<sup>[117]</sup>. Secondly, migration of cases have been observed, changing in response to age and industrialization level of the country. The demographic changes occurred in the past decades worldwide from west to east, and specially in China will likely result in increased cases of PD in the future<sup>[118]</sup>. Thirdly, there is an exponential growth of people suffering from this disease, for which there is no cure or immunization<sup>[119]</sup>.

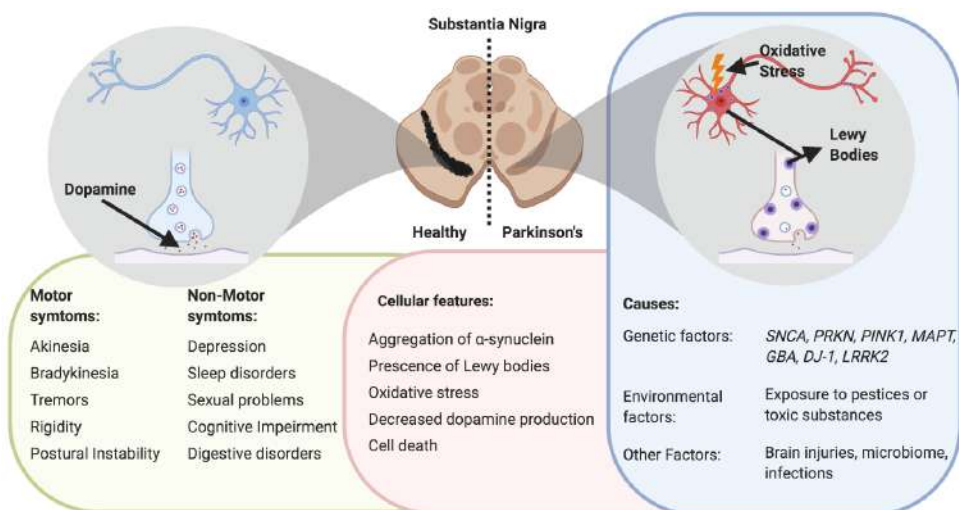
PD is a chronic disease characterized by the progressive death of dopaminergic neurons, specifically located in the substantia nigra pars compacta (figure 4). The substantial loss of these neurons leads to partial or full impairment, depending on the severity of PD, of primary motor functions including akinesia (loss of ability to move your muscles voluntarily), bradykinesia (slowness of movement), tremor, rigidity and postural instability<sup>120</sup>. Secondary motor symptoms such as gait disturbances, micrographia (abnormally small and cramped handwriting), precision grip impairment and speech problems<sup>[120]</sup>. Even though dopaminergic neurons are innately responsible for movement capabilities, more evidence have shed light onto the complex interaction these neurons may have with other neuronal structures and neurochemical systems, including the cerebellum, prefrontal cortical areas, and the serotonergic, glutamatergic and cholinergic systems in order to affect motor functions<sup>[121]</sup>.

The cellular and molecular mechanisms leading to PD are numerous, however, it is accepted that dopamine metabolism, oxidative stress, endoplasmic reticulum stress, impairment of protein degradation, mitochondrial dysfunction and neuroinflammation are the leading causes of dopaminergic neuron death<sup>[122]</sup>. Perhaps, one of the most studied proteins in PD is  $\alpha$ -synuclein, due to its significant contribution to cellular stress and death. Aggregates of this protein among others, represent the hallmark sign of PD, Lewy bodies (LB)<sup>[123]</sup>. Lewy bodies are thought to form due to mutations affecting normal functionality of  $\alpha$ -synuclein. Therefore, giving rise to uncontrolled phosphorylation and polymerization<sup>[124]</sup>, interaction with other binding proteins<sup>[125]</sup>, compensatory cellular mechanisms such as upregulation of ubiquitinating-proteasome system<sup>[126]</sup> and chaperons<sup>[127]</sup>, among others. This agglomeration triggers a chain reaction-like effect, that subsequently impairs vital cellular mechanisms such as dopamine metabolism incrementing over oxidized derivatives of dopamine, causing oxidative stress and leading to mitochondrial and lysosomal dysfunction<sup>[128]</sup>. Similarly, autophagy, which plays a critical role clearing accumulated misfolded proteins and damaged organelles is impaired<sup>[129]</sup>. This inhibition, boosts  $\alpha$ -synuclein secretion and transport to other neurons, through extracellular vesicles, thus extending the progression of the disease<sup>[130]</sup>.

Parkinson's disease, as described briefly in the last section, has been extensively studied, however, there is still no consensus on what exactly causes the disease in its sporadic form<sup>[131]</sup>.

Mutations in genes such as *SNCA*, *PRKN*, *PINK1*, *MAPT*, *GBA*, *DJ-1*, *LRRK2* among others have been identified to be responsible for the familial form of the disease. Nevertheless, these alterations only explain 30% of the monogenetic and 3-5% of the genetically complex cases<sup>[132]</sup>, making evident our lack of knowledge in terms of the genetic background or possible pleiotropic effect of mutations in PD.

Environmental factors have also been correlated with PD, their concentration and exposure time may play a major role with regards to the onset of the disease<sup>[133]</sup>. Factors such as smoking<sup>[134]</sup>, coffee drinking<sup>[135]</sup>, vigorous exercise<sup>[136]</sup>, ibuprofen use<sup>[137]</sup>, and plasma urate<sup>[138]</sup> have been inversely associated. On the other hand, factors such as pesticide exposure<sup>[139]</sup>, use of pesticides<sup>[140]</sup> and traumatic brain injury<sup>[141]</sup> have been positively associated. Air pollutants have also been assessed in this context, the respiratory tract represents an important gateway for chemicals that are able to cross the brain blood barrier, and has been shown as a pathway for pathogenic  $\alpha$ -synuclein transport to the brain<sup>[142]</sup>. Thus, enabling potential role to drive inflammation and neurodegenerative diseases<sup>[143]</sup>.



**Figure 4.** Overview of Parkinson's Disease.

In the past decade the gut microbiome has gained tremendous relevance due to its contribution towards different aspects of the host's physiology, affecting uptake of nutrients, medications and environmental chemicals<sup>[144]</sup>. Furthermore, evidence is building up, supporting the hypothesis that the microbiome could have the potential to alter various aspects of brain activity, neurological functions and behaviors<sup>[144]</sup>. Interestingly, a hypothesis proposed by Braak in 2003, states that sporadic PD may be a result of a virus or bacterial infection in the gut<sup>[145,146]</sup>. Following studies, pointed out a dual-hit hypothesis, describing that the sporadic form of PD could start from neurons in the nasal cavity or the gut and then spread into the CNS in specific patterns across the olfactory tract and the vagal nerve<sup>[147]</sup>. Furthermore, the microbiome composition of PD patients and control subjects were compared, observing an increase of proinflammatory strain composition in the gut microbiome<sup>[148, 149]</sup>. Interestingly, a recent study has highlighted the role of *Bacillus subtilis* PXN21,

a probiotic strain, to clear and inhibit  $\alpha$ -synuclein aggregations in an established model of synucleinopathy in the nematode *Caenorhabditis elegans*<sup>[150]</sup>. Recent observations, have also noted the effect that occurring members of the gut microbiome have on PD treatment. L-DOPA, has been the gold standard drug for treating PD, because of its ability to cross the BBB and be converted into dopamine in the brain. Bacterium species such as *Enterococcus faecalis* and *Eggerthella lenta* have been shown to interact and degrade the L-DOPA by an interspecies catabolic pathway converting it into tyramine<sup>[151]</sup>. Even though the gut microbiome adds another layer of complexity regarding the efforts to understand and decipher the causes of PD, it also opens a potential window for more effective therapy compositions and treatments.

### E. Advanced Microbiome Therapeutics

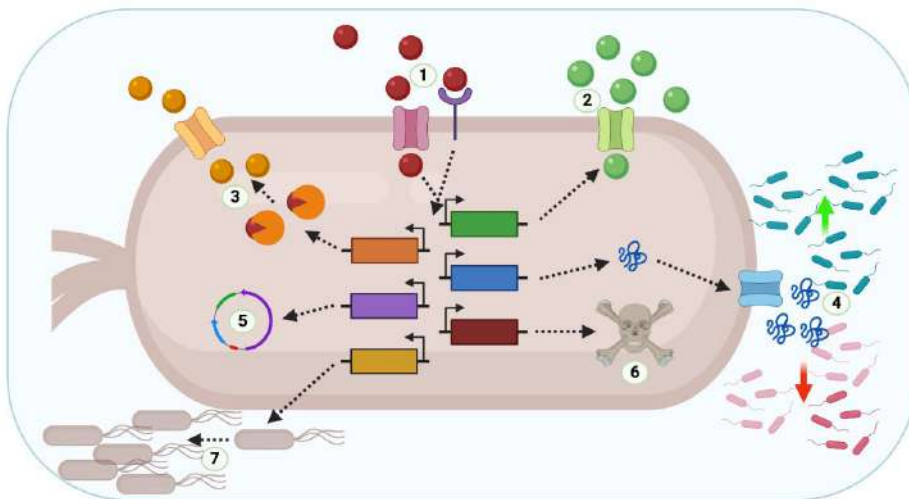
Microbial species are probably the living organisms most well adapted to any particular niche. As simple as we might think they are, they have evolved clever mechanisms to withstand several different adverse conditions, from deep sea thermal vents<sup>[152]</sup>, extremely acidic swamps<sup>[153]</sup> and radioactive places<sup>[154]</sup>, to being hard working “guests” in our bodies<sup>[155]</sup>. It is this high degree of specialized adaptations, towards their environment, that makes them immensely attractive for developing living drugs that are able to localize to specific areas in our bodies and deliver specialized therapeutic treatments, which otherwise would be difficult or inefficient by conventional technologies. Thus, bypassing conventional limits of traditional therapies such as dose, pharmacokinetics, side effects, patient compliance and inter-patient variation<sup>[156]</sup>

Advanced microbiome therapeutics (AMTs, figure 5), also known as living biotherapeutic products (LBPs) are already being constructed and tested<sup>[157]</sup>. In fact, there are ongoing clinical trials for therapeutic indications such as oral mucositis, gastrointestinal inflammation in primary immunodeficiency, type 1 diabetes Mellitus, lung cancer, HPV-associated cancers, metastatic prostate cancer, solid tumors, cancer therapy-associated rashes, phenylketonuria (PKU) and progressive glioblastoma<sup>[157]</sup>. Several companies such as Oragenics, ActoBio Therapeutics, Advaxis Immunotherapies, Anaeropharma Science, Azitra, Symvivo, Synlogic, VAXIMIM, among others are driving the development and test of AMTs<sup>[157]</sup>. Even though AMTs have been design for different therapeutic purposes targeting different locations, the present the present section will focus in AMTs aimed to the intestinal environment.

Since its first isolation in 1884, by Theodor Escherich, *Escherichia coli* has become an immensely important model organism to study the mysteries of biology<sup>[158]</sup>. A couple of decades after, a probiotic version of this bacterium was isolated, *E. coli* Nissle 1917 (EcN)<sup>[159]</sup>, and since then, it has been widely used to treat diverse gastrointestinal conditions such as, irritable bowel syndrome and inflammatory bowel disease<sup>[160]</sup>. Furthermore, it has the ability to compete and inhibit the growth of opportunistic pathogens, including *Salmonella species* and other enteropathogenic coliforms, through the production of iron-sequestering siderophores<sup>[161]</sup> and bacteriocins<sup>[162]</sup>. Besides these features, it has also been observed that EcN stimulates anti-inflammatory mechanisms by interacting with the intestinal epithelium<sup>[163]</sup>. These qualities, together with the accumulated knowledge of *E. coli*, with regards to its physiology, metabolism and ample tool set to genetically modify it, has enabled EcN to be an optimal bacterial chassis for the construction of AMTs.

Examples of engineered versions of EcN have already been described for intestinal-acting antimicrobial agents. For instance, delivered anti-biofilm enzyme, dispersin B (DspB), by EcN showed reduction of pre-colonized *P. aeruginosa* abundance in murine and nematode models<sup>[164]</sup>.

Antimicrobial properties against *Salmonella*<sup>[165]</sup> and Enterococcal species<sup>[166]</sup> have also been engineered into EcN, enabling improved protection against pathogens as well as the potential to clear specific niches within the intestine by the expression of heterologous bacteriocins. Metabolic disorders have also been addressed by the construction of AMTs. A publication by Chet et al. showed that intestinal production of N-acylphosphatidylethanolamine (NAPE) by a genetically modified strain of EcN could reduce the symptoms related to high-fat diet in mice, decreasing adipose tissue formation, insulin resistance and hepatosteatosis<sup>[167]</sup>. Another group modified EcN by introducing genes for fructose metabolism, a common sugar additive in western diet, and for pyrroloquinoline quinone (PQQ) production, an antioxidant and cellular pathway mediator, demonstrated protection against metabolic syndrome using rats<sup>[168]</sup>.



**Figure 5.** Overview of an AMT. 1) Environmental cues are sensed or taken up and trigger a panel of responses. 2) Biotherapeutic production and delivery. 3) Degradation or inactivation of compound of interest. 4) Modulation of microbiome species by producing antimicrobial peptides. 5) Memory recording mechanisms. 6) Biocontainment strategies or kill switches. 7) AMT abundance regulation.

Perhaps, one of the best examples to describe the process of development of AMTs is SYN1618, an EcN strain constructed by Synlogic to treat phenylketonuria<sup>[169]</sup>. PKU is a disease caused by a mutation in the gene encoding phenylalanine hydroxylase (PAH), affecting 1 in 10 000 newborns. Without functional PAH, phenylalanine builds up in the blood and brain to concentrations that can result in neurological damage. If left untreated, PKU leads to seizures, mental retardation, involuntary movements among others, as a consequence of impaired phenylalanine metabolism<sup>[170]</sup>. However, if identified on time, severe effects of the disease can be avoided, treatments normally include diet restrictions, reducing intake of phenylalanine containing foods, and complementing the diet with special formulas<sup>[171]</sup>. Though this can essentially save the life of patients, it also restricts them for life, affecting their life quality. Synlogic's approach, based on an engineered EcN capable of efficiently taking up and metabolizing phenylalanine, reducing levels of the amino acid and relieving the patient from the potential effects of the disease. If proven successful in clinical trials, this could be an elegant strategy that could help improve treatments and life quality of patients.

There are numerous diseases that would benefit from the approach described above. In addition, this could enable the capacity to personalize treatments even further. Thanks to synthetic biology, AMTs could be modified to respond to *in vivo* conditions such as presence of specific food molecules<sup>[172]</sup>, temperature<sup>[173]</sup>, inflammation<sup>[174,175]</sup>, tumors<sup>[176]</sup>, or respond to supplemented inducers<sup>[177,178]</sup>. This could titrate the expression levels of genes involved in the production of the therapeutic molecule, modulating the levels of drug delivery and specificity. A recent example of novel uses of biosensing capabilities was published by Taketani and collaborators this year (2020). In the investigation, they uncovered novel bile acid responsive genetic elements and generated elaborated logic gates that would enable therapeutic bacteria to activate different programs of gene expression in different circumstances or stages<sup>[179]</sup>. This would make the AMT a robust platform for responding to complex stimuli, which is often the case in *in vivo* systems.

Biocontainment is another crucial aspect of AMTs, the engineered bacteria should be able to withstand the passage through the digestive tract, colonize the desired niche, deliver the necessary therapeutic action, and all of this restricting its ability to survive outside the host. Otherwise, the AMT could easily spread among people, who might not need it and might cause an adverse effect on their health. For this reason, it is essential that robust mechanisms are put in place to ensure containment of the AMT in the desired environment, being incapable of surviving anywhere else. One strategy is to introduce specific auxotrophies that would limit the replication of the AMT when the specific metabolite is missing. These metabolites could be nucleosides<sup>[180]</sup>, amino acids<sup>[181]</sup> or cell wall components<sup>[169]</sup>. Another approach is to implement synthetic kill switched sensitive to particular stimuli<sup>[182]</sup>. However, these synthetic circuits must be redundant as mutations can easily be selected for, enabling the AMT to survive in other environments. Gallager and collaborators were able to reduce the escape frequency by constructing a multilayered genetic safeguard, containing engineered riboregulators of essential genes, and an engineered addiction module based on nucleases<sup>[183]</sup>. This approach allowed the reduction of the escape frequency to  $<1.3 \times 10^{-12}$ .

Often, treatments for various diseases involve oral administrations of standardized tablets and doses, overlooking many physiological features of the patient<sup>[184]</sup>. In neurodegenerative diseases such as PD, severe motor complications due to dopaminergic treatments are often considered worse than the actual disease, by some patients. Pharmacokinetic studies of L-DOPA, the drug used to treat PD, show that it is rapidly taken up in the small intestine, contributing to a sharp increase in the blood<sup>[185]</sup>. This spike in L-DOPA, is accompanied by undesirable side effects such as uncontrollable movements that pose a greater inconvenience to patients than the actual symptoms of PD<sup>[186]</sup>. Extended-release strategies, involving gel-like matrixes for the delivery of L-DOPA have proven inefficient<sup>[187]</sup>. Direct administration of L-DOPA in the small intestine, by peristaltic pumps, has proven beneficial, reducing OFF times significantly<sup>[188]</sup>. Nevertheless, it is a very invasive alternative and life quality of the patient is greatly compromised. Enabling microbial delivery in the intestine could decrease the severity of side effects and even achieve better pharmacokinetics and pharmacodynamics, as these organisms could constantly produce specific amounts of the drug, which could be controlled by regulatory genetic circuits or modulating the amount of AMT within the gut<sup>[189]</sup>. Thus, improving immensely the life quality and progression of these patients.

In the case of diseases characterized by unbalanced patterns of bile acids, it is first necessary to understand whether modulating and regulating specific bile acids by an AMT could be useful for treating the chronic states. Most diseases in this area are triggered as a result of exposure to SBAs for long periods of time, in this particular case AMTs capable of degrading, neutralizing or blocking re-uptake of these compounds, in a constant and progressive manner, would offer the best strategy. In



this way, secondary bile acids would be lost at a greater rate than the rate at which they are generated, protecting the host from their cumulative effect. Nonetheless, an important aspect would be the fine tuning and degree of conversion, as SBAs are also beneficial to other physiological roles and most likely this response is dependent on the person treated.

Overall, AMTs have the potential to allow significant improvements with regards to treatment strategies for different diseases, taking into consideration their ability to colonize their niche and respond to their environment. These are features exquisitely favorable for developing personalized treatments. Therefore, this represents a higher level of regulation that could be exploited to improve therapies and life quality. Patients suffering of chronic diseases, which are medicated for life could have a greater degree of freedom, without being restricted by continuous dosing regimens. Furthermore, AMTs would also contribute to a more sustainable pharmaceutical industry, as processes would be greatly simplified and replaced with fermentations to grow and harvest AMT strains, rather than having countless chemical processes.

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## II. Bile Acids Are Not a Native Product of Microbial Marine Species

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### Abstract

Bile acids are essential for proper digestion and uptake of fatty acids and lipo-soluble molecules. Advances towards understanding bile acid metabolism and signaling, and their interactions with members of gut microbiome has shed light onto the complex physiological regulations that these molecules possess. Thus, a great industrial interest for developing manufacturing technologies for efficiently producing bile acids and derivatives of interest, which could be applied as therapeutics. Numerous studies have reported the identification and characterization of bacterial isolates capable of producing bile acids, making the identification of the pathway highly desired for driving biotechnological applications. In the present study, we investigate further the ability of three marine bacterial isolates (*Maribacter dokdonensis*, *Dokdonia donghaensis* and *Myroides pelagicus*), which were previously reported to produce bile acids. We conclude that while these bacteria can in fact modify bile acids by deconjugating the amino acid moiety, they are unable to synthesize these compounds. Furthermore, we identify putative bile salt hydrolases, which could be responsible for the different bile acid profile observed. Lastly, a steroid catabolic pathway is delineated in *Rhodococcus marinonascens*.

Key words: Bile acids, bile salt hydrolase, marine bacteria, bile acid modification, bile acid production.

### Introduction

Bile acids are amphipathic molecules that aid the digestion and uptake of fatty acids in vertebrates<sup>[1]</sup>. The amphipathic nature is a result of the opposing hydroxyl radicals in the structure, therefore, helping solubilize hydrophobic compounds by the formation of micelles<sup>[2]</sup>. These molecules are synthesized *de novo* from cholesterol in the liver of various vertebrates and are further modified by bacterial species in the gut. The first and most important modification of bile acids, also known as the “gatekeeper of bile acid metabolism”, involves de-conjugating the amino acid moiety from the steroid body, which is performed by bile salt hydrolase (BSH) positive bacteria<sup>[3]</sup>, allowing further metabolism of bile acids by other species. BSHs have been shown to be enriched in gut microbiome species, however, their presence in marine metagenomes has been functionally identified as well<sup>[4]</sup>.

Reports of bacteria capable of producing bile acids were published by several groups (Table 1), some claiming that bile acids were synthesized *de novo* by their isolates. Bile acids are a product of a complex metabolic pathway in vertebrates, where at least 14 different enzymes are involved<sup>[5]</sup>. Up to date, there is no clear explanation of the beneficial role these molecules could have for the bacteria



producing them. Claims that these molecules improve the uptake of hydrophobic carbon sources or inhibit possible competitors in their given environment have not yet been validated experimentally.

Implications of microbial production of bile acids are of high biotechnological interest, as several diseases are influenced by endogenous levels of these molecules<sup>[24]</sup>. Bile acid variants have also proven to be therapeutic and are the base of different treatments to address pathologies affecting the liver and gallbladder<sup>[25,26]</sup>. Thus, elucidating a microbial biosynthetic pathway could enable the development and implementation of bile acid producing cell factories for sustainable production, without being limited by the supply of animal derived products.

In the present study, we investigate the bile acid production capabilities of three previously characterized bile acid producing strains: *Maribacter dokdonensis*, *Dokdonia donghaensis* and *Myroides pelagicus* (*Myroides* SM-1). Our observations indicate that bile acids are not produced by these bacteria. Nonetheless, we further investigated the source of bile acids and suggest possible explanations for the varying bile acid profile detected among the studies.

## Materials and Methods

### Strains and Culture Conditions

Marine isolates *Maribacter dokdonensis* (DSM No. 17201) and *Dokdonia donghaensis* (DSM No. 17200) were ordered from DSMZ catalogue, *Myroides pelagicus* (KCTC No. 12661) was ordered from the Korean Collection for Type Cultures (KCTC). All strains were ordered as freeze-dried cultures and were reconstituted in the recommended media. Cultivations were carried out in Marine Broth (MB) Difco 2216 and mineral salt media (MM)<sup>[6]</sup>. Strains were incubated at 30°C and 250 RPM in a shaking incubator. All strains were confirmed by 16S sequencing, to rule out any contamination.

### Fermentations

Fermentations were performed as previously described<sup>[7,8]</sup>. A single colony was inoculated into 5 ml of MB or MM and was grown until saturation for 48 hours. Saturated culture was then used to inoculate 250 ml of MB or MM in 1 L baffled shake flask. Culture samples were taken every 8 hours for OD and total bile acid quantification. Fermentations were carried out in biological triplicates for all strains, including a negative control strain (*E. coli*). For mineral media fermentations, the presence of cholesterol was tested, in order to decipher whether bile acids were synthesized *de novo* using cholesterol. Stock concentration of cholesterol was dissolved in absolute ethanol and supplemented in the media at 1 g/L, according to previous study<sup>8</sup>. Sampling strategy was the same as the one described for marine broth fermentations.

### Fluorometric quantification of total bile acids

Fluorometric quantification of total bile acids from media supernatants was performed using “Bile Acid Assay Kit” (Sigma-Aldrich) and following the manufacturer’s instructions. Biological triplicates were used and all samples were processed at the same time.

### LC-MS/MS quantification of bile acids

50µl of bacterial broth were extracted with 3 volumes of methanol containing deuterated internal standards (d<sub>4</sub>-TCA, d<sub>4</sub>-GCA, d<sub>4</sub>-GCDCA, d<sub>4</sub>-GUDCA, d<sub>4</sub>-GLCA, d<sub>4</sub>-UDCA, d<sub>4</sub>-CDCA, d<sub>4</sub>-LCA; 50nM of each). After 10 minutes of vortex and 10 minutes of centrifugation at 20 000g, the supernatant was diluted 1:50 in methanol:water [1:1]. Bile acids were analyzed using ultra-

performance liquid chromatography-tandem mass spectrometry (UPLCMS/MS) according to previous work<sup>[18]</sup>. Briefly, after injection (5µL) the bile acids were separated on a C18 column (1.7µ, 2.1 x 100mm; Kinetex, Phenomenex, USA) using water with 7.5mM ammonium acetate and 0.019% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). The chromatographic separation started with 1 minute isocratic separation at 20%B. The B-phase was then increased to 35% during 4 minutes. During the next 10 minutes the B-phase was increased to 100%. The B-phase was held at 100% for 3.5 minutes before returning to 20%. The total runtime was 20 minutes. Bile acids were detected using multiple reaction monitoring (MRM) in negative mode on a QTRAP 5500 mass spectrometer (Sciex, Concord, Canada).

#### Identification of putative bile salt hydrolases

We identified putative BSHs using the COG database<sup>[20]</sup> (COG3049), which has been associated with bile acid salt hydrolase<sup>[4]</sup>. For the genome considered in this study, we extracted the ORFs using GenMarkS<sup>[21]</sup>. The ORFs were subsequently blasted against the bile acid salt hydrolase proteins using TBLASTN. Multiple alignment of the translated ORFs were performed using MUSCLE<sup>[22]</sup>. The Newick tree was generated from the alignment profiles via fasttree<sup>[23]</sup>. The tree figure generated via figtree (<http://tree.bio.ed.ac.uk/software/figtree/>).

#### Identification of a putative *Rhodococcus marinonascens* steroid degradation pathway

Complete genome of *Rhodococcus jostii* RHA1 was downloaded from NCBI with accession number NC\_008268.1. Previously reported<sup>[14]</sup> locus tags associated with steroid degradation pathway in *Rhodococcus jostii*, were extracted and blasted, using tblastx, to the genome of *Rhodococcus marinonascens* (accession number: BCXB00000000) to identify genes associated with steroid degradation in this specie.

#### Statistical analysis and software tools

Design of genetic parts was carried out using online lab notebook tool Benchling<sup>[19]</sup>. Statistical analysis was carried out in GraphPad Prism 7 (La Jolla California, USA. [www.graphpad.com](http://www.graphpad.com)). Figures were created using Biorender (BioRender.com).

## Results

#### Undetectable bile acid production by bacteria in marine broth Difco 2216

For determining whether marine isolates *M. dokdonensis*, *D. donghaensis* and *M. pelagicus* had bile acid production capabilities, fermentations in MB broth were carried out, keeping careful similarity to the setup described in the original publications. Nonetheless, fluorometric quantification analysis of the supernatants of different time points (24 and 48 hours) did not show any significant difference compared to the 0 hours sample, among all the strains tested, including the negative control (Fig. 1). Interestingly, 0 hour samples from all strains contained high amounts of bile acids ( $\approx 15 \mu\text{M}$ ), including the negative control, suggesting presence of bile acids in the initial culture media (Marine Broth Difco 2216) and not from the bacteria cultured. Furthermore, there is no difference in terms of bile acid concentrations among strains was noticeable (Fig. 1).

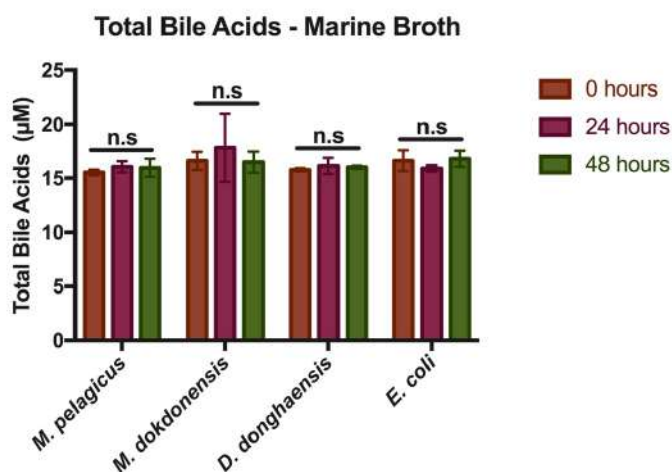


Figure 1. Total bile acid quantification from marine broth at different time points.

table 1. Reported species capable of producing bile acids or bile acid derivatives.

Strain	Isolated From	Condition	Media	Peptone		Reference
				Present		
<i>Aeromicrobium halocynthiae</i>	<i>Halocynthia roretzi</i> (Marine ascidian)	Aerobic	A1+C Broth	4 g/L		11
<i>Streptococcus faecium</i>	Soil	Anaerobic	C-SPY	5 g/L		10
<i>Psychrobacter sp</i>	<i>Stelletta sp</i> (Marine ascidian)	Aerobic	Zobell 2216 Broth	5 g/L		9
<i>Myroides odoratus</i>	Marine sample	Aerobic	Marine Broth 2216	5 g/L		8
<i>Myroides odoramitimus</i>	Marine sample	Aerobic	Marine Broth 2216	5 g/L		8
<i>Myroides pelagicus</i> SM1	Marine sample	Aerobic	Marine Broth 2216	5 g/L		8
<i>Dokdonia donghaensis</i>	Sea water	Aerobic	Marine Broth 2216	5 g/L		7
<i>Polaribacter dokdonensis</i>	Sea water	Aerobic	Marine Broth 2216	5 g/L		7
<i>Donghaena dokdonensis</i>	Sea water	Aerobic	Marine Broth 2216	5 g/L		7
<i>Maribacter dokdonensis</i>	Sea water	Aerobic	Marine Broth 2216	5 g/L		7
<i>Hahella chejuensis</i>	Sea water	Aerobic	Marine Broth 2216	5 g/L		7
<i>Rhodococcus marinonascens</i>	Sea water	Aerobic	Marine Broth 2216	5 g/L		7
<i>Haslibacter halocynthiae</i>	<i>Halocynthia roretzi</i> (Marine ascidian)	Aerobic	A1+C broth	4 g/L		12
<i>Penicillium sp.</i>	young stems of <i>Scurulla atropurpurea</i>	Aerobic	Glucose Peptone medium	10 g/L		13

Bile acids are not produced *de novo* from cholesterol

To decipher whether bile acids were produced *de novo* from cholesterol<sup>[8]</sup>, marine strains were grown on MM supplemented with cholesterol. From all the strains tested, only *M. pelagicus* and *E. coli* (negative control) were capable of growing in mineral media. The addition of cholesterol did not

affect growth parameters significantly (Supp. Fig. 1). However, no bile acids were observed from any of the samples taken along the fermentation.

#### Marine isolates deconjugate bile acids present in the culture media

In order to validate the different bile acid profiles among the marine strains, that were previously reported, samples were analyzed using liquid chromatography coupled with mass spectrometry (LC-MS/MS). No significant difference was observed in the concentration of total bile acids, validating previous bile acid quantification by fluorometric analysis (Fig. 2A). Even though no production of bile acids took place, modification of these molecules was variable among the marine strains investigated. *M. dokdonensis* showed the most activity hydrolyzing amide bonds from glycine conjugates of primary (Fig. 2C-D) and secondary bile acids (Fig. 3C-D), *D. donghaensis* and *M. pelagicus* were also capable of performing this modification, however, to lesser extent. On the other hand, taurine conjugates did not seem to be greatly affected by *M. dokdonensis* and *D. donghaensis*, with the exception of tauroolithocholic acid, in which *D. donghaensis* was able to deconjugate roughly 25%. *M. pelagicus*, on the contrary, had activity against most of these conjugates. None of the strains tested seemed to be able to deconjugate taurocholic acid, a comprehensive overview of all modification can be seen in Table 2. In the case of *M. dokdonensis* and *D. donghaensis*, the decrease in conjugated bile acids correlates to the increase of the deconjugated fractions, validating the bioconversion of these molecules.

Table 2. Deconjugation capacity of marine bacteria.

Bile Acid	Specie	Deconjugation of glycine conjugate (%)	Deconjugation of taurine conjugate (%)
cholic acid	<i>M. dokdonensis</i>	91.28	0
	<i>D. donghaensis</i>	14.69	0
	<i>M. pelagicus</i>	2.29	0
chenodeoxycholic acid	<i>M. dokdonensis</i>	99.99	0
	<i>D. donghaensis</i>	74.86	1.79
	<i>M. pelagicus</i>	35.32	32.93
deoxycholic acid	<i>M. dokdonensis</i>	88.02	0
	<i>D. donghaensis</i>	78.39	4.45
	<i>M. pelagicus</i>	37.58	38.11
lithocholic acid	<i>M. dokdonensis</i>	100	7.81
	<i>D. donghaensis</i>	90.06	25.89
	<i>M. pelagicus</i>	17.17	85.90
ursodeoxycholic acid	<i>M. dokdonensis</i>	100	0
	<i>D. donghaensis</i>	35.69	0
	<i>M. pelagicus</i>	8.8	9.8

#### Putative bile salt hydrolases identified in marine isolates

To determine whether the deconjugation of bile acids observed was a result of endogenous bile salt hydrolases, bioinformatic analysis were performed. Six different bile salt hydrolases (BSH)s homologs were identified in total (table 3). Only one putative BSH gene was identified in *D. donghaensis*, while two and three were identified in *M. pelagicus* and *M. dokdonensis*. Closest genes from the reference consisted in BSHs from soil, fresh and marine water environments, belonging to members of *Sphingobacteriaceae* and *Flavobacteriaceae*. Lowest and highest identity (34.9% and 56.2) were observed for a putative BSHs from *M. dokdonensis* and *M. pelagicus* respectively.

Table 3. Putative BSH genes identified in marine isolates.

Species	Putative BSH Accession	Reference BSH Accession	Species	AA Identity (%)	E-value
			<i>Solitalea canadensis</i> DSM 3403		
<i>D. donghaensis</i>	NZ_CP015125.1	YP_006258575.1	( <i>Sphingobacteriaceae</i> )	46.215	1.03e-87
<i>M. pelagicus</i>	NZ_WMJY01000026.1	YP_004317200.1	<i>Sphingobacterium</i> sp. 21 ( <i>Sphingobacteriaceae</i> )	55.620	1.48e-149
<i>M. pelagicus</i>	NZ_WMJY01000026.1	YP_004580371.1	<i>Lacinutrix</i> sp. 5H-3-7-4 ( <i>Flavobacteriaceae</i> )	56.262	0
<i>M. dokdonensis</i>	NZ_FNTB01000001.1	YP_004163530.1	<i>Cellulophaga algicola</i> DSM 14237 ( <i>Flavobacteriaceae</i> )	36.250	1.79e-06
<i>M. dokdonensis</i>	NZ_FNTB01000001.1	YP_004163530.1	<i>Cellulophaga algicola</i> DSM 14237 ( <i>Flavobacteriaceae</i> )	34.940	5.10e-07
<i>M. dokdonensis</i>	NZ_FNTB01000001.1	YP_004163530.1	<i>Cellulophaga algicola</i> DSM 14237 ( <i>Flavobacteriaceae</i> )	40.789	1.97e-09

#### Identification of a putative steroid degradation pathway in *Rhodococcus marinonascens*

In order to understand the reason why low concentrations of bile acids were attributed to *R. marinonascens* fermentations, in previous reports of [7], despite the significant presence of bile acids in MB, we sought to identify if *R. marinonascens* shared any steroid catabolic genes with a close relative *R. jostii*, a known cholate degrader. All 38 genes associated with the cholate degradation pathway in *R. jostii* were identified in *R. marinonascens*, however, with varying degree of amino acid (AA) identity (table 4). Identity above 75% was observed for genes *hsaF3*, *kshA3* and *casG*, which encode for cholate catabolic enzymes for side-chain and AB-ring degradation. The gene having the least identity (28.4%) was *casA*, a transcriptional regulator.

## Discussion

Numerous studies have reported production of bile acids and derivatives by marine bacteria<sup>[7-12]</sup>, and a fungal specie<sup>[13]</sup>. Surprisingly, there has not been follow up studies trying to identify and uncover the bacterial or fungal biosynthetic route for producing these metabolites, even though this would have important relevance in the biotechnological and biomedical fields.

The production of bile acids compromises a lengthy and complex pathway, which is both energy demanding and toxic to some extent, due to the production of hydrophobic molecules with surfactant properties. The fact that there might be organisms capable of this significant sacrifice would be possible, only if the advantages of producing such molecules are substantially higher for their survival or for competing in their given environment. Many theories have been formulated in order to explain the production of these molecules by these species, mostly relating to availability of insoluble carbon sources<sup>[7]</sup>, competitive inhibition<sup>[10]</sup> and symbiotic relations with insects and fishes<sup>[8]</sup>. However, there has not been any study up to date confirming these mechanisms.

Table 4. Identity of cholate degrading genes in *R. marinonascens*

Gene	<i>R. jostii</i> Locus Tag	<i>R. marinonascens</i> Genome Accession	AA Identity (%)	Start Position	End Position	E-value
	ro05790	NZ_BCXB01000025.1	50.000	38639	38926	1.35e-40
<i>hsd4A3</i>	ro05791	NZ_BCXB01000001.1	61.224	234032	234472	3.97e-85
<i>camM</i>	ro05792	NZ_BCXB01000054.1	32.787	10853	11218	9.09e-25
	ro05793	NZ_BCXB01000022.1	39.655	25121	24948	1.36e-08
	ro05794	NZ_BCXB01000013.1	46.667	15902	16036	2.31e-05
<i>kstR3</i>	ro05795	NZ_BCXB01000015.1	30.000	125373	124954	5.08e-15
<i>hsaB3</i>	ro05796	NZ_BCXB01000001.1	46.296	128815	129300	2.34e-45
<i>hsaD3</i>	ro05797	NZ_BCXB01000001.1	48.485	127695	127892	1.05e-49
<i>kstD3</i>	ro05798	NZ_BCXB01000001.1	40.191	120565	119939	1.38e-142
<i>hsaE3</i>	ro05799	NZ_BCXB01000001.1	67.939	120783	121568	2.30e-115
<i>hsaG3</i>	ro05800	NZ_BCXB01000001.1	74.751	121579	122481	7.01e-135
<i>hsaF3</i>	ro05801	NZ_BCXB01000001.1	75.143	122496	123545	1.35e-176
<i>hsaA3</i>	ro05802	NZ_BCXB01000012.1	66.182	143396	142572	2.30e-171
<i>hsaC3</i>	ro05803	NZ_BCXB01000001.1	45.304	127913	128455	2.33e-80
	ro05810	NZ_BCXB01000004.1	37.931	60671	60411	7.93e-08
<i>kshA3</i>	ro05811	NZ_BCXB01000001.1	76.159	125640	125188	1.56e-176
<i>hsaA3b</i>	ro05812	NZ_BCXB01000001.1	50.638	126307	127011	3.91e-122
<i>kstD3b</i>	ro05813	NZ_BCXB01000001.1	55.556	119242	119000	1.57e-52
<i>casA</i>	ro05814	NZ_BCXB01000001.1	28.415	375053	375601	4.79e-24
<i>casB</i>	ro05815	NZ_BCXB01000001.1	67.268	217301	216138	0
<i>casC</i>	ro05816	NZ_BCXB01000001.1	65.035	92210	92638	3.45e-162
<i>casD</i>	ro05817	NZ_BCXB01000001.1	51.667	118446	118087	5.07e-82
<i>casE</i>	ro05818	NZ_BCXB01000023.1	40.476	82552	82427	4.26e-04
<i>casF</i>	ro05819	NZ_BCXB01000022.1	58.462	20406	20795	6.99e-60
<i>casG</i>	ro05820	NZ_BCXB01000006.1	86.520	168790	169746	0
<i>casH</i>	ro05821	NZ_BCXB01000021.1	53.034	72382	71246	1.67e-133
<i>casI</i>	ro05822	NZ_BCXB01000001.1	46.923	229985	229596	1.40e-84
<i>casJ</i>	ro05823	NZ_BCXB01000013.1	51.351	16013	16123	5.17e-10
<i>casK</i>	ro05824	NZ_BCXB01000008.1	48.276	647	733	0.055
<i>casL</i>	ro05825	NZ_BCXB01000001.1	58.049	233145	232531	1.39e-108
<i>casM</i>	ro05826	NZ_BCXB01000001.1	47.059	96170	96424	1.07e-34
<i>casN</i>	ro05827	NZ_BCXB01000001.1	46.259	231403	230963	1.40e-79
<i>casO</i>	ro05828	NZ_BCXB01000001.1	56.911	96517	96885	2.85e-43
<i>casP</i>	ro05829	NZ_BCXB01000001.1	71.200	97329	98078	2.98e-159
<i>casQ</i>	ro05830	NZ_BCXB01000001.1	55.556	118398	118291	2.55e-13
	ro05831	NZ_BCXB01000001.1	65.041	208144	208512	2.85e-57
	ro05832	NZ_BCXB01000025.1	61.233	38255	38935	1.01e-94
<i>kshB3</i>	ro05833	NZ_BCXB01000002.1	69.362	239768	240472	7.01e-130

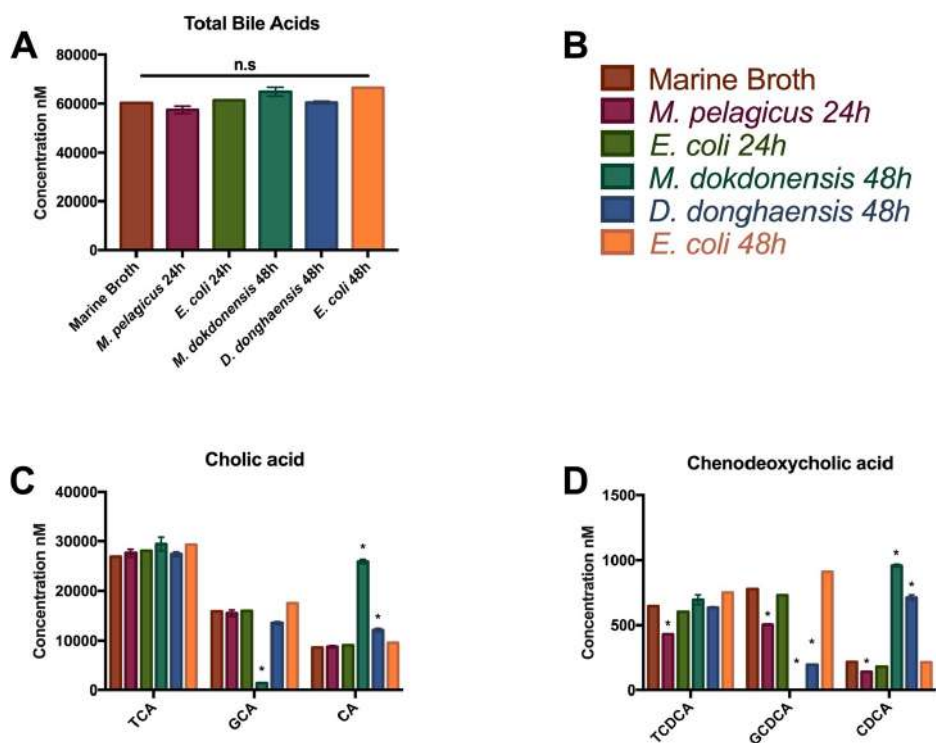


Figure 2. Quantification of total bile acids and primary bile acid deconjugation using LC-MS. A) Total bile acids. B) Legends for the different data shown. C and D) Conjugated and unconjugated profiles of primary bile acids, cholic and chenodeoxycholic acid. Taurocholic acid (TCA), glycocholic acid (GCA), cholic acid (CA), taurochenodeoxycholic acid (TCDCA), glycochenodeoxycholic acid (GCDCA), chenodeoxycholic acid (CDCA). Each column represents the mean and error bars represent the standard deviation. Biological duplicates were analyzed for each marine strain, only one replicate was analyzed for *E. coli* and marine broth samples. All comparisons were made against MB broth, Two-way ANOVA, 95% CI,  $P < 0.05$ .

Data from our quantification analysis shows no significant increase of bile acids compared to marine broth alone. Intriguingly, most of the studies mentioned do not measure total bile acids from marine broth Difco 2216 alone, assuming bile acids come only from the different bacterial species. Only one study<sup>[8]</sup> shows a picture from a thin layer chromatography, demonstrating no bile acids from the media used. Our data demonstrates clear abundance of bile acids in this media. Nevertheless, having bile acids in culture media is not uncommon, taking into account the different components used in the formulation and their source. Most probably, bile acids identified are coming from the peptone fraction, as we demonstrated (Sup. Fig. 1). Further communication with the supplier confirmed that the composition of the marine broth Difco 2216 had not changed over the last 20 years and that the source of peptone is bovine and porcine. With regards to mineral media fermentations and bile acid production *de novo* from cholesterol by *M. pelagicus*<sup>[8]</sup>, we believe that the bile acids seen by the

authors, most probably, comes from contamination by the inoculum fraction used, possibly done in marine broth. Investigation of further strains was thought to be irrelevant, as most studies have used media composed of peptone or used marine broth for their respective experiments.

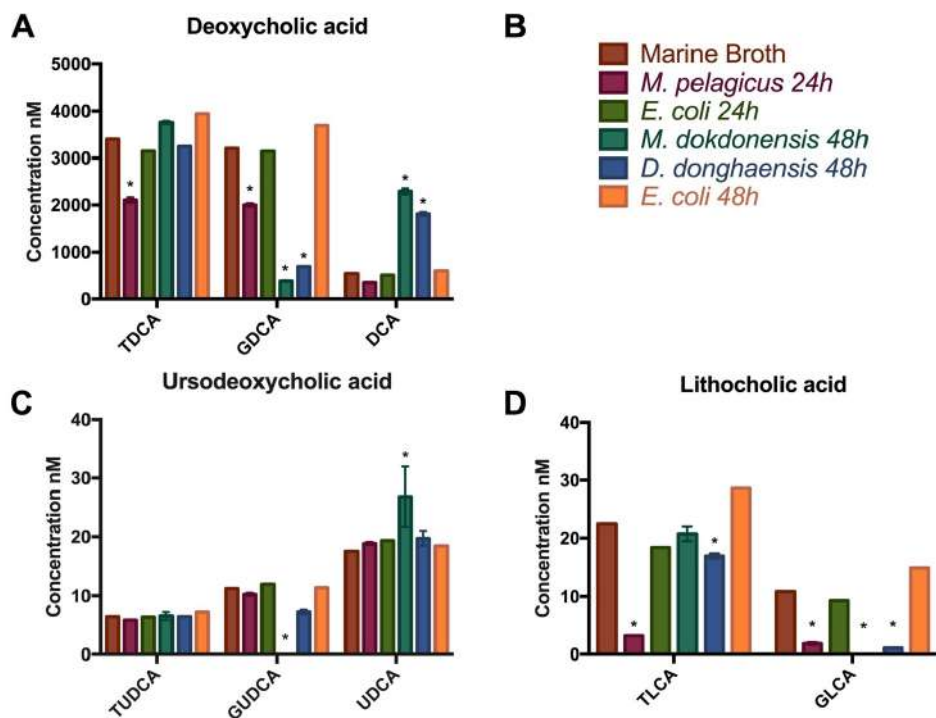


Figure 3. Quantification of Secondary bile acid deconjugation using LC-MS. A, C and D) Conjugated and unconjugated profiles of secondary bile acids, deoxycholic, ursodeoxycholic and lithocholic acid. B) Legends for the different data shown. Taurodeoxycholic acid (TDCA), glycodeoxycholic acid (GDCA), deoxycholic acid (DCA), tauroursodeoxycholic acid (TUDCA), glyoursodeoxycholic acid (GUDCA), ursodeoxycholic acid (UDCA), tauroolithocholic acid (TLCA), glycolithocholic acid (GLCA). Each column represents the mean and error bars represent the standard deviation. Biological duplicates were analyzed for each marine strain, only one replicate was analyzed for *E. coli* and marine broth samples. All comparisons were made against MB broth, Two-way ANOVA, 95% CI,  $P < 0.05$ .

The different bile acid profiles observed, however, could be a result of the combination of a differential preference of their respective bile salt hydrolases and the lack of standards used in their analytical methods. In this manner, an inadequate assessment can be easily drawn, and one could conclude that bacterial species have different bile acid production profiles.

Another aspect to take into consideration is the use of specific strains as negative controls, in the case of this study<sup>[7]</sup>, a *Rhodococcus* specie was used in order to demonstrate that no bile acids were produced in the given media. However, important steroid degradation capabilities have been



identified and demonstrated in this genera<sup>[14,15]</sup>. Our data, also suggests the presence of these capabilities in *Rhodococcus marionascens*, a strain used to demonstrate low bile acid production in the same study.

Bacterial species investigated showed capabilities to de-conjugate mostly the glycine fraction of the primary and secondary bile acids. A previous study has shown the abundance and diversity of bile salt hydrolases (BSHs) in nature by performing functional metagenomic analyses of different environmental samples<sup>4</sup>. Bile salt hydrolases have been studied in detail in probiotic strains, as it seems to be an important factor determining colonization capability in the gut environment<sup>[16]</sup>, it is likely that these species isolated from marine samples could be commensals of the digestive tract of fishes or aquatic organisms, employing BSHs as a mean to obtain valuable amino acids from bile acids produced by the host organism and thus, enabling them to colonize. It is also possible that free living marine bacteria use these enzymes to scavenge for amino acids from bile salts excreted from fishes, through feces. Recent studies have uncover that the roles of bile acids in the aquatic environment could exceed far beyond their digestive potential, as they could be used as behavioral cues by fish species<sup>[17]</sup>.

## Conclusions

Having dissected the different articles showing bile acid production in bacteria, and taking together the data generated in this study, it is unlikely that bile acids are a product of the metabolism of marine bacteria and other isolates. The source of the different bile acids identified seem to be from the peptone fraction present in the culture media used (Marine Broth Difco 2216). Furthermore, differences in bile acid profiles observed among the different isolates reported in the literature are potentially a result of the preference of their respective bile salt hydrolases, steroid degrading pathways, novel bile acid modifying enzymes and the narrow selection of bile acids used as standards in their respective analytical methods. Further investigation is still required in order to properly decipher the role of novel BSHs in marine bacteria and their ecological importance.

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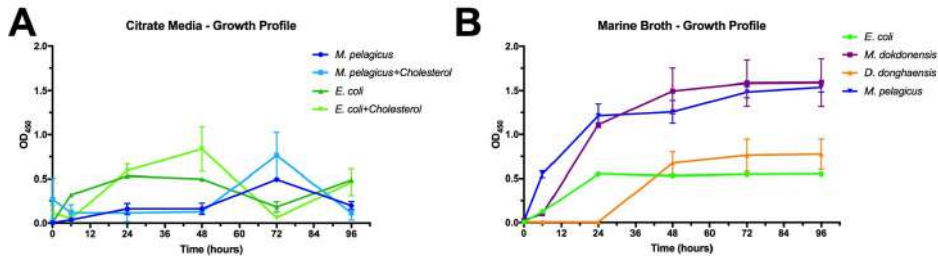
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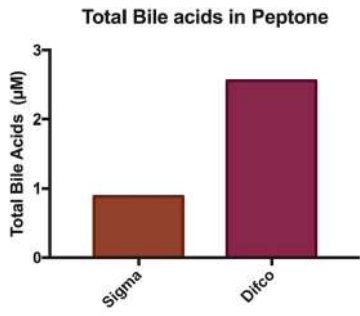
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## Supplemental Materials

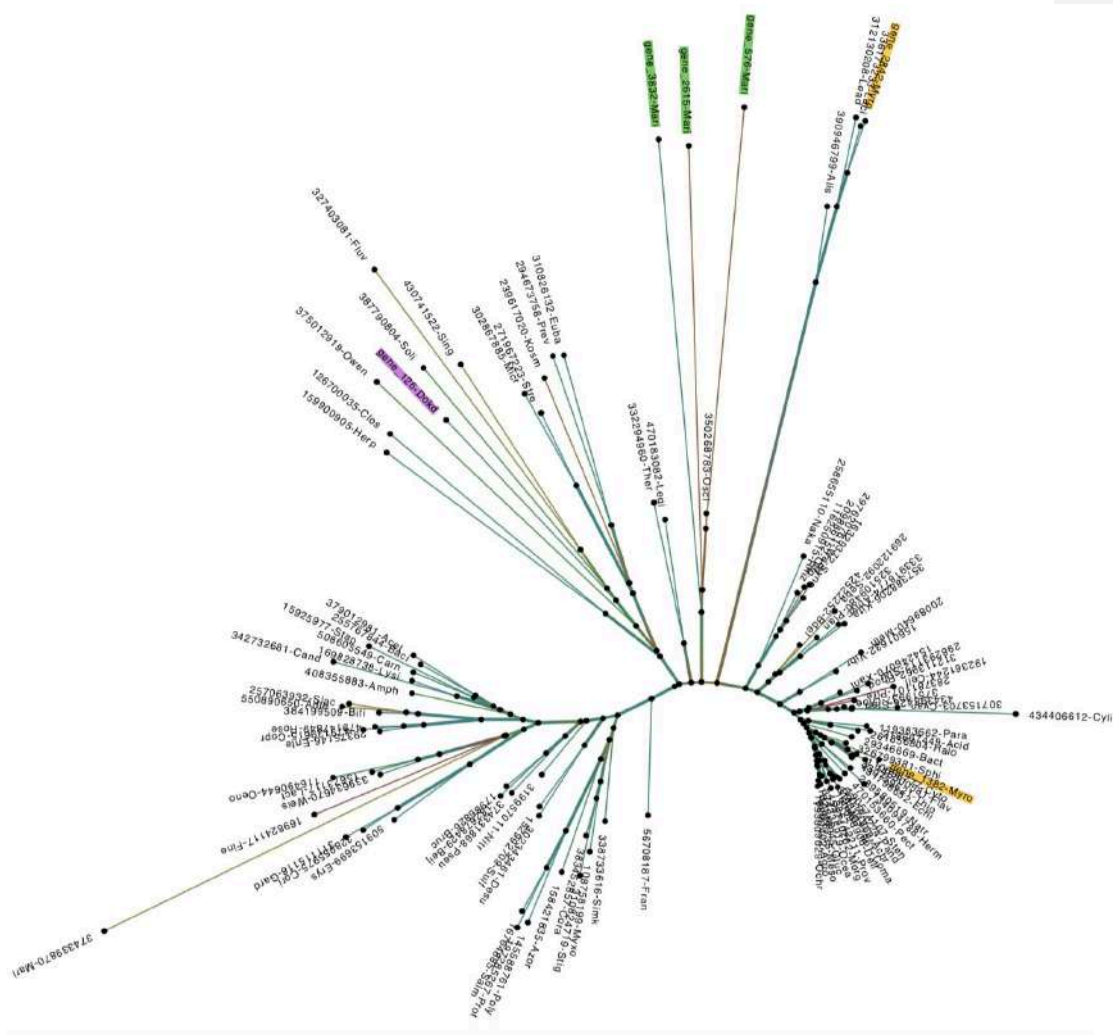
### Supplemental Figures



Supplemental figure 1. Growth profiles of marine bacteria and *E. coli* in citrate minimal media (A) and marine broth (B). Each data point represents the mean of biological triplicates, error bars represent the standard deviation.



Supplemental figure 2. Total bile acid quantification from commercially available peptone dissolved in MQ water (5 g/L).



Supplemental figure 3. Phylogenetic tree of known BSHs together with identified BSHs enzymes from *M. pelagicus*, *D. donghaensis*, *M. dokdonensis* (highlighted in yellow, purple and green, respectively).

### III. Exploring Strategies for Secondary Bile Acid Degradation and Neutralization

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#### Abstract

Bile acids have become a major focus point in recent years because of their important features, not only as important digestive molecules but also because of their relevance towards regulating key physiological aspects. The gut microbiota plays a crucial role on bile acid homeostasis, as they are able to deconjugate primary bile acids and further modify their structures into secondary bile acids. These modified versions, depending on their structure, have been reported to be toxic, have pro-inflammatory and carcinogenic effects, and be immunomodulatory. In the present study, we explore three different approaches: 1) Functional metagenomic screening, 2) Isolation of natural degraders, and 3) Bacterial sulfonation of bile acids. These approaches has led us to the discovery of genes involved in withstanding bile acid stress in bacteria, the isolation of novel species capable of consuming deoxycholic acid as the sole carbon source, and the construction of a probiotic strain of *E. coli* for sulfonating secondary bile acids. Overall, valuable information and understanding has been acquired in order to develop and efficient advanced microbiome therapeutic capable of modulating secondary bile acid profiles *in vivo*.

Key words: Bile acids, deoxycholic acid, lithocholic acid, sulfonation, functional metagenomics, bacterial isolation, sulfotransferase.

#### Introduction

Bile acids are key digestive molecules, that enable fat emulsification for appropriate absorption of fatty acids and liposoluble molecules through the intestine<sup>[49]</sup>. Besides this well documented and understood role, bile acids are also important signaling molecules. By their interaction with several receptors<sup>[37]</sup>, this molecules can affect energy expenditure, lipid and glucose metabolism, and the immune system<sup>[31,32]</sup>. These effects, however, are very dependent on the bile acid nature or its structure, as different conformations have different affinities towards the given receptors.

Bile acids are the end product metabolism of cholesterol, they are synthesized in the liver from the contribution of at least 14 different enzymes<sup>[48]</sup>. Primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA) are normally conjugated with glycine or taurine, forming bile salts. After the ingestion of food, bile salts that had been stored in the gall bladder are secreted into the duodenum, where they exert their digestive role dissolving fat droplets and enabling the uptake of fatty acids and lipo-soluble molecules<sup>[49]</sup>. First microbial modification involve hydrolyzing the amino acid moiety from the bile acid body by bile salt hydrolases (BSH) positive bacteria. This step is thought to be the gate keeper for further microbial bile acid modifications<sup>[51]</sup>. After digestion, 95%

of bile acids are actively taken up by enterocytes through transporter, in the well documented enterohepatic circulation, decreasing intestinal concentrations dramatically<sup>[52]</sup>. Primary unconjugated bile acid leftovers are further metabolized by microorganisms in the large intestine producing secondary bile acids (SBA)s, deoxycholic acid (DCA) and lithocholic acid (LCA) by 7 $\alpha$ -dehydroxylation of CA and CDCA, respectively<sup>[53]</sup>. Even though no active transport of bile acids takes place in the large intestine, secondary bile acids can still translocate into the blood stream by passively diffusing through the enterocytes<sup>[54]</sup>.

Several studies have pointed out the harmful effects of secondary bile acids in gut and peripheral organs exposed to these molecules, including gall-stones<sup>[55]</sup>, inflammatory diseases<sup>[34,56]</sup> and the onset of hepatic<sup>[29-31]</sup>, intestinal<sup>[28]</sup> and colon cancer<sup>[57]</sup>, as a consequence of their poor solubility, cytotoxicity and immunomodulatory traits. These findings have raised considerable attention regarding the importance of gut microbiota towards health and disease, and have reshaped previously established paradigms, concerning the mechanisms that trigger the disease, as well as medical strategies for new treatments.

In vertebrates, sulfonation is a key player in biotransformation pathways of peptides and small molecules. More specifically, cytosolic sulfotransferases (SULT)s are responsible for the sulfonation of small molecules such as neurotransmitters, steroids, xenobiotics and bile acids<sup>[74]</sup>. This action is thought to decrease their bioactivity and increase the solubility of such molecules, channeling them to known excretion routes<sup>[26]</sup>. Previous, endeavors have successfully expressed active sulfotransferases in bacteria<sup>[43,50,75-79]</sup>, in order to characterize the enzymes or produce sulfated products. Nonetheless, little to none research have focus on employing these enzymes for detoxification purposes mediated by bacteria; having an untapped potential, yet to be unlocked.

Strategies to identify novel microbial activities have been extensively reviewed. Among these, the use of functional metagenomic screens has pathed the way for the discovery of novel genes<sup>1</sup> including transporters<sup>[58]</sup>, antibiotic resistance genes<sup>[59]</sup>, Cas9 inhibitors<sup>[60]</sup>, among others. On the other hand, conventional and novel culturing techniques have also contributed significantly to the isolation of novel bacterial species capable of growing or utilizing different carbon sources of interest such as, hydrocarbons<sup>[61]</sup>, organic pollutants<sup>[62]</sup>, steroids<sup>[13,14]</sup> and bile acids<sup>[63]</sup>.

In the present study, we explore different strategies in order to identify approaches by which to degrade or neutralize toxic secondary bile acids (SBA)s, deoxycholic acid (DCA) and lithocholic acid (LCA). A functional metagenomic screening and classical culturing isolation techniques are employed for the identification of genes and bacterial species that could have the potential for degrading these molecules. Additionally, we investigate various sulfotransferases, and engineer the first bacterial probiotic specie capable of sulfonating secondary bile acids, describing its potential use as an advanced microbiome therapeutic (AMT).

## Methods

### *E. coli* strains, culture conditions and genetic material

All cloning procedures were performed using *E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA) and standard Luria Bertani (LB) media supplemented with appropriate antibiotics, unless otherwise stated. Liquid cultures and plates were incubated at 37°C for 16h (250 rpm for liquid cultures), unless otherwise stated. *E. coli* Nissle 1917 (EcN) variant was obtained from commercially available Mutaflor® product. Sulfotransferase plasmid library, plasmid pCBJ368 and components for

optimized sulfonation were kindly provided by Christian B. Jendresen and Prof. Alex T. Nielsen at the Novo Nordisk Center for Biosustainability at DTU. These materials also form part of a recent publication<sup>[43]</sup>. Metagenomic libraries utilized (Sup. Table 1) were constructed and published in previous studies<sup>[58,59,69]</sup>. pZE21-GFP was provided by Ruben V. Uribe from the Sommer Lab at the Novo Nordisk Center for Biosustainability at DTU and has also been published previously<sup>[60]</sup>.

#### Plasmid construction and EcN transformation

DNA fragments for plasmid construction were obtained by using Phusion U High-Fidelity PCR master mix (ThermoFisher Scientific). Fragments were purified using GeneJet PCR purification Kit (ThermoFisher Scientific), following manufacturer's instructions. Cloning reaction was performed using 1 µl of USER enzyme (NEB), 1 µl of DpnI (ThermoFisher Scientific), 1 µl of 10X Cut Smart buffer (NEB) and 200 ng of DNA fragments and MQ water, for a total reaction volume of 10 µl. Mixture was incubated at 37°C for 30 minutes, followed by 15 minutes at 15°C. 5 µl of the USER reaction was used to transform chemically competent *E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA) by heatshock at 42°C. 1 ml of SOC media was used to recover the transformed cells for 1 hour, and 50 µl were plated in LB plates supplemented with the appropriate antibiotic. Plates were incubated at 37°C overnight. Next day colonies were screened through colony PCR, using OneTaq Quick-Load 2x Master Mix (NEB). Positive colonies were inoculated into 5 ml of 2xYT medium (containing 16 g/L Tryptone, 10 g/L Yeast Extract and 5 g/L NaCl) and were incubated overnight. Next day, plasmids were purified using NucleoSpin Plasmid EasyPure purification kit (Macherey-Nagel), following manufacturer's instructions. Concentration of purified plasmids were measured with NanoDrop (ThermoScientific™), and later sequenced using Eurofins overnight sequencing service. Cultures of colonies harboring the correct plasmid were stored at -80°C as glycerol stocks.

A single colony of EcN was inoculated in LB media overnight. next day 100 µl of the overnight culture was used to inoculate 10 ml of 2xYT. Optical density (OD) was followed and cultures were harvested between OD<sub>600</sub> = 0.4 - 0.5, using a prechilled centrifuged at 4500 g for 10 minutes. Pellets were washed 3 times with cold 10% glycerol/MQ H<sub>2</sub>O solution. Lastly, 100 ng of the desired plasmid was transferred onto the pellets and 50 µl of 10% glycerol/MQ H<sub>2</sub>O solution was used for resuspension. Resuspended cells were then transferred to a cold 0.1 cm Gene Pulser electroporation cuvette (Bio-Rad) and were electroporate (BioRad MicroPulser) at 1.8 kV. Cells were recovered using 1 ml of SOC media for 1 hour in a shaking incubator at 37°C, before plating. Schematic representations of the plasmids constructed in this study can found in figure 3 of supplemental Materials.

#### Fermentations

Small scale fermentations were performed by inoculating strains, in biological duplicates, unless otherwise stated, into 500 µl of M9 media (Sup. Table 7) (0.4% glucose) supplemented with appropriate antibiotics in 96-deep well plates. Preculture was allowed to grow until saturation (24 hours), after which an aliquot of 5 µl was taken to inoculate the production culture (500 µl), using the same setup. After 22 hours, optical density was measured and plates were centrifuged at 4500 rpm for 10 min. Supernatants were then frozen until further LC-MS/MS preparations were performed. For fermentations of KRX strains, 2xYT was used for preculture, and rhamnose (0.1%) and IPTG (0.1 mM) was added to the production culture to induce expression T7 RNA polymerase.

#### pOSIP mediated genomic integrations

Integration of large DNA fragments into EcN was performed via pOSIP<sup>[83]</sup>. Backbone of pOSIP-KO and DNA fragment to be integrated were amplified with Phusion U DNA polymerase and cloned by



USER cloning. 5 µl of the USER reaction was transformed, via heat-shock, into chemically competent EcN cells. Cells were allowed to recover for 1 hour in SOC media at 37°C, before plating in LB agar supplemented with kanamycin. Plates were incubated at 30°C overnight. Following day, colony PCR was performed to verify appropriate integration of the fragment of interest. In order to excise the integration cassette, positive clones were transformed with pE-FLP and plated in LB supplemented with ampicillin at 30°C. following day, colonies were streak in LB plates with and without antibiotics to confirm loss of the integration cassette and pE-FLP plasmid. To confirm the strain, the integrated fragment was PCR amplified and sequenced.

#### Generation *E.coli*ΔtolC::amp

*E. coli* TOP10 harboring the pTK-RED<sup>[67]</sup> plasmid was grown overnight at 30°C in LB supplemented with spectinomycin. Next day, 5 ml of LB were inoculated with 50 µl of overnight culture and grown at 30°C in a shaking incubator. 50 µl of Arabinose 1M were used to induce the expression of Lambda Red recombinases at OD<sub>600</sub> = 0.4, culture was allowed to grow for another 30 min and was then placed on ice for 10 min. Culture was washed 3 times using a pre-chilled centrifuged at 4500 g for 10 minutes, and cold MQ water, 100 ng of previously amplified and purified ampicillin cassette with 50 bp homolog upstream and downstream sequences to the tolC gene were transferred onto the pellet, and 50 µl of cold MQ water was used to resuspend the cells. Resuspended cells were then transferred to a cold 10 mm electroporation Gene Pulser electroporation cuvette (Bio-Rad) and were electroporate (BioRad MicroPulser) at 1.8 kV. Cells were recovered using 1 ml of SOC media for 1 hour in a shaking incubator at 30°C, before plating. Colonies were screen based on their sensitivity towards bile acids, *tolC* locus was amplified and sequenced to confirming deletion of *tolC*. Recombineering plasmid was cured by streaking the cells and incubating at 42°C. Next day colonies were tested for resistance against spectinomycin.

#### Identifying optimal range of DCA

Optimal range of DCA concentration for functional metagenomic screening was identified by performing minimum inhibitory concentration (MIC) tests on different concentrations of DCA in 2 LB and M9 minimal media. In short, Strains to be tested were grown overnight in 5 ml of either LB or M9 at 37°C in a shaking incubator. Next day, cells were diluted 1:100 and 0.8 µl was used to inoculate single wells of a 96-well microtiter plates, containing different concentrations of DCA. Growth was monitored using a BioTek ELx202 microtiter plate reader for 24 hours. Inhibition % was calculated as follows:

$$\text{Inhibition \%} = 100 - \left[ \frac{(\text{Growth on media containing DCA} * 100)}{\text{Growth on media}} \right]$$

Identified MIC and neighboring concentrations were tested in LB and M9 solid media to validate inhibition. In short, 1 ml of overnight grown cells to be tested was washed once with MQ water, serially diluting 10 fold in a 96-well microtiter plate, 5 µl were spotted on the agar plates containing different concentrations of DCA, spot assays.

#### Functional metagenomic screening and Nanopore sequencing

*E. coli* TOP10 was grown overnight at 37°C in 5 ml of M9 medium in a shaking incubator. Next day, 250 µl of overnight culture was used to inoculate 25 ml of M9 medium in a 250 ml baffled shake flask. The culture was harvested at OD<sub>600</sub> = 0.4 – 0.5, electrocompetent cells were prepared as mentioned previously. 100 ng of each metagenomic library was electroporated and cells were

recovered in M9 media for 1 hour. Recovered cells were plated in 10 x 10 cm square petri dishes. Plates were incubated at 37°C overnight. Following day, colonies were scraped of using 1ml of PBS, cells were then centrifuged and plasmids were extracted using NucleoSpin® Plasmid EasyPure purification kit (Macherey-Nagel). Phusion High-Fidelity PCR 2x Master Mix (ThermoFisher Scientific) was used to amplify metagenomic inserts from each purified plasmid library, using specific bar coded primers. PCR products were purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel), following manufacturer's instructions. Purified fragments were quantified using Qubit and equal concentrations were pooled together.

#### Long-read (Nanopore) DNA sequencing and analysis

Barcoded amplicons from pooled metagenomic libraries were diluted to 1.5 µg in 45 µl of nuclease-free water. DNA quality and concentration were determined on a Qubit Fluorometer (Thermo Fisher Scientific). DNA libraries for long-read Nanopore sequencing were prepared using the SQK-LSK108 ligation sequencing kit (Oxford Nanopore Technologies), according to the manufacturer's instructions. In short, the pooled barcoded amplicons were combined with 8.5 µl of nuclease-free water, 6.5 µl of FFPE Repair Buffer (New England BioLabs), and 2 µl of FFPE Repair Mix (New England BioLabs) followed by incubation for 15 minutes at 20°C. The FFPE repaired DNA was cleaned up with AMPure XP beads (Beckman Coulter Life Sciences), resuspended in 46 µl of nuclease-free water and quantified on a Qubit fluorometer. For end repair and dA-tailing, 7 µl of Ultra II End-prep reaction buffer (New England BioLabs), 3 µl Ultra II End-prep enzyme mix (New England BioLabs), and 5 µl of nuclease-free water were added to 45 µl of repaired DNA, followed by incubation at 20°C for 5 minutes and 65°C for 5 minutes in a thermal cycler. The end-prepped DNA was cleaned up with AMPure XP beads and resuspended in 31 µl of nuclease-free water. Sequencing adapters were ligated to the DNA by addition of 20 µl of Adapter mix 1D (AMX-1D) and 50µl of Blunt/TA ligation master mix to 30µl of end-prepped DNA and subsequent incubation for 10 minutes at room temperature. The adapter ligated DNA mix was cleaned with AMPure XP beads, mixed with adapter binding buffer (ABB) and finally resuspended in 15 µl Elution buffer (ELB). Finally, 12 µl of the final DNA library was diluted in 35 µl RBF buffer, 2.5 µl nuclease-free water, and 25.5 µl of library loading beads (LLB) and gently loaded onto a primed sequencing flow cell (R9.4.1; FLO-MIN106, Oxford Nanopore Technologies) according to the manufacturer's instructions. Sequencing was performed with live basecalling using the MinKNOW software and stopped after approximately 72 hour. Specifics with regards to processing of sequencing results can be found in supplemental materials and methods.

#### Bioinformatic processing of nanopore sequencing data

Given a set of raw reads, we identified the ORF using GenMarkS<sup>[86]</sup>. ORFs were clustered at 95% identity, and the longest one per cluster was chosen. ORFs were then translated into proteins using EMBOSS package. The protein sequences are then blasted using blastp against NCBI ref-protein database and the top hit per protein is chosen to be the functional annotation. The proteins were then ranked based on the number of reads, excluding TolC and other membrane proteins as false positive candidates. The top 100 non-hypothetical proteins were considered for manual curation. Manually curated genes as well as hypothetical genes observed in at least 30 reads had their reads clustered at 80% identity. The representative reads with more than ten reads in their clusters were used to generate the primers for ORF amplification.

#### Validation of functionally identified genes and DCA quantification

Identified ORFs were amplified and cloned into the same backbone plasmid used for the functional metagenomic screening (pZE21)<sup>[68]</sup>, following previously described methods. After verification,

constructed plasmids were transformed into *E. coli*  $\Delta$ tolC::amp and MIC validating experiments were performed. Colonies were cultured in the same fashion as for MIC experiments.

#### LC-MS/MS quantification of bile acids

50  $\mu$ l of bacterial broth was extracted with 3 volumes of methanol containing deuterated internal standards (d<sub>4</sub>-TCA, d<sub>4</sub>-GCA, d<sub>4</sub>-GCDCA, d<sub>4</sub>-GUDCA, d<sub>4</sub>-GLCA, d<sub>4</sub>-UDCA, d<sub>4</sub>-CDCA, d<sub>4</sub>-LCA; 50nM of each). After 10 minutes of vortex and 10 minutes of centrifugation at 20 000g, the supernatant was diluted 1:50 in methanol:water [1:1]. Bile acids were analyzed using ultra-performance liquid chromatography-tandem mass spectrometry (UPLCMS/MS) according to previous work<sup>[65]</sup>. Briefly, after injection (5  $\mu$ L) the bile acids were separated on a C18 column (1.7  $\mu$ m, 2.1 x 100mm; Kinetex, Phenomenex, USA) using water with 7.5mM ammonium acetate and 0.019% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). The chromatographic separation started with 1 minute isocratic separation at 20%B. The B-phase was then increased to 35% during 4 minutes. During the next 10 minutes the B-phase was increased to 100%. The B-phase was held at 100% for 3.5 minutes before returning to 20%. The total runtime was 20 minutes. Bile acids were detected using multiple reaction monitoring (MRM) in negative mode on a QTRAP 5500 mass spectrometer (Sciex, Concord, Canada).

#### Isolation of DCA degrading strains and 16S sequencing

Sterile containers were given to healthy volunteers for acquiring fecal samples. Fecal samples were then frozen at -20°C until further processing. A small scoop of feces  $\approx$  0.1 g was homogenized in 1 ml of PBS, and 100  $\mu$ l were plated onto M9 minimal agar with either 0.5 g or 1 g of DCA as the only carbon source, in both aerobic and anaerobic conditions. Plates were re-streaked on the same media and conditions 3 times until single colonies could easily be noticed, this was performed every 24 hours (aerobic conditions) and 48 hours (anaerobic conditions). In addition, Anaerobic media was supplemented with 10 g/L of KNO<sub>3</sub>, as an electron acceptor. Figure 4A shows an overview of the experimental workflow. After the final streak, 4 isolates per fecal sample were tested for growth on M9 minimal broth, having the same concentrations of DCA as the previous experiment, all conditions remained the same. Colonies were inoculated in a 96-deepwell plate and growth was followed using a BioTek™ ELx202 microtiter plate reader for aerobic cultures and BioTek™ Epoc-2 Microplate reader for anaerobic samples. Isolate showing growth were stored as glycerol stock at -80°C. Colony PCR of isolates was performed as described previously, PCR products were diluted 1:80 and 15  $\mu$ l were used for sanger sequencing (Eurofins overnight sequencing service).

#### Preliminary DCA degradation cultures

Aerobic and anaerobic isolates were inoculated into 10 ml of M9 minimal broth containing either 0.5 g/L or 1 g/L DCA, as the sole carbon source. Cultures were grown under the same conditions and growth was followed as described previously. Supernatant samples for bile acid quantification were taken every 24 hours by centrifuging 500  $\mu$ l of the culture at 10 000 g for 1 minute.

#### Sulfotransferase screening for sulfonation of DCA and LCA

Biological duplicates of *E. coli* KRX strains harboring sulfotransferase plasmid and pCJB368 were inoculated into 350  $\mu$ l of 2xYT, supplemented with the appropriate antibiotics, using a 96-deepwell plate. The plate was incubated at 37°C for 24 hours in a shaking incubator. After 24 hours, the culture was diluted 1:100 into M9 minimal media complemented with 0.4% glucose and supplemented with 50  $\mu$ M LCA or 100  $\mu$ M DCA. Plate was incubated for 24 additional hours, before harvesting the supernatant by centrifugation at 4500 g for 10 minutes. Samples were then frozen at -20°C until

further processing for LC-MS/MS analysis. Same procedure was followed for further sulfonation experiments, with some minor variations in media composition.

#### Genomic modification via no-SCAR recombineering

Genomic modification of the PUWA promoter was carried out using the Scarless Cas9 Assisted Recombineering (no-SCAR) system<sup>[73]</sup>. Briefly, strains transformed with plasmid pHM156, containing a thermosensitive ORI, the  $\lambda$ -Red system under an arabinose inducible promoter, the *cas9* gene under a constitutive promoter (BBa\_J23105), and a pMB-targeting gRNA under the regulation of the tetO promoter, were inoculated from a fresh plate into 3 ml of LB supplemented with 100  $\mu$ g/ml ampicillin (amp) and were incubated in a shaking incubator at 30°C and 250 rpm. Next day, cells were inoculated 1:50 into 10 ml of 2xYT broth, supplemented with ampicillin, and were grown at 30°C until OD<sub>600</sub> of 0.3-0.4. At this OD, 10  $\mu$ l of 10% arabinose was used to induce the expression of  $\lambda$ -Red system and cells were allowed to grow for another 30 min. The culture was cooled using ice for 10 min before cells were prepared for electroporation. Cells were electroporated with A) plasmid pMB-X, containing a gRNA specific to the target (X) under constitutive expression, and B) an overlapping DNA fragment for knock out or knock in modifications. Once electroporated cells were recovered in 1 ml of SOC for 1 hour at 30°C, after which 4 ml of SOC and 5  $\mu$ l of ampicillin and chloramphenicol (cam) were added. Tubes were incubated overnight at 30°C in a shaking incubator (250 rpm). Following day, 50  $\mu$ l were plated onto LB plates containing amp and cam, and were grown overnight at 30°C. Positive clones, confirmed by colony PCR, were inoculated in 2ml of LB broth supplemented with amp and ATc (200 ng/ml) and were grown at 30°C in a shaking incubator overnight. Next day, cells were streaked on LB and grown at 37°C overnight, for curing pHM156. Plasmid curing was verified by streaking single colonies in amp and cam. Guide RNAs were designed using CRISPy-web<sup>[70]</sup>.

#### Construction of inducible and constitutive sulfonation plasmids

Plasmids (Sup. Fig. 6) were constructed following standard cloning methods, described previously. Fragments of interest containing the sulfotransferase gene, the genes encoding for the recycling enzymes (CysDNCQ) and sulfate transporters (CysPUWA from *E. coli* or CysP from *B. subtilis*) were amplified and cloned using USER cloning, as a single operon. Promoters were incorporated in the reverse primer used for amplifying the backbone fragment from the pMUT plasmid. For the inducible approach, the T7 promoter was used to drive expression of the sulfotransferase, recycling enzymes and the transporters. Additionally, a plasmid having the T7 polymerase repressed by *lacI* had to be transformed in the strains harboring the inducible sulfonation system. On the other hand, a strong constitutive promoter, developed in house, was used for driving expression of the constitutive sulfonation approach.

#### Statistical analysis and software tools

Design of genetic parts was carried out using online lab notebook tool Benchling<sup>[66]</sup>. Statistical analysis was carried out in GraphPad Prism 7 (La Jolla California, USA. [www.graphpad.com](http://www.graphpad.com)). Figures were created using Biorender (BioRender.com).

## Results

#### Identification of optimal concentration of DCA for functional metagenomic screening

In order to identify an optimal concentration for the functional metagenomic screening using *E. coli* deletion strains for *tolC* and *acrAB* were tested on a range of DCA concentrations in both LB and M9 media (Sup. Fig. 1 & 2). The *tolC* deletion made the bacterium highly sensitive towards DCA. The

minimum concentration having 100% inhibition was determined to be 75  $\mu$ M. On the same concentration, the *tolC* KO strain expressing an isomerization pathway for DCA (ISO2), a “positive control” for DCA degrading enzymes, showed to have less than 50% inhibition (Sup. Fig. 1A). Further confirmation experiments on solid media demonstrated that 75  $\mu$ M was appropriate for the functional metagenomic screening, as growth of the positive control (ISO2) was observed while no growth was seen from the negative control (GFP) (Sup. Fig. 1B).

#### Identification of candidate genes for DCA resistance

For identifying genes involved in secondary bile acid resistance from metagenomic libraries, a functional metagenomic screening was performed on DCA. From all libraries tested only human fecal metagenomic libraries gave transformants, using 0.031 g/L (75  $\mu$ M) of DCA (Sup. Table 2). The highest amount of colonies was seen in the library H3, accounting for approximately 2440. On the other hand, the libraries with least amount of colonies were H1 and H5 with 84 and 100 colonies respectively.

To narrow down possible ORFs involved in DCA degradation, sequencing data was extracted using GeneMarkS<sup>[86]</sup>, giving a total of 89118 ORFs. Exclusion of pseudogenes and sequences with less than 300 base pairs resulted in a total of 65668 ORFs total annotated 51868. These ORFs were then clustered using cd-hit at 95% of identity and coverage and the longest sequence was annotated using tblastx against COGS database with a minimum E-value of 1E-5, obtaining 51868 annotations. Of which, 15113 belonged to membrane proteins (11496 to TolC), 36755 to other annotated genes. A detailed count and percentage of the function of genes obtained is shown in supplemental table 9. ORFs obtained were further trimmed to remove false positive (*tolC* and membrane transporters) and genes unlikely to perform catabolic function (structural membrane proteins, transcription factors, motility factors, etc.), and a threshold of at least 30 hits was established to reduce the number of candidate genes to validate. Lastly, if contiguous ORFs were observed in the same contig, the full sequence was extracted and also validated. Table 1 shows final trimmed candidates that were cloned and validated experimentally.

TolC encoding ORFs accounted for the largest fraction of annotated ORFs (11.02%), however, none of the *tolC* genes identified belonged to the genus *Escherichia*. Two genera accounted for more than 90% of the *tolC* genes observed, *Parabacteroides*, *Bacteroides* and *Serratia* (Sup. Table 10), demonstrating compatibility among their efflux systems.

Table 1. Trimmed candidate ORFs identified in 75  $\mu$ M DCA functional metagenomic screening.

Candidate	Gene	Name	Source	% Identity	Library
1	<i>ispG</i>	(E)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase DUF896 domain-containing protein	<i>Clostridium innocuum</i>	96.8	(H2)
2.1	FOC47_01470		<i>Enterocloster bolteae</i>	95.21	(H4)
2.2	<i>proB</i>	Glutamate-5-kinase	<i>Enterocloster bolteae</i>	93.15	(H4)
2.3	<i>proA</i>	Glutamate-5-semialdehyde dehydrogenase	<i>Enterocloster bolteae</i>	100	(H4)
4	G4D55_16645	GNAT family N-acetyltransferase	<i>Clostridium innocuum</i>	96.52	(H2)
5	G4D55_05670	Alpha/Beta hydrolase	<i>Clostridium innocuum</i>	98.87	(H4)
7.1	GMB04_08740	phosphotransferase tRNA (guanosine(46)-N7)-methyltransferase	<i>Lactobacillus rhamnosus</i>	100	(H4)
7.2	<i>trmB</i>		<i>Lactobacillus rhamnosus</i>	99.1	(H4)
8	BACOV975_01021	glycosyl hydrolase	<i>Bacteroides ovatus</i>	100	(H4)
9.1	<i>plsY</i>	acyl-phosphate glycerol 3-phosphate acyltransferase	<i>Enterocloster bolteae</i>	100	(H1)
9.2	<i>gpsA</i>	NAD(P)-dependent glycerol-3-phosphate dehydrogenase	<i>Enterocloster bolteae</i>	100	(H1)
10	N597_06180	Cyclase NADPH-dependent FMN reductase	<i>Streptococcus sp.</i>	100	(H4)
14	A4V08_14035		<i>Lachnoclostridium sp.</i>	100	(H4)

#### Validation of identified of ORFs and Quantification of DCA

MIC profiles of *E. coli* $\Delta$ *tolC*::amp harboring plasmids with individually cloned ORFs (Table 1) for validation showed no major difference when compared to negative control along the different concentrations tested at 24 hours (Fig. 1A). At 48 hours, however, a more spread profile of resistance was observed for 75  $\mu$ M DCA (Fig. 1B). Furthermore, when analyzing specifically the 75  $\mu$ M data point, significant differences were observed for 5 of the 13 cloned ORFs (Fig. 1C).

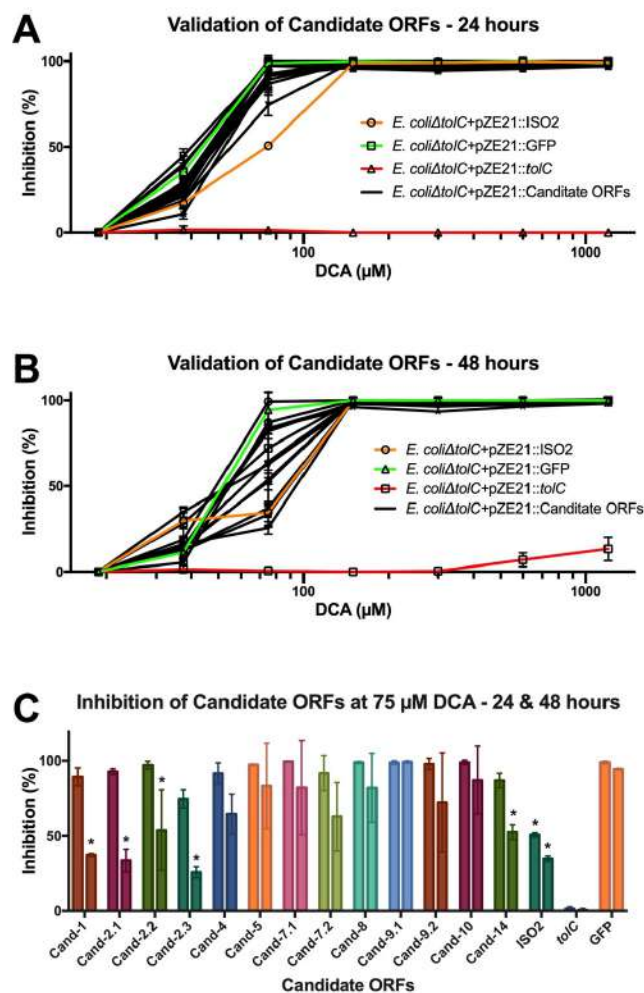


Figure 1. Validation of identified ORFs through MIC tests against DCA. A) MIC experiment values at 24 hours. B) MIC experiment values at 48 hours. C) Further observation of inhibition percentage of candidate ORFs in 75  $\mu$ M DCA at 24 hours (first column) and 48 hours (second column). Each column represents the mean of biological triplicates, error bars show standard deviation. Two-way ANOVA (Dunnett's multiple comparison test) against GFP control, with 95% CI was used to determine significance,  $P < 0.05$ .

Deoxycholic acid quantification analysis after 48 hours of cultivation showed no difference for most candidates with the exception of candidate 5, in which a small increase of DCA was observed. Positive control strain, harboring DCA isomerization pathway, showed a significant decrease, all strains were compared to the GFP expressing control. Interestingly, low amounts of DCA were

measured for the TolC expressing strain (Fig. 2A). Having witnessed loss of DCA, most likely due to growth, a Pearson correlation analysis was performed comparing OD values to DCA levels of control strains, identifying a strong negative correlation of  $r^2 = 0.9332$  (Sup. Fig. 3C). Corrected values for total DCA were generated by adding the expected loss of DCA due to biomass formation with the measured DCA concentration. Interestingly, candidate 2.1, coding for a DUF896 domain-containing protein of an unknown function, was the only candidate ORF to show a small but significant decrease ( $P = 0.0326$ ) compared to the GFP control (Fig. 2B). This result, however, needs to be treated with caution, as the estimation for bile acids lost is not fully validated, being just an approximation. At first view there seems to be a linear relation between OD and DCA, however, if more data points are integrated this might not be the case and might affect DCA corrected values. Nevertheless, these results show that the majority of genes identified may be playing an indirect role for increasing DCA resistance in *tolC* deleted *E. coli*.

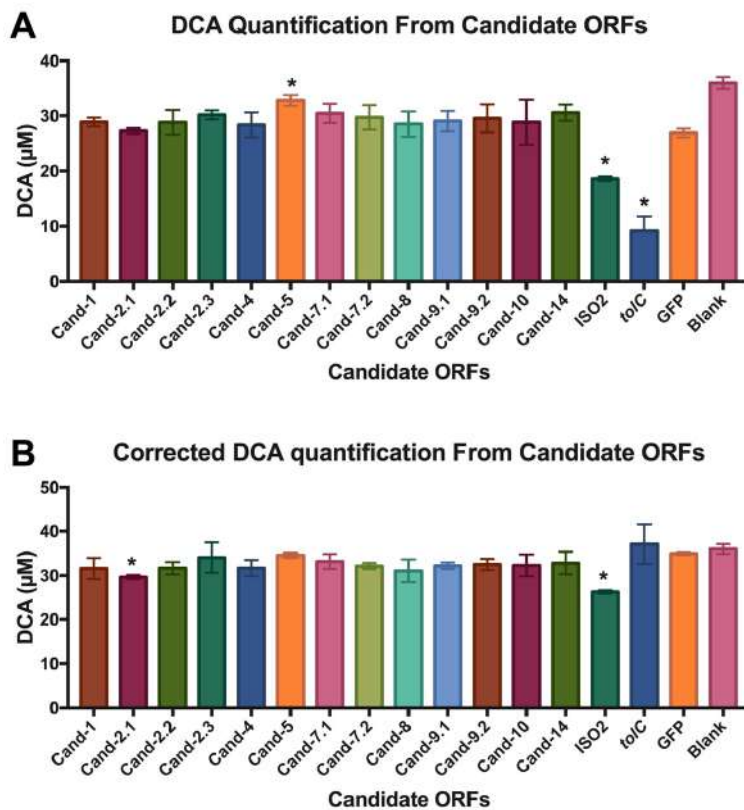


Figure 2. DCA quantification of candidate ORFs. A) Deoxycholic acid quantification from the supernatant of 48 hour cultures of strains harboring ORFs plasmids in M9 media supplemented with 37.5 μM DCA . B) Shows corrected values for DCA quantification, based on expected loss of DCA



due to growth. Each column represents the mean of biological triplicates, error bars show standard deviation. One-way ANOVA (Dunnett's multiple comparison test) against GFP control, with 95% CI was used to determine significance at  $P < 0.05$ .

#### Isolation of natural DCA degrading isolates

In order to isolate novel species capable of DCA degradation, conventional culturing procedures were employed using M9 base media complemented with DCA, as the sole carbon source. A schematic representation of the isolation procedure is shown in Sup. Fig. 4A. This led to the isolation of 112 strains, 28 strains from each condition tested (Sup. Table 3). Initial testing showed no growth in liquid media of any of the strains isolated from 0.5 g/L DCA in aerobic conditions (data not shown). Nevertheless, strains isolated from the remaining conditions showed varying growth. Best performing strains, which showed sustained growth over days were selected for further experiments in a larger volume (Sup. Fig. 4B-D).

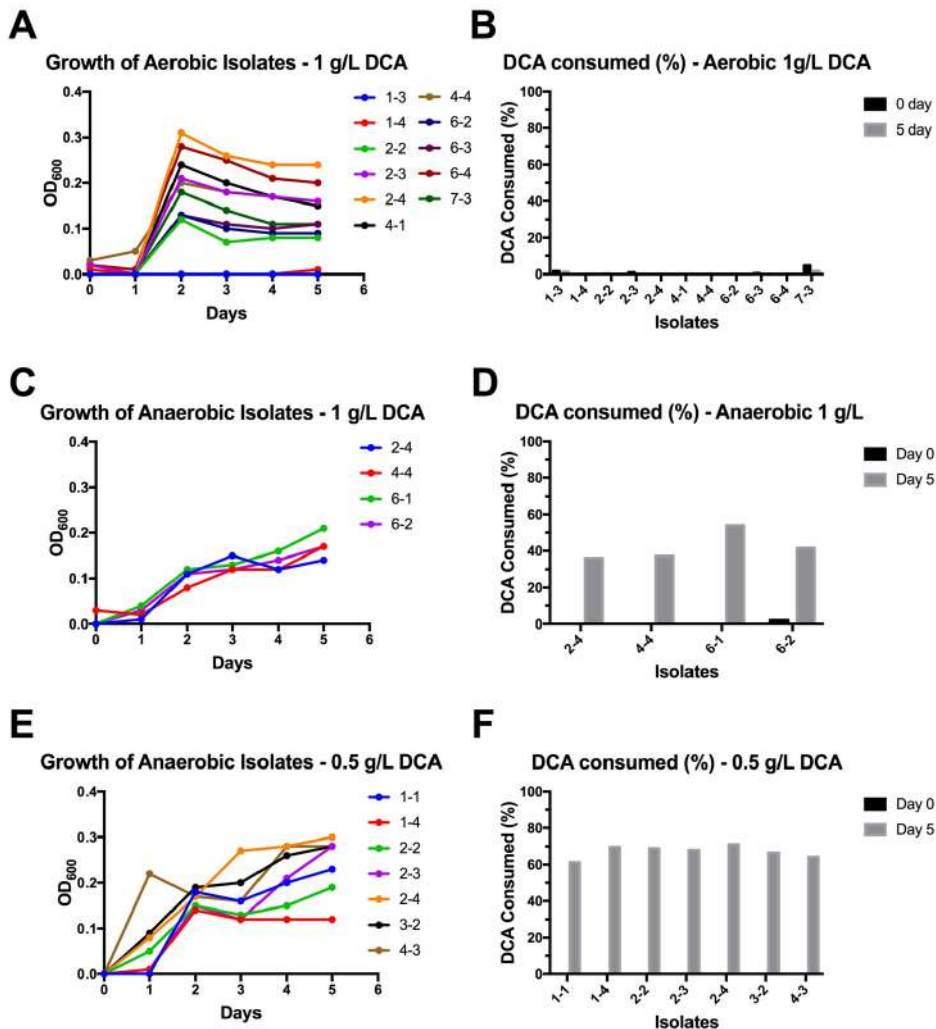


Figure 3. Preliminary growth and percentage of DCA consumption from strains isolated using aerobic conditions and 1 g/L DCA (A & B), anaerobic conditions and 1 g/L DCA (C & D), and anaerobic conditions and 0.5 g/L DCA (E & F). Same concentrations of DCA as in isolation conditions were used. Only 1 biological replicate was used per isolate. Percentage of DCA consumed was calculated having the blank sample (no inoculum) of the corresponding day as a reference.

A total of 22 strains were considered as best performers based on the results of the initial growth experiment. From these, 11 belonged to the 1 g/L DCA aerobic condition and the rest to the anaerobic condition of both 0.5 g/L and 1 g/L DCA. From these, only the strains from anaerobic conditions

showed degradation of DCA (Fig. 3B,D & F), consisting of 7 strains isolated using 0.5 g/L of DCA (Sup. Table 4) and 4 using 1 g/L of DCA (Sup. Table 5). Interestingly, there were no aerobic isolates capable of degrading DCA, even though growth was observed (Fig. 5A,B). This could be due to the isolated bacteria utilizing another carbon source in the media. Isolates were inoculated from a glycerol stock, being likely that glycerol was preferred over DCA for growth. On the other hand, anaerobic isolates seem to degrade DCA, despite the presence of glycerol. Unlike the aerobic isolates, which show a strong growth profile over the first 2 days of culture, anaerobic isolates show a steady growth, supporting the idea that DCA is being utilized over time. Sequencing results revealed a predominant presence of the genera *Escherichia*, accounting for 6 out of the 11 isolates. Uncultured bacteria accounted for 2 isolates, of which one was an uncultured *Klebsiella* strain. Additionally, 1 unclassified bacteria, 1 *Cronobacter* and a *Shigella* species were also isolated.

#### Sulfonation of secondary bile acids in *E. coli*

To determine whether *E. coli* was a suitable host for sulfonating bile acids, a library of 43 sulfotransferases<sup>[43]</sup> were expressed and tested for *in vitro* inducible sulfonation using *E. coli* KRX (Sup. Table 6). This led to the identification of 16 variants capable of sulfonating DCA, from which 2 had activity against LCA as well (Fig. 4A & Table 2). Sulfotransferases shown to sulfate DCA and/or LCA came from a broad source of organisms, belonging to mammalian, fish, insect and algal species and included putative enzymes (pst42 and pst48). Sulfonation activity was then tested in EcN using the same inducible system but cloned into a native plasmid of the strain, and incorporating 2 new sulfotransferases, pst50 and pst51, which encode for the native and codon optimized human SULT2A1. These mentioned sulfotransferases showed to be the only ones having activity in this setup (Fig. 4B). Interestingly, the non-codon optimized version outperformed the optimized version for both DCA and LCA sulfonation. However, when switching the expression towards a constitutive system, having both the sulfotransferase and the cofactor recycling genes *cysDNCQ* driven by a strong constitutive promoter, pst51 showed much greater activity towards DCA and LCA than the non-optimized variant (Fig. 4C), reaching similar sulfonation values for both secondary bile acids.

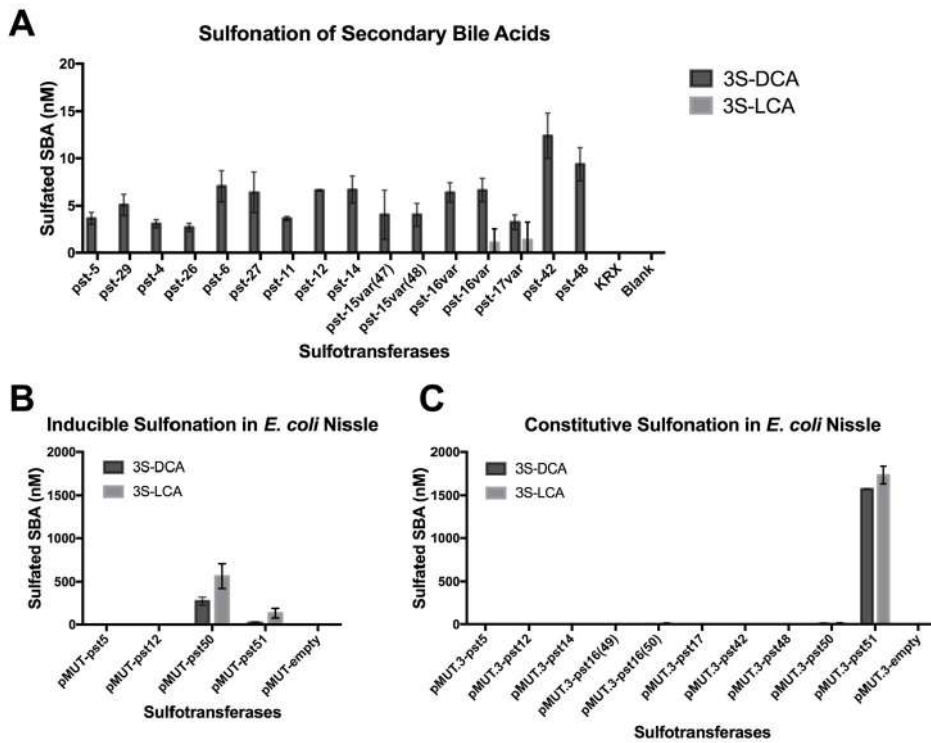


Figure 4. A) Shows sulfonation of secondary bile acids, DCA and LCA, by *E. coli* KRX expressing a panel of sulfotransferases. B & C) Shows sulfonation of *E. coli* Nissle 1917 expressing different sulfotransferases in an inducible and constitutive approach, respectively.

Lastly, improved sulfate uptake capabilities were tested in order to assess whether the native uptake system was a limitation for optimal secondary bile acid sulfonation. Integration of a low-mid strength Anderson promoter (BBa\_J23110) upstream of the *cysPUWA* operon, which forms part of *E. coli*'s native sulfate/thiosulfate uptake machinery<sup>[71]</sup>, was introduced. Another approach tested consisted of integrating the *cysP* gene from *B. subtilis*, which is part of the inorganic phosphate transporter family and that has been shown to restore sulfate starvation<sup>[72]</sup>. When tested together with the constitutive expression of *pst51* and *cysDNCQ*, only CysP from *B. subtilis* seemed to improve significantly sulfonation of DCA (Fig. 5A). LCA, on the other hand, showed no significant improvements (Fig. 5B).

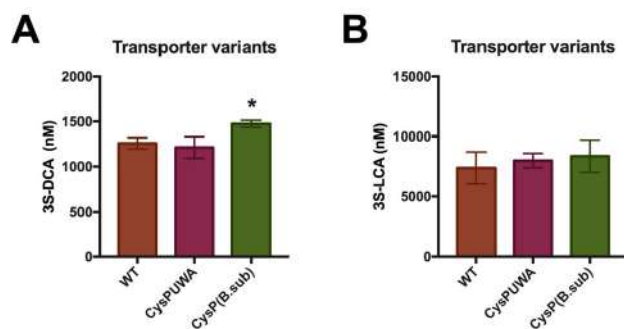


Figure 5. Sulfonation capacity of transporter variants integrated in the genome of *E. coli* Nissle 1917 harboring pMUT.3-pst51. Strains were culture in biological triplicates in M9 media supplemented with 100  $\mu$ M DCA (A) or 50  $\mu$ M LCA (B) and 0.2% Casamino acids. One-way ANOVA (Dunnett's multiple comparison test) with 95% CI was used to determine significance,  $P < 0.05$ .

Table 2. Sulfotransferases shown to sulfonate secondary bile acids DCA and LCA.

Sulfotransferase ID	Source	Enzyme	Bile acid sulfonated
pst-5	<i>R. norvegicus</i>	SULT1A1	DCA
pst-29	<i>D. rerio</i>	SULT1ST1	DCA
pst-4	<i>H. sapiens</i>	SULT1A1	DCA
pst-26	<i>R. norvegicus</i>	SULT1A1	DCA
pst-6	<i>D. melanogaster</i>	dmST1	DCA
pst-27	<i>D. Melanogaster</i>	dmST1 - clone2	DCA
pst-11	<i>E. caballus</i>	SULT1A1	DCA
pst-12	<i>G. gallus domesticus</i>	SULT1E1	DCA
pst-14	<i>S. scrofa domesticus</i>	SULT1A1	DCA
pst-16var (CBJ1149)	<i>G. gallus domesticus</i>	SULT1B1-predicted - clone1	DCA
pst-16var (CBJ1150)	<i>G. gallus domesticus</i>	SULT1B1-predicted - clone2	DCA, LCA
pst-17var (CBJ1151)	<i>G. gallus domesticus</i>	SULT1C1	DCA, LCA
pst-42	<i>Zostera marina</i>	KMZ74024.1	DCA
pst-48	<i>Zostera marina</i>	KMZ73756.1	DCA
pst-50	<i>H. sapiens</i>	SULT2A1	DCA, LCA
pst-51	<i>H. sapiens</i>	SULT2A1 (codon-opt)	DCA, LCA

#### Identification of metabolic engineering targets for improved sulfonation in *E. coli*

In order to identify metabolic engineering targets for increasing sulfonation capabilities in *E. coli*, a plasmid expressing only pst50, SULT2A1 non-codon optimized, was transformed into several KEIO strains. These strains had KO for known and putative sulfatases, and sulfatase maturing enzymes (Table 3). One-way ANOVA analysis showed that KO of *ydeN*, *ydeM*, *aslB* and *hdhA* increased significantly the sulfonation of DCA, none of the KOs showed to decrease sulfonation, compared to

a *E. coli* MG1655 WT control (Fig. 6A). On the other hand, LCA sulfonation seemed to be mostly decreased by the presence of the KOs, with the exception of *yjcS* (Fig. 6B), transforming the data to log values decreased variability and evidenced a significant decrease in LCA sulfonation by *yidJ* and *ydeM* KO strains. An aspect to take into consideration is that MG1655 is not the background strain of the KEIO collection, having few genetic difference.

Table 3. Genes of interest for further metabolic engineering

Gene	Function
<i>yjcS</i>	SDS sulfatase
<i>aslA</i>	putative Ser-type sulfatase
<i>ydeN</i>	putative Ser-type sulfatase
<i>yidJ</i>	YidJ is a putative Cys-type sulfatase
<i>ydeM</i>	member of the anaerobic sulfatase maturation enzyme subfamily of the Radical SAM superfamily of enzymes
<i>aslB</i>	AslB is a member of the anaerobic sulfatase maturation enzyme subfamily of the Radical SAM superfamily of enzymes
<i>hdhA</i>	7- $\alpha$ -hydroxysteroid dehydrogenase catalyzes the dehydroxylation of cholic and chenodeoxycholic acids

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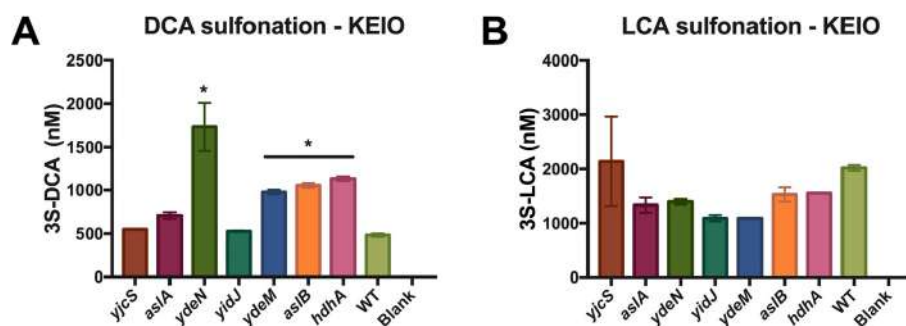


Figure 6. Identification of metabolic engineering targets for optimization of sulfonation capabilities in DCA (A) and LCA (B), by using KEIO strains. Strains were cultured in biological triplicates in M9 media supplemented with either 100  $\mu$ M DCA or 50  $\mu$ M LCA. One-way ANOVA (Dunnnett's multiple comparison test) against WT control, with 95% CI was used to determine significance,  $P < 0.05$ .

## Discussion

Functional metagenomic library screening as an approach for the identification of DCA degrading enzymes

Even though functional metagenomic screenings have been widely used for the identification of novel genes and mechanisms<sup>[1]</sup>, this approach may not be the best for the identification of catabolic genes involved in bile acid breakdown. Known metabolic pathways for the degradation of steroid compounds have been catalogued to be lengthy and complex<sup>[2]</sup>. This makes it very unlikely to find steroid catabolic genes, screening through genomic libraries containing fragment sizes of 1.5 – 2 kb. Nevertheless, candidate 2.1 seem to reduce DCA levels, with a yet to be understood mechanisms.

The protein encoded in this ORF is relatively small (74 aa), conserved among Firmicutes and is predicted to interact with DNA<sup>[84]</sup>. Intracellular presence of DCA has been reported to activate SOS response and cause DNA damage<sup>[4,85]</sup>. Even though the role of this protein with regards to degradation of DCA is elusive, it may have a role protecting DNA from bile acids. It is likely that remaining candidate ORFs are increasing tolerance of *E. coli* towards deoxycholic acid by mediating resistance mechanisms related to osmolarity and oxidative stress, instead of a direct interaction towards DCA.

Mutations leading to feedback resistance variants of *proAB*, which increase proline biosynthesis, have been shown to be osmoprotective<sup>[3]</sup>. Increased concentrations of proline in the cytosol, restores the osmotic differential between the extracellular and intracellular space, conferring resistance to adverse osmotic conditions. Bile acids are known for their biosurfactant activity, affecting the proteins and phospholipids of the cell membrane, disrupting cellular homeostasis that could lead to leakage of the cytosolic components<sup>[4]</sup>. In addition, the presence of bile acids intracellularly can lead to low pH stress on cells affecting ion transport, and extracellular presence of these molecules are able to chelate calcium and iron, resulting in low bioavailability of these ions and osmotic effects<sup>[5,6]</sup>. Overexpression of *proA* and *proB* (candidates 2.2 and 2.3), a similar effect compared to the feedback inhibition mutants could be taking place in order to increase osmolarity resistance by the overproduction of proline. The mechanism of candidate 2.1, giving raise to DCA resistance still remains unclear as these family of proteins are of unknown function. However, it is possible that its role is linked to osmotic regulation, as it was present in the same contig were *proA* and *proB* were identified. Additionally, Candidate 1 (*ispG*) is a member of the non-mevalonate pathway or MEP pathway, catalyzing the conversion of 2C-methyl-D-erythritol 2,4-cyclodiphosphate into 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate and responsible for production of the building blocks for isoprenoid biosynthesis. In *B. subtilis*, isoprene biosynthesis is increased under heat, salt and oxidative stress conditions<sup>[7]</sup>, likely enabling resistance working as membrane stabilizing agent and/or antioxidant<sup>[9]</sup>.

Bile acids may also cause oxidative stress by altering the membrane permeability. In a previous mentioned study<sup>[4]</sup>, three genes of *Propionibacterium freudenreichii*: cysteine synthase, oxidoreductase and superoxide dismutase were identified to contribute in oxidative damage remediation. Another study<sup>[9]</sup>, identified an atypical flavin reductase with oxidative stress protection capacity from *Paracoccus denitrificans*, which was upregulated under induced oxidative stress conditions. On the same note, our identified NAD(P)H dependent FMN reductase (A4V08\_14035) might be having a similar role protecting the cell from the oxidative stress caused by DCA. It is known that oxidation of NADH and FADH<sub>2</sub> back to NAD<sup>+</sup> and FAD, in related to the conservation of energy through the electron transport chain and ATP generation<sup>[10]</sup>. In that sense, this mechanism deals with the possible energetic distress caused by bile acids and could use reductases as a way to counter act cellular energy depletion.

Even though some ORFs were demonstrated to confer resistance against DCA, none of them were able to interact directly with DCA, accentuating for the diversity of mechanisms microorganisms have developed in order to withstand bile acid stress. A possible explanation behind the reason why several ORFs were not shown to confer any resistance even though they showed up in the functional metagenomic screen could be explained by the extraction of the specific ORF from its native context. Neighboring regions to the gene are known to possess important regulatory roles allowing appropriate expression of the genes<sup>[11]</sup>, by removing these vital regulatory domains expression patterns might have altered prompting mutations or lesions to withhold the given gene product.

#### Isolating natural DCA degraders from the human gut

Complete degradation of steroid molecules have been reported to take place in members belonging to the phyla Actinobacteria<sup>[13]</sup> and Proteobacteria<sup>[14]</sup>. Aerobic degradation of steroids is oxygenase mediated, requiring molecular oxygen to activate and initiate the side chain and sterane body catabolism<sup>[2]</sup>. On the other hand anaerobic degradation of steroid structures is mediated by hydratase/dehydrogenase reactions using H<sub>2</sub>O as the oxygen donor and a molybdenum cofactor<sup>[15]</sup>. The oxygen independent pathway for steroid degradation has been studied in detail in only two species of bacteria, *Steroidobacter denitrificans*<sup>[16,17]</sup> (Gammaproteobacteria) and *Sterolibacterium denitrificans*<sup>[15,18]</sup> (Betaproteobacteria), having outlined important catabolic steps of the pathway. Taking into account the preliminary results presented in this study, anaerobic steroid degradation could be wider spread than previously thought. The gut microbiota represents a rich source of diversity of microbial species, that has not been fully exploited due to limitations in culturing capabilities<sup>[19]</sup>. Additionally, these species interact continuously with dietary molecules, including steroidal compounds such as bile acids, hormones and cholesterol under varying conditions. Nonetheless, further research is necessary to elucidate the mechanisms by which these bacteria are breaking down deoxycholic acid and whether this has meaningful implications in health or disease.

#### Bacterial sulfonation of secondary bile acids

A quite diverse number of sulfotransferases (SULT)s were identified to have sulfonating capabilities towards secondary bile acids DCA and to a lesser extent LCA, possibly due to the difference in hydrophobicity of the molecules. Nonetheless, proving for the first time bacterial sulfonating action towards bile acids in an *in vitro* set up. It still remains unclear which structural features allow these enzymes to have activity towards secondary bile acids, thus comparing the active sites could give important clues to which amino acid residues play a key role on determining the given specificity. Sulfonating action from sulfotransferases extracted from *D. melanogaster* and *Z. marina*, both being non bile acid producing organisms and having low possibility of encountering substantial amounts of bile acids, supports the theory that these enzymes could act on a variety of substrates, therefore having some degree of promiscuity. All sulfated bile acids identified, were sulfated only in position C-3 of in both DCA and LCA. Nevertheless, future mining and testing of other sulfotransferase could enable sulfonation of bile acids in different positions, for instance, hagfish of the class Myxini and sea lampreys are known to sulfate bile alcohols in 2 positions<sup>[20]</sup>. Additionally, nature represents an interesting and rich source of sulfotransferases, as organisms also sulfate diverse compounds for defense mechanisms<sup>[22]</sup>, especially in marine environments, where sulfate salt accounts for third most abundant element in seawater<sup>[21]</sup>. Interestingly, natural sulfated products from marine species have been shown to have a variety of therapeutic uses<sup>[23]</sup>, making these metabolites attractive targets for future AMTs designs.

#### AMT driven secondary bile acid sulfonation and its prospective

In vertebrates sulfonation represents an inactivation/detoxification route by which a sulfuryl group from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) is transferred to undesired compounds, that could be harmful or induce adverse effects<sup>[24]</sup>. By performing this action, SULTs increase their substrate solubility and channel them towards excretory pathways<sup>[25]</sup>. Therefore, sulfonation of SBAs contributes to bile acid homeostasis under pathological conditions, allowing excretion of surplus concentrations<sup>[26]</sup>. Having successfully transferred SBA sulfonating capabilities to a probiotic strain (*E. coli* Nissle 1917), could lead to the development of novel therapeutic strategies for neutralizing or diminishing SBA pool build up, produced by known 7 $\alpha$ -dehydroxylating bacterial species<sup>[27]</sup>. This action could have important repercussions for a range of diseases influenced or triggered by increased pools of secondary bile acids such as, intestinal<sup>[28]</sup> and liver<sup>[29-31]</sup> carcinogenesis, obesity<sup>[29,30]</sup>, among



others. In fact, a recent study<sup>[32]</sup> highlighted the importance of converting administered DCA into a less hydrophobic version (isoDCA) by epimerizing the C-3 hydroxyl group by a synthetic bacterial consortia, promoting the generation of peripheral Treg cells and an overall anti-inflammatory state, which could have great implications for treating inflammatory diseases.

An ideal advanced microbial therapeutic with sulfonation capabilities could perform similar actions, decreasing harmful concentrations of DCA and LCA, neutralizing inflammatory effects and protecting peripheral organs from induced tumorigenesis. Furthermore, this strategy might also be advantageous for treating non-alcoholic fatty liver disease (NAFLD), as studies have shown that patients have elevated concentrations of secondary bile acids<sup>[33]</sup>. These elevated levels of SBAs are thought to increase inflammation<sup>[64]</sup> in the liver and promote steatosis, worsening the disease, eventually causing fibrosis and cirrhosis<sup>[34]</sup>. Since sulfonation is a broad strategy to deal with several toxic compounds, an AMT with these capabilities could easily be designed for dealing with xenobiotics of interest that pose a health risk<sup>[35,36]</sup>, by changing the sulfotransferase expressed. In this way, these molecules could be neutralized before being absorbed by enterocytes and entering peripheral circulation. Translocating sulfonation capabilities to the intestine would reduce the load on hepatic demand and limit its exposure to damaging molecules.

It is well documented that bile acids are potent agonist for relevant receptors, modulating important physiological features<sup>[37]</sup>. Even though sulfonation is thought to reduce bioactivity, it is possibly that this modification enables novel signaling signatures of sulfated bile acids peripherally and in the gut environment, making this a tremendous target for uncovering unknown physiological implications of this detoxification mechanism.

#### Sulfonation differences among *E. coli* strains and effect of sulfate importers

Differences seen in sulfonation between KRX and Nissle 1917 strains could be attributed to different characteristics of the strains. *E. coli* Nissle 1917 was isolated from a human sample and since then it has been used as a probiotic strain, without suffering any artificial modification<sup>[38]</sup>. On the other hand, *E. coli* KRX has been modified to be efficient with regards to plasmid transformation and propagation, and protein expression<sup>[39]</sup>. These modifications enable the KRX strain to withstand the pressure exerted by maintaining the plasmid and the expression of the different components. On the counter part, Nissle 1917 could be more sensitive to this pressures and might opt to mutate, recombine or modify components of the plasmid that hinder optimal growth or causes a significant fitness cost, this has been thoroughly evaluated in industrial setups<sup>[40]</sup>.

Contrary differences in sulfonation capacity were witnessed between pst50 and pst51 under different expression systems used (inducible vs. constitutive), being the non-optimized version better in the inducible setup and the optimized version better in the constitutive setup. Codon usage has been reported as the factor of most importance in prokaryotic gene expression<sup>[41]</sup>. Consequently, T7 polymerase driven expression of the codon optimized version could be too strong for *E. coli* Nissle, reducing fitness and triggering rearrangements in the plasmid that might result in poor levels of sulfonation. On the other hand, under a constitutive expression system a non-codon optimized gene might be the source of fitness cost, from the time it is first incorporated in the cell, as it is continuously draining rare codons, affecting translation of other necessary proteins.

Limiting concentrations of dissolved sulfate or its bioavailability could also affect sulfonation of molecules. By incorporating a sulfate importer from *B. subtilis* (CysP), DCA sulfonation was enhanced, however, not to the extent of LCA. This could be a result of the high reported specific activity of the enzyme towards LCA<sup>[42]</sup>. Interestingly, this importer did not increase the sulfonation

of LCA, presumably because the production has reached a bottleneck by some other factor. Another study demonstrated that improving supply of sulfate leads to increased production of zosteric acid<sup>[43]</sup>. However, this might not be the case, as expression of additional sulfate importers did not increase production. Media composition could be playing a role in limiting sulfonation, as no extra sulfate was added when performing the experiments, this may explain why there seems to be an upper limit in production of 3S-LCA. Same phenomenon was observed when metabolic engineering targets were identified, demonstrating it is most likely not an artifact.

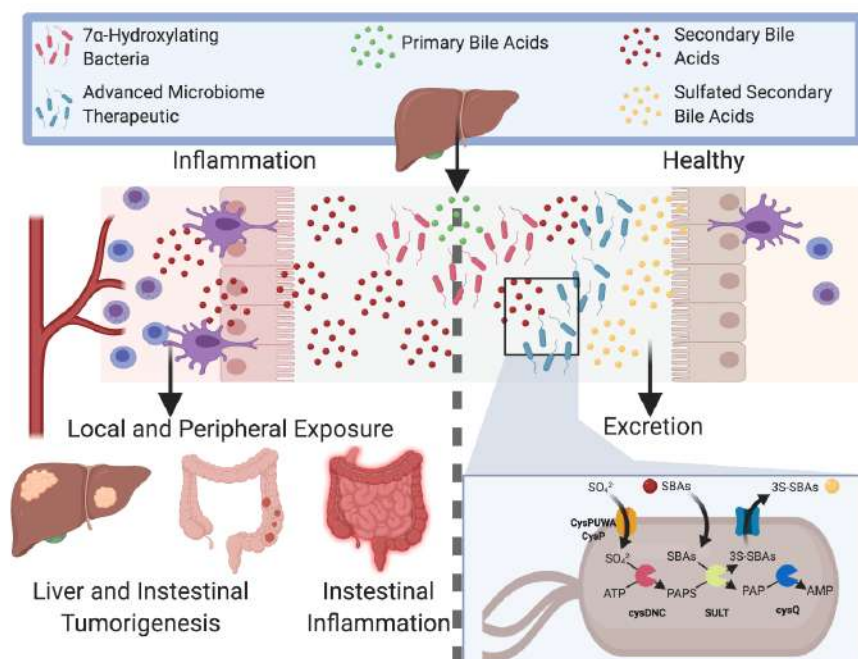


Figure 10. Delineation of the inflammatory and carcinogenic effect of secondary bile acids and treatment with an advanced microbiome therapeutic for sulfonating secondary bile acids. Primary bile acids are secreted into the intestine. After deconjugation in the small intestine, primary bile acids are further metabolized into secondary bile acids. Under pathological conditions these secondary bile acids accumulate, causing inflammation and cancer. When an AMT for secondary bile acid sulfonation is used, SCBs are sulfated, resulting in reduced hydrophobicity. This enables SBAs to be easily excreted without causing pathological outcomes.

#### Metabolic engineering for improved sulfonation

Sulfonation performance in KEIO strains clearly show a positive effect of knocking out sulfatase maturation units (*ydeM* and *aslB*) and *ydeN*, a gene encoding for a putative sulfatase. In contrast to a previous study<sup>[44]</sup> that considered *E. coli* to be deprived from active sulfatases, here we show (indirectly) possible native sulfatase activity on 3S-DCA by YdeN, a putative Ser-type sulfatase<sup>[45]</sup>. Moreover, anaerobic sulfatase maturing enzymes (YdeM and AslB)<sup>[46]</sup> seem to also play a role on aerobic sulfatase capabilities. However, it remains unclear whether these maturing enzymes are

acting on YdeN or other unidentified sulfatases, and if this is mediated by oxygen levels. Other efforts for increasing sulfonation in *E. coli* have focused in increasing sulfate<sup>[43]</sup> and the coenzyme PAPS<sup>[50]</sup>, however, to our knowledge, this is the first attempt to evaluate whether sulfatases and sulfatase maturing enzymes could be used as targets for metabolic engineering. Another interesting knock-out target that increases sulfonation of DCA is *hdhA*. It catalyzes the dehydroxylation of both cholic acid and chenodeoxycholic acid in position C-7<sup>[47]</sup>, producing 7-oxocholic and 7-oxochenodeoxycholic acids, respectively. Presumably, this modified structure could be decreasing the affinity of the bile acid towards the sulfotransferase, revealing that there may be less specific activity of the enzyme towards these oxo variants. These findings represent the first report of increased production of sulfated compounds via modification of native sulfatases and sulfatase maturing enzymes. Nonetheless, further omics approaches could be highly beneficial for identifying other more complex bottlenecks for increasing sulfated bile acid production.

### Conclusion and outlook

Overall, three different strategies were employed for identifying approaches for secondary bile acid neutralization or degradation. Employing functional metagenomics screenings have been a popular strategy for identifying novel genes. Even though, for the present study it proved to be unsuccessful, it widened our understanding of the mechanisms used by bacterial species for dealing with different stress responses caused by bile acids. This insights, could later be used for the implementation of robust mechanisms to withstand bile acid stress in a future AMT.

Several bacterial species were isolated with preliminary great potential for degradation of DCA. It still remains unclear how these microbes are catabolizing this molecules and whether this is a widespread functionality of the gut microbiota. Nevertheless, confirmation of this capability is still necessary, as only one biological replicate was used for most of the experiments due to space limitations in the anaerobic environment. If proven to be true, this would be the first report of bacterial strains isolated from the gut environment capable of utilizing bile acids as the sole carbon source. This would reinforce the importance that the gut microbiome has on regulating health and disease, as bile acids are important molecules with transcendence at many different levels in human physiology.

Finally, we report for the first time bacterial sulfonation of secondary bile acids. This newly entrusted capability could revolutionize the development of future AMTs, switching the focus from degradation to neutralization of toxic compounds. This feature could make engineering of AMTs that target the removal of toxic compounds more feasible, as once an optimal chassis is developed, the sulfotransferase giving the specificity could be easily replaced for another one that has greater specificity towards the molecule of interest. Even though great advantages exists for using this approach, there are future challenges that would need to be addressed for properly applying this technology *in vivo*. Sulfotransferases are able to sulfate a range of molecules, this implies that stringent enzymatic engineering would be needed for having specific sulfonation and reduced off target activity, that could lead to activation of carcinogenic xenobiotics<sup>[80]</sup>. Furthermore, members of the gut microbiota has been reported to be able to hydrolyze sulfated compounds for sulfate scavenging and are often related in inflammatory aspects of diseases<sup>[81,82]</sup>. This characteristic would affect the envisioned AMT directly, as it would be competing for sulfate and hydrolyzing the sulfated compound produced. Nonetheless, strategies employing bacteriocins against these species could be proven beneficial. On the other hand, understanding better the dynamics of sulfate metabolism in the gut could aid in the design of future AMTs, insights regarding sulfate scavenging mechanisms could be transferred to desired AMTs or with the appropriate tools suitable members of the gut microbiota

could be repurposed for AMT applications. Lastly, as in any natural environment, fierce competition takes place in the gut, most likely the developed AMT would need to compete with other microbial species for resources and establishment in the intestine.

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## Supplemental materials and methods

### Nanopore sequencing processing

Resulting fast5 files were base-called with Albacore (Oxford Nanopore Technologies) using the following command:

```
>read_fast5_basecaller.py --flowcell FLO-MIN106 --kit SQK-LSK108 --output_format fast5,fastq --input directory_of_input_fast5_files --save_path directory_for_outputfiles --worker_threads number_of_worker_threads
```

After filtering out reads that failed the Albacore quality control, adapter sequences were trimmed off the passed reads using Porechop (<https://github.com/rrwick/Porechop>) with the following command:

```
>porechop -i directory_of_passed_input_fastq_files -o save_path directory_for_output_files -t number_of_worker_threads
```

For Porechop demultiplexing of barcoded samples the following command was used:

```
>porechop -i directory_of_passed_input_fastq_files -b save_path directory_for_output_files
```

Canu (<https://github.com/marbl/canu>) was used for draft genome assembly using the following command:

```
>canu -p prefix_of_created_files -d save_path directory_for_output_files genomeSize=4434543 -nanopore-raw / directory_of_input_fastq.gz_files batThreads=number_of_worker_threads
```

Finally, draft genome annotation was performed using PROKKA (<https://github.com/tseemann/prokka>) with the following command:

```
> prokka --outdir 'directory_for_output_files' --prefix prefix_of_fa_files 'directory_of_input_files'
```

Albacore basecalling produced 48.75 GB passed reads and 8.96 GB failed reads.

Porechop produced 12 Mb, 10Mb, 9Mb, 10Mb, 8Mb, 9Mb, 11Mb, 7Mb, 6Mb, 9Mb, 10Mb, 10Mb, 7Mb, for barcodes, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, respectively.

### Supplemental tables

Supplemental table 1. Origin of the different metagenomic libraries.

Library ID	Origin
H1	Human feces
H2	Human feces
H3	Human feces
H4	Human feces
H5	Human feces
Pig	Pig feces
Cow	Cow feces
Soil	Soil

Supplemental table 2. Approximate CFU count obtained of the different metagenomic libraries screened.

75 $\mu$ M DCA	CFU (approx.)
H1	84
H2	180
H3	2440
H4	340
H5	100
PIG	0
COW	0
MARI (SOIL)	0

Supplemental table 3. Conditions used for the isolation of natural degraders of DCA

No	Condition	DCA (g/L)
1	Aerobic	0.5
2	Aerobic	1
3	Anaerobic	0.5
4	Anaerobic	1

Supplemental table 4. Strain according to 16S sequencing from isolates of 0.5 g/L DCA.

Isolate	Specie
1-1	<i>Escherichia coli</i> strain MS7163
1-4	<i>Escherichia coli</i> strain MS7163
2-2	<i>Cronobacter sakazakii</i> strain M.D.E.NA4-2
2-3	<i>Escherichia coli</i> strain TU-6
2-4	Bacterium YC-LK-LKJ9
3-2	Uncultured bacterium clone contig55882
4-3	Uncultured <i>Klebsiella sp.</i> clone F5mar.28

Supplemental table 5. Strain according to 16S sequencing from isolates of 0.5 g/L DCA.

Isolate	Specie
---------	--------

2-4	<i>Escherichia coli</i> strain HCD22-1
4-4	<i>Escherichia coli</i> strain NRC76
6-1	<i>Escherichia fergusonii</i> strain CGS24
6-2	<i>Shigella flexneri</i> strain 74-1170

Supplemental table 6. Sulfotransferases tested

Sulfotransferase ID	Source	Enzyme	Reference
pst-5	<i>R. norvegicus</i>	SULT1A1	43
pst-29	<i>D. rerio</i>	SULT1ST1	43
pst-31	<i>D. rerio</i>	SULT6B1	43
pst-4	<i>H. sapiens</i>	SULT1A1	43
pst-26	<i>R. norvegicus</i>	SULT1A1 – clone 2	43
pst-6	<i>D. melanogaster</i>	dmST1	43, 73
pst-27	<i>D. Melanogaster</i>	dmST1 - clone2	43, 73
pst-8	<i>D. melanogaster</i>	dmST3 - variant A	43, 73
pst-10	<i>D. melanogaster</i>	dmST4	43, 73
pst-11	<i>E. caballus</i>	SULT1A1	43
pst-12	<i>G. gallus domesticus</i>	SULT1E1	43
pst-13	<i>C. lupus familiaris</i>	SULT1A1	43
pst-14	<i>S. scrofa domesticus</i>	SULT1A1	43
pst-15var (CBJ1146)	<i>G. gallus domesticus</i>	SULT1B1 - clone1	43
pst-15var (CBJ1147)	<i>G. gallus domesticus</i>	SULT1B1 - clone2	43
pst-15var (CBJ1148)	<i>G. gallus domesticus</i>	SULT1B1 - clone3	43
pst-16var (CBJ1149)	<i>G. gallus domesticus</i>	SULT1B1-predicted -clone1	43
pst-16var (CBJ1150)	<i>G. gallus domesticus</i>	SULT1B1-predicted -clone2	43
pst-17var (CBJ1151)	<i>G. gallus domesticus</i>	SULT1C1	43
pst-18var (CBJ1152)	<i>G. gallus domesticus</i>	SULT2B1-variant1	43
pst-18var (CBJ1153)	<i>G. gallus domesticus</i>	SULT2B1-predicted	43
pst-18var (CBJ1154)	<i>G. gallus domesticus</i>	SULT2B1-variant2	43
pst-25	<i>R. norvegicus</i>	SULT1A1 (codon-opt)	43
pst-28	<i>C. elegans</i>	SSU-1	43
pst-30	<i>D. rerio</i>	SULT4A1	43
pst-32	<i>A. Thaliana</i>	AtSOT12	43
pst-33	<i>Streptomyces</i>	Cpz8	43
pst-34	<i>Streptomyces</i>	LipE	43
pst-35	<i>S. punctatus DAOM BR117</i>	SPPG_07427	43
pst-36	<i>H. ochraceum DSM 14365</i>	Hoch_6098	43
pst-37	<i>R. radiotolerans</i>	RradSPS_0172	43
pst-38	<i>Zostera marina</i>	KMZ76263.1	43
pst-39	<i>Zostera marina</i>	KMZ64288.1	43
pst-40	<i>Zostera marina</i>	KMZ76264.1	43

pst-41	<i>Zostera marina</i>	KMZ59959.1	43
pst-42	<i>Zostera marina</i>	KMZ74024.1	43
pst-43	<i>Zostera marina</i>	KMZ64284.1	43
pst-44	<i>Zostera marina</i>	KMZ72298.1	43
pst-45	<i>Zostera marina</i>	KMZ72292.1	43
pst-46	<i>Zostera marina</i>	KMZ72296.1	43
pst-47	<i>Zostera marina</i>	KMZ76265.1	43
pst-48	<i>Zostera marina</i>	KMZ73756.1	43
pst-49	<i>Zostera marina</i>	KMZ69186.1	43
pst-50	<i>H. sapiens</i>	SULT2A1	This study
pst-51	<i>H. sapiens</i>	SULT2A1 (codon-opt)	This study

Supplemental table 7. M9 media composition

Stock Solution/ Compounds	Per 1 Liter	Comments
MQ H <sub>2</sub> O	Up to 1 L	Autoclaved
20% (w/v) glucose	20 ml for 0.4%	Filter sterilized
10X M9 salts	100 ml	House stock
2M MgSO <sub>4</sub>	1 ml	House stock
Trace elements	500 µl	Sup. Table 4
Wolfe's vitamin	1 ml	Reference 87
1M CaCl <sub>2</sub>	100 µl	House stock

Supplemental table 8. Trace element solution

Compound	Amount per 1 L
Disodium EDTA	15 g
ZnSO <sub>4</sub> 7H <sub>2</sub> O	4.5 g
MnCl <sub>2</sub> 4H <sub>2</sub> O	0.7 g
CoCl <sub>2</sub> 6H <sub>2</sub> O	0.3 g
CuSO <sub>4</sub> 2H <sub>2</sub> O	0.2 g
Na <sub>2</sub> MoO <sub>4</sub> 2H <sub>2</sub> O	0.4 g
CaCl <sub>2</sub> 2H <sub>2</sub> O	4.5 g
FeSO <sub>4</sub> 7H <sub>2</sub> O	3 g
H <sub>3</sub> BO <sub>3</sub>	1 g
KI	0.1 g

Supplemental table 9. Function, number of genes observed and percentage from the functional metagenomic screening on 0.031 g/L of DCA.

Function	Number of genes	Percentage
----------	-----------------	------------

Amino_acid_transport_and_metabolism	1170	1.31
Carbohydrate_transport_and_metabolism	3940	4.42
Cell_cycle_control,_cell_division,_chromosome_partitioning	939	1.05
Cell_motility	438	0.49
Cell_wall/membrane/envelope_biogenesis	14019	15.73
Coenzyme_transport_and_metabolism	830	0.93
Defense_mechanisms	5662	6.35
Energy_production_and_conversion	3517	3.95
Function_unknown	2420	2.72
General_function_prediction_only	1986	2.23
Inorganic_ion_transport_and_metabolism	3030	3.4
Intracellular_trafficking,_secretion,_and_vesicular_transport	1154	1.29
Lipid_transport_and_metabolism	512	0.57
Mobilome:_prophages,_transposons	1715	1.92
Nucleotide_transport_and_metabolism	652	0.73
Posttranslational_modification,_protein_turnover,_chaperones	1682	1.89
Replication,_recombination_and_repair	2701	3.03
Secondary_metabolites_biosynthesis,_transport_and_catabolism	150	0.17
Signal_transduction_mechanisms	1400	1.57
Transcription	888	1
Translation,_ribosomal_structure_and_biogenesis	3062	3.44
Others	37250	41.8

Supplemental table 10. TolC from other genera compatible with *E. coli*.

<b>Genera</b>	<b>Percentage</b>
<i>Parabacteroides</i>	48.08
<i>Bacteroides</i>	35.82
<i>Serratia</i>	8.8
<i>Stenotrophomonas</i>	3.35
<i>Alistipes</i>	2.67
<i>Rahnella</i>	1.25

Supplemental figures

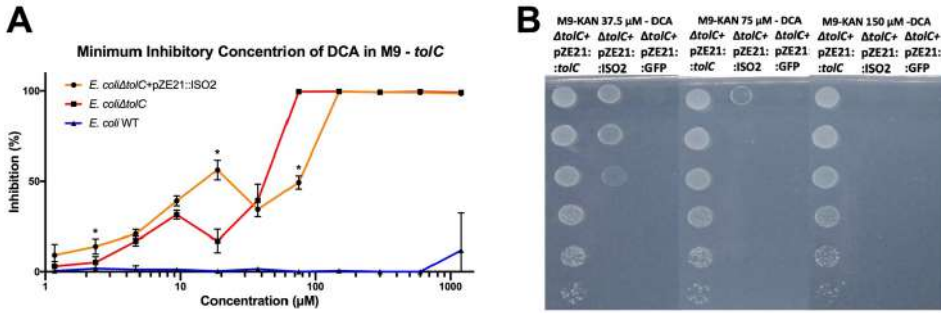
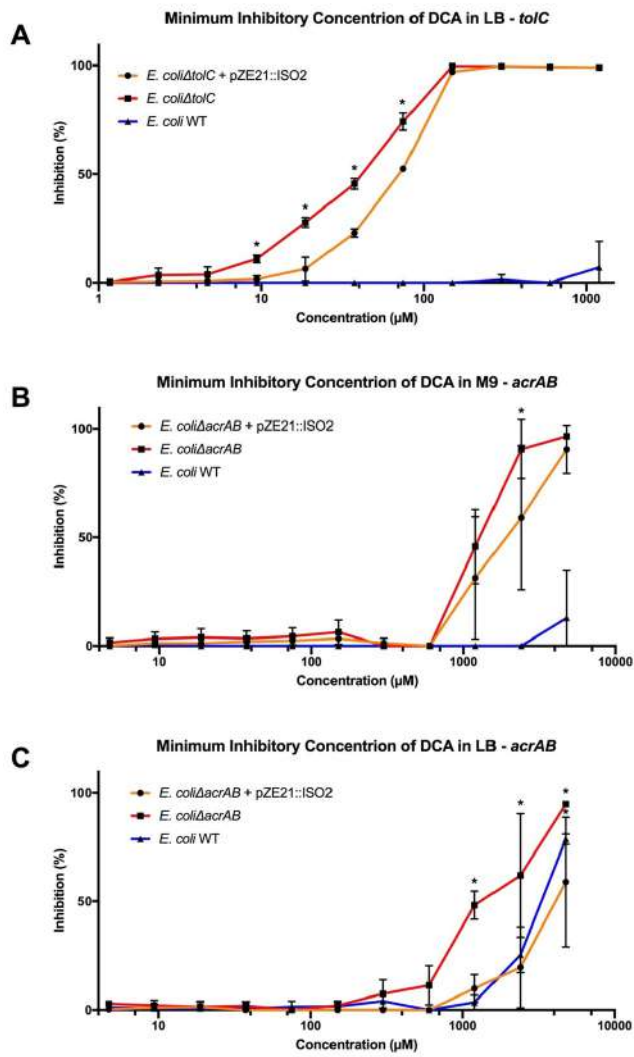
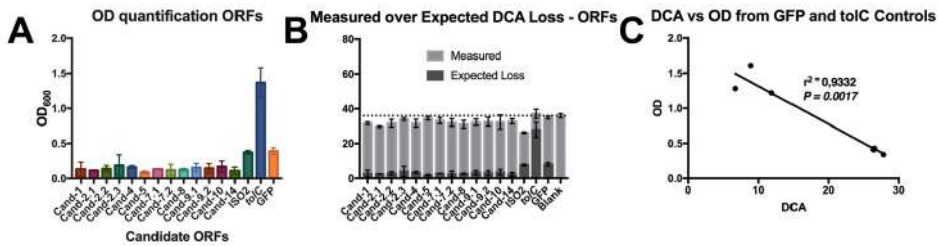


Figure 1. Identification of optimal parameters for DCA functional metagenomic screening in M9 liquid media (A) and in M9 solid media (B). Each data point represents the mean of biological triplicates, error bars show standard deviation. Two-way ANOVA with 95% CI was used to determine significance at  $P < 0.05$ .

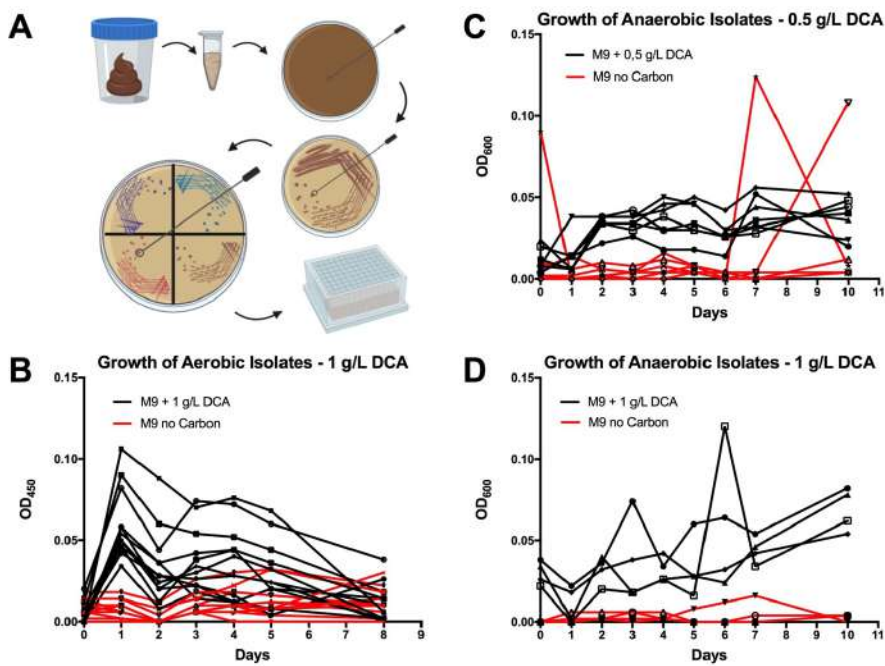


Supplemental figure 2. DCA minimum inhibitory concentration tests of: A) *toIC* KO on LB, B) *acrAB* KO in M9 and C) *acrAB* KO in LB.

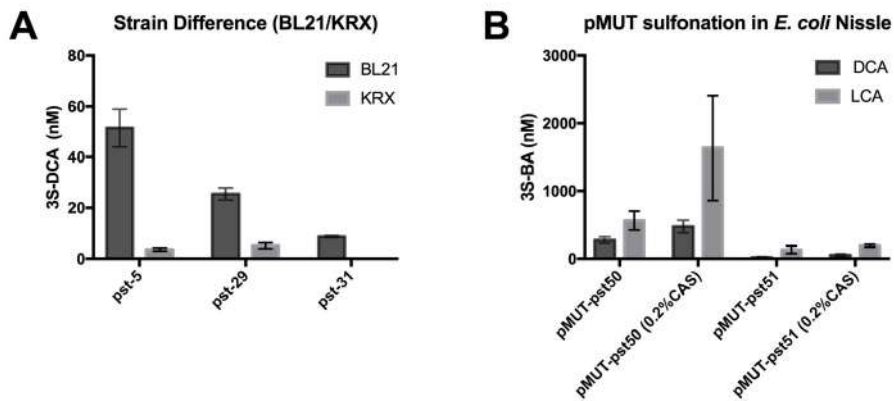




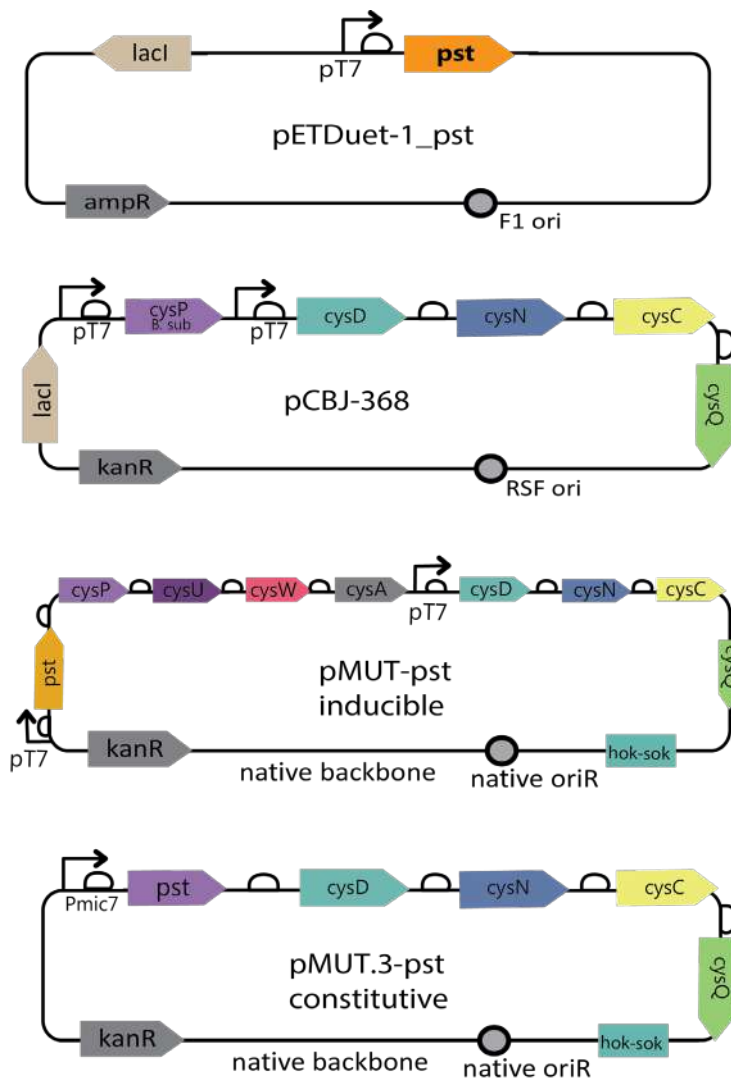
Supplemental figure 3. A) OD<sub>600</sub> measurements of *tolC* KO *E. coli* harboring candidates ORFs after 48 hours of cultivation in M9 media supplemented with 37.5 μM of DCA. B) Stacked values of measured DCA and expected loss of DCA due to biomass formation. C) Pearson's correlation test of OD vs DCA concentration from GFP and TolC expressing controls (*tolC* KO background).



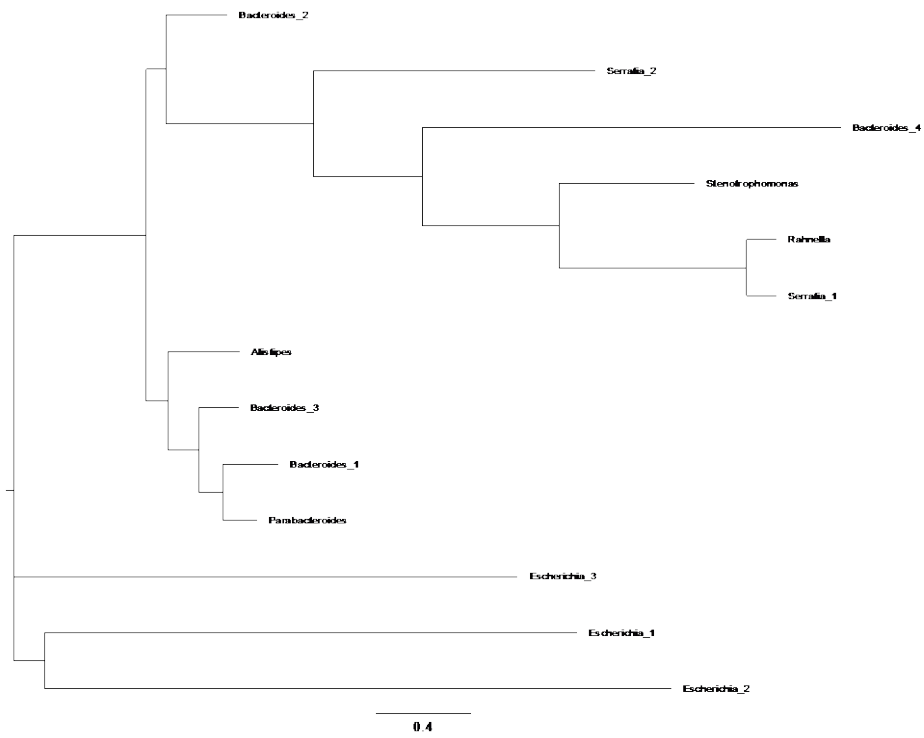
Supplemental figure 4. A) Shows an overview of the culturing method for the isolation of DCA degrading strains. B-C) Shows initial growth experiments of strains inoculated in M9 with complemented with DCA as a carbon source (black lines) or control with no carbon source added (red lines).



Supplemental figure 5. A) Sulfonation of DCA by *E. coli* BL21 or KRX expressing 3 different sulfotransferases. B) Sulfonation of DCA and LCA by *E. coli* Nissle expressing human sulfotransferase SULT2A1 (pst50) and its codon optimized version (pst51) in M9 media and M9 media supplemented with 0.2% Cas amino acids.



Supplemental figure 6. Overview of plasmids used for sulfonation of secondary bile acids.



Supplemental figure 7. Phylogenetic tree of the *tolC* genes obtained from the functional metagenomic screening and 3 *E. coli* reference *tolC* genes. Tree was generated by *fasttree*<sup>[88]</sup> and visualized using *figtree* (<http://tree.bio.ed.ac.uk/software/figtree/>).

#### IV. Engineering Advanced Microbiome Therapeutics for Production and Delivery of L-DOPA and Dopamine

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##### Abstract

Neurotransmitters play a fundamental role transducing signals among neurons and other effector cells. Catecholamines, neurotransmitters derived from tyrosine, are involved in the modulation of different physiological mechanisms and represent a major therapeutic target for a range of neurological diseases. Parkinson's disease, characterized by the progressive neurodegeneration of dopaminergic neurons in the substantia nigra, has become the fastest growing neurological disease worldwide. Common treatments involve the oral administration of L-DOPA, a dopamine precursor, and carbidopa, a decarboxylase inhibitor. Even though this treatment has proven to be beneficial for recovering movement capabilities, side effects linked inadequate dosing or treatment complications due to disease progression trigger uncontrolled movements worsening as the disease proceeds. This side effect is often described as worse than the actual disease by numerous patients, affecting their life quality deeply. Hence, developing more efficacious treatments is of great importance. In the present study, an advanced microbiome therapeutic is engineered and tested for the production and delivery of L-DOPA in the gut, in order to overcome common dosing limitations seen in conventional treatments. Engineering of such strain was achieved by cloning a truncated version of the rat tyrosine hydroxylase and optimizing co-factor availability by upregulating the tetrahydromapterin biosynthetic pathway. Furthermore, another strain was constructed for the co-expression of bacteriocins in order to inhibit *Enterococcus faecalis*, limiting the conversion of L-DOPA into dopamine that normally decreases treatment efficacy in PD patients. Lastly, several dopamine decarboxylases were screened in order to identify an enzyme with reduced by-product formation, for the development of a dopamine-producing advanced microbiome therapeutic.

Key words: Parkinson's disease, L-DOPA, *E. coli* Nissle 1917, *E. faecalis*, dopamine, microbiome, therapeutics.

##### Introduction

Besides being involved in neuromodulation of cognitive functions, reward and motivation<sup>[56-57]</sup>, dopamine (DA) has a key role in motor control<sup>[58]</sup>. Dopamine, as part of the catecholamine family, is produced from L-tyrosine by two enzymatic steps<sup>[59]</sup>. The first step of conversion involves the hydroxylation of L-tyrosine into L-DOPA by the tyrosine hydroxylase enzyme (TH). Subsequently, L-DOPA is decarboxylated by the aromatic L-amino acid decarboxylase (AAAD or AADC), also known as DOPA decarboxylase (DDC), into dopamine. In the brain, it is produced in the ventral tegmental area, substantia nigra and hypothalamus<sup>[60]</sup>, nevertheless, it is also known to be produced in considerable quantities in the gastrointestinal tract<sup>[40]</sup>. In Parkinson's Disease (PD), dopaminergic

neurons in the substantia nigra undergo degeneration, resulting in reduced dopamine levels over time<sup>[61]</sup>. Diminished concentrations of DA impair execution of normal voluntary movements and are a clear sign of PD progression<sup>[62]</sup>, resulting in slowed movement (bradykinesia), tremor, rigid muscles, impaired posture and balance, among others<sup>[63]</sup>. The administration of L-DOPA has been the gold standard treatment of PD for decades<sup>[64]</sup>. When administered, L-DOPA crosses the brain blood barrier (BBB) and undergoes decarboxylation in the brain, restoring levels of DA. Even though PD patients are able to recover voluntary movement capabilities, when given this treatment, the dosing regimen needs to be tightly controlled, as too little might not have any effect, and too much could trigger undesirable side effects<sup>[65,66]</sup>. In fact, the therapeutic window of L-DOPA treatment is reduced as the disease progresses, needing more doses with higher concentrations of L-DOPA. Another interesting aspect observed from long term treated patients is the increased abundance of certain species of bacteria in the gut<sup>[49]</sup>. *Enterococcus faecalis* and *Eggerella lenta* have been reported to work in consortia in order to utilize and degrade the L-DOPA administered<sup>[50]</sup>. Additionally, a common DDC inhibitor co-administered with L-DOPA (carbidopa) does not act on these bacterial decarboxylases, emphasizing the need for alternate strategies for efficacious delivery of L-DOPA in the gut environment. Inhibition of vancomycin resistant *Enterococcus* species in mice has been shown previously by an engineered *E. coli* Nissle 1917 (EcN)<sup>[55]</sup> expressing single and combined bacteriocins, proving to be a possible alternative for modulating bacterial species in gut environment.

Recently, numerous efforts to engineer the probiotic bacterium *E. coli* Nissle 1917 for therapeutic purposes have been reported<sup>[67-70]</sup>. These studies address the potential of developing therapeutics using bacteria and outline their many advantages. In the PD area, a patent application<sup>[42]</sup>, published in 2019 (parallel to our investigations), outlined the use of EcN for the intestinal delivery of L-DOPA, expressing HpaB and HpaC, known to hydroxylate a wide array of aromatic compounds. Furthermore, they tested efficacy to treat PD by gavaging their strain into a genetic mouse model of PD (MitoPark mice)<sup>[71]</sup>, witnessing a significant increase in motility and recovery of other functions. Even though the findings have not been published in a peer-reviewed article, they suggest the possibility of treating PD by employing engineered probiotic EcN with L-DOPA production capabilities.

*E. coli* has been one of the work horses in the metabolic engineering field, being subject to a myriad of modifications<sup>[72]</sup>. Previous reports have shown production of L-DOPA<sup>[10,11]</sup> and dopamine<sup>[54]</sup> in laboratory strains. Several attempts and strategies have been performed in *E. coli* to increase the carbon flux through the shikimate pathway and the aromatic amino acid biosynthetic pathway to increment tyrosine production<sup>[1-9]</sup>, leading to increased titers in bioreactor fermentations. Nevertheless, most studies have used the native HpaBC machinery to perform the hydroxylation of L-tyrosine, and only one reports the use of a truncated rat TH<sup>[12]</sup>.

In the present study, we successfully engineered *E. coli* Nissle 1917 for the production of L-DOPA, envisioning its use as an advanced microbiome therapeutic (AMT) for treating PD. Moreover, we achieved significant increase of L-DOPA production by the overexpression of genes involved in tetrahydromapterin (MH4) biosynthesis. Additionally, this strain is tested in mice as a proof of concept for delivering L-DOPA *in vivo*. Steps towards addressing undesired *in vivo* L-DOPA metabolism by *Enterococcus faecalis* are also considered and tested by the co-expression of bacteriocins. Lastly, an initial dopamine AMT is engineered by testing several decarboxylases for the production of dopamine.

## Methods

### *E. coli* strains, culture conditions and gene fragments

All cloning procedures were performed using *E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA) and standard Luria Bertani (LB) media supplemented with appropriate antibiotics, unless otherwise stated. Liquid cultures and plates were incubated at 37°C for 16h (250 rpm for liquid cultures), unless otherwise stated. *E. coli* Nissle 1917 was obtained from commercially available Mutaflor® product. *E. faecalis* was ordered from DSMZ (DSM 20478) and was cultured according to guidelines in a GMO class 2 laboratory. Initial plasmids containing bacteriocins H1 and F3 were kindly provided by Carola Rosenkilde. The rat (*Rattus norvegicus*) truncated TH containing plasmids and DDCs (A-K) were kindly provided by Hao Luo and Lei Yang from the Iloop team at the Center for Biosustainability at DTU led by Markus Herrgard and Andreas Worberg. Enterocin A, DDCs L and M were ordered from IDT. Genes involved in MH4 biosynthetic pathway were amplified from *E. coli* Nissle 1917.

### Plasmid construction and *E. coli* Nissle 1917 transformation

DNA fragments for plasmid construction were obtained by using Phusion U High-Fidelity PCR master mix (ThermoFisher Scientific). Fragments were purified using GeneJet PCR purification Kit (ThermoFisher Scientific), following manufacturer's instructions. Cloning reaction was performed using 1 µl of USER enzyme (NEB), 1 µl of DpnI (ThermoFisher Scientific), 1 µl of 10X Cut Smart buffer (NEB) and 200 ng of DNA fragments and MQ water, for a total reaction volume of 10 µl. Mixture was incubated at 37°C for 30 minutes, followed by 15 minutes at 15°C. 5 µl of the USER reaction was used to transform chemically competent *E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA) by heatshock at 42°C. 1 ml of SOC media was used to recover the transformed cells for 1 hour, and 50 µl were plated in LB plates supplemented with the appropriate antibiotic. Plates were incubated at 37°C overnight. Next day colonies were screened through colony PCR, using OneTaq Quick-Load 2x Master Mix (NEB). Positive colonies were inoculated into 5 ml of 2xYT medium (containing 16 g/L Tryptone, 10 g/L Yeast Extract and 5 g/L NaCl) and were incubated overnight. Next day, plasmids were purified using NucleoSpin Plasmid EasyPure purification kit (Macherey-Nagel), following manufacturer's instructions. Concentration of purified plasmids were measured with NanoDrop (ThermoScientific™), and later sequenced using Eurofins overnight sequencing service. Cultures of colonies harboring the correct plasmid were stored at -80°C as glycerol stocks.

A single colony of *E. coli* Nissle 1917 was inoculated in LB media overnight. The next day 100 µl of the overnight culture was used to inoculate 10 ml of 2xYT medium. Optical density (OD) was followed and cultures were harvested between OD<sub>600</sub> = 0.4 - 0.5, using a prechilled centrifuge at 4500 g for 10 minutes. Pellets were washed 3 times with cold 10% glycerol/MQ H<sub>2</sub>O solution. Lastly, 100 ng of the desired plasmid was transferred onto the pellets and 50 µl of 10% glycerol/MQ H<sub>2</sub>O solution was used for resuspension. Resuspended cells were then transferred to a cold 0.1 cm Gene Pulser electroporation cuvette (Bio-Rad) and were electroporated (BioRad MicroPulser) at 1.8 kV. Cells were recovered using 1 ml of SOC media for 1 hour in a shaking incubator at 37°C, before plating. Schematic representations of the plasmids constructed in this study can be found in figure 3 of supplemental Materials.

### Genomic modification via no-SCAR recombineering

Genomic modifications were carried out using the Scarless Cas9 Assisted Recombineering (no-SCAR) system<sup>[73]</sup>. Briefly, strains transformed with plasmid pHM156, containing a thermosensitive ORI, the  $\lambda$ -Red system under an arabinose inducible promoter, the *cas9* gene under a constitutive promoter (BBa\_J23105), and a pMB-targeting gRNA under the regulation of the tetO promoter, were inoculated from a fresh plate into 3 ml of LB supplemented with 100  $\mu$ g/ml ampicillin (amp) and were incubated in a shaking incubator at 30°C and 250 rpm. Next day, cells were inoculated 1:50 into 10 ml of 2xYT broth, supplemented with ampicillin, and were grown at 30°C until OD<sub>600</sub> of 0.3-0.4. At this OD, 10  $\mu$ l of 10% arabinose was used to induce the expression of  $\lambda$ -Red system and cells were allowed to grow for another 30 min. The culture was cooled using ice for 10 min before cells were prepared for electroporation. Cells were electroporated with A) plasmid pMB-X, containing a gRNA specific to the target (X) under constitutive expression, and B) an overlapping DNA fragment for knock out or knock in modifications. Once electroporated cells were recovered in 1 ml of SOC for 1 hour at 30°C, after which 4 ml of SOC and 5  $\mu$ l of ampicillin and chloramphenicol (cam) were added. Tubes were incubated overnight at 30°C in a shaking incubator (250 rpm). Following day, 50  $\mu$ l were plated onto LB plates containing amp and cam, and were grown overnight at 30°C. Positive clones, confirmed by colony PCR, were inoculated in 2ml of LB broth supplemented with amp and ATc (200 ng/ml) and were grown at 30°C in a shaking incubator overnight. Next day, cells were streaked on LB and grown at 37°C overnight, for curing pHM156. Plasmid curing was verified by streaking single colonies in amp and cam. Guide RNAs were designed using CRISPy-web<sup>[76]</sup>.

#### Fermentations

Small scale fermentations were performed by inoculating strains, in biological triplicates, into 500  $\mu$ l of M9 media (Supp. Table 3) (0.4% glucose) supplemented with appropriate antibiotics in 96-deep well plates. Preculture was allowed to grow until saturation (24 hours), after which an aliquot of 5  $\mu$ l was taken to inoculate the production culture (500  $\mu$ l), using the same setup. After 22 hours, optical density was measured and plates were centrifuged at 4500 rpm for 10 min. Supernatants were then frozen until further HPLC was performed. For LC-MS/MS samples, supernatants were filtrated using 96-well filter plates (AcroPrep™) prior being frozen. For dopamine fermentations, 0.2% Casamino acids (Teknova) was also supplemented to the M9 media. Growth was monitored by transferring 150  $\mu$ l into a 96-well microtiter plate and using a BioTek ELx202 microtiter plate reader.

#### Competition experiments

*E. faecalis* and L-DOPA EcN strains expressing different bacteriocins were grown overnight in Brain Heart Infusion (BHI) broth (NutriSelect™), without supplementation of antibiotics. Following day, EcN cultures were washed once and resuspended in PBS. Cultures were diluted accordingly to have a concentration of 10<sup>7</sup> and 10<sup>6</sup> CFU/ml of EcN and *E. faecalis* respectively in 10 ml of BHI. Throughout the experiment 200  $\mu$ l were taken periodically for CFU plating and 1ml for future HPLC quantification. Samples for HPLC quantification were centrifuged at 10 000 g for 3 min, supernatant was transferred into a 96-well plate. Before HPLC quantification the supernatants were filtered using a 96-well filter plate (AcroPrep™). Culture dynamics were followed by transferring 200  $\mu$ l of the competition culture into a 96-well microtiter plate and running a kinetic experiment measuring OD and GFP in a fluorescent microtiter plate reader (Synergy H1). Competition experiment was performed for 48 hours.

#### Animal experiments

Female mice (NMRI, supplied by Taconic Biosciences, 6 weeks of age) were group-housed on a 12h light:dark cycle at constant temperature with ad libitum access to food and water in a Specific Pathogen Free (SPF) facility. Upon delivery, mice were given 5 days to adjust to new location. Cohort



size was 8 animals, and 4 different cohorts were tested. All animals received Streptomycin (5 g/L) in the drinking water to ensure colonization, 3 days before being gavaged and throughout the experiment. A single oral gavage of  $10^8$  cells was administered of either L-DOPA-producing (designated 'EcN\_L-DOPA') or a control *E. coli* Nissle ('EcN\_EMPTY') strain without expression of the tyrosine hydroxylase gene. Samples were taken for the following 7 days, after which animals were euthanized and final blood samples and gut content samples were collected. 2 of the 4 cohorts were also treated with the DDC inhibitor Carbidopa via intraperitoneal injection (10 mg/kg body weight) every 24h. Fresh fecal samples were collected daily for 7 days to quantify colonization and metabolite levels. Plasma samples were taken on day 2 (submandibular sampling) and day 7 (*vena cava*) after gavage, and urine samples were taken on day 3 and 6. A detailed scheme of the setup and sampling be found in supplemental figure 1.

#### Gavage preparation

A single colony was grown in LB medium, supplemented with 50 µg/ml kanamycin, overnight at 37°C (approximately 18 hours) in a shaking incubator (250 rpm). Next day, cultures were centrifuged at 4000 g for 5 min, washed once and resuspended in sterile PBS to initial volume. Optical density was measured and resuspension was diluted to achieve  $OD_{600} = 0.35$  ( $5 \times 10^8$  CFU/ml). Diluted cells were kept at room temperature, 200 µl of the gavage solution was used for each mice.

#### *In vivo* metabolite analysis from plasma and urine

Plasma and urine were analyzed for tyrosine, 3,4-dihydroxyphenylalanine (L-DOPA), dopamine, 3-O-Methyl-dopa (3-OMD), 3-Methoxytyramine (3-MT), homovanillic acid (HVA), serotonin, 5-hydroxytryptophan, tryptamine, 5-Hydroxyindoleacetic acid (5-HIAA) and tryptophan. For plasma: blood samples were collected via the submandibular vein on day 2 and heart puncture on day 7 post gavage, kept on ice for 20-30 min in Li-Heparin Microtainers. Plasma was then separated by centrifugation at 10 000 g at 4°C for 90 sec. Plasma fraction was transferred into a new microcentrifuge tube and snap-frozen in ethanol bath and dry ice. Samples were then placed in dry ice, protected from light, and then moved to -80°C freezer. Urine samples were collected by placing the animal in a glass container and rapidly transferring the fresh urine into a microcentrifuge tube, which was then kept frozen at -80°C. An internal standard buffer containing 0.9% NaCl, 0.2% Ascorbic acid, and 20 mg/L C-13-labelled tryptophan was to plasma and urine samples, after thawing. Proteins were precipitated using methanol extraction, supernatant was transferred into a new tube and dried using a vacuum centrifuge protected from light. Dried pellets were reconstituted in 50 µl of MQ water for LC-MS/MS analysis.

#### Colony forming units from feces and gut content

For fecal samples were placed in the a pre-weighed 1.5 ml Eppendorf tube with 1 ml PBS. Samples were then docked using a microcentrifuge tube rack until the fecal pellet dissociated, each sample was then weighed and placed on ice for 10 min. Samples were docked again and centrifuged at 0.5 g for 1 minute at 4°C. An 200 µl aliquot was transferred into a 96-well microtiter plate. 10 fold serial dilutions and plating was performed employing a liquidator. Samples were plated in LB supplemented with streptomycin and kanamycin, for calculating AMT abundance in the sample. Samples of 2 time points were also plated in LB supplemented only with Strep to calculate plasmid loss.

#### HPLC quantification

Quantitative analysis of L-DOPA, dopamine, L-tyrosine and tyramine in cell-free supernatant was performed by High-Performance Liquid Chromatography (HPLC) on an UltiMate 3000 UHPLC system (ThermoScientific). The system consisted of an LPG-3400RS quaternary pump and a WPS-

3000RS autosampler with a TCC-3000 column oven and a DAD-3000 diode array detector. Samples were run at a pressure of 600 bar using a CORTECS column (1.6  $\mu\text{m}$ , 2.1x150 mm) at 30°C with an injection volume of 1  $\mu\text{l}$  and a flowrate of 0.350 ml/min in 10 mM ammonium formate as mobile phase. Data analysis was performed using Chromeleon 7.2.9 (ThermoScientific). HPLC calibration standards contained L-DOPA, L-Tyrosine, tryptamine and dopamine which were dissolved in 0.1% formic acid and subsequently suspended in filter sterilized, spent M9 media or MQ water, calibration curve ranging from 0 to 200 mg/L.

#### LC-MS quantification

Detection and quantification of L-DOPA, dopamine, tyrosine, tyramine, phenethylamine, serotonin, tryptamine, tryptophan, and 5-HTP were conducted by liquid chromatography mass spectrometry (LC-MS) measurements on a Dionex UltiMate 3000 UHPLC (Fisher Scientific, San Jose, CA) connected to an Orbitrap Fusion Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA). The system used an Agilent Zorbax Eclipse Plus C18 2.1 x 100 mm, 1.8  $\mu\text{m}$  column kept at 35°C. The flow rate was 0.350 mL/min with 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B) as mobile phase. The gradient started as 5% B and followed a linear gradient to 35% B over 1.5 min. This solvent composition was held for 3.5 min after which it was changed immediately to 95% B and held for 1 min. Finally, the gradient was changed to 5% B until 6 min. The sample (1  $\mu\text{l}$ ) was passed on to the MS equipped with a heated electrospray ionization source (HESI) in positive-ion mode with sheath gas set to 60 (a.u.), aux gas to 20 (a.u.) and sweep gas to 2 (a.u.). The cone and probe temperature were 380°C and 380°C, respectively, and spray voltage was 3500 V. Scan range was 50 to 500 Da and time between scans was 100 ms. Quantification of the compounds was based on calculations from calibration standards analyzed before and after sets of 24 samples. All reagents used were of analytical grade.

#### Statistical analysis and software tools

Design of genetic parts was carried out using online lab notebook tool Benchling<sup>[75]</sup>. Statistical analysis was carried out in GraphPad Prism 7 (La Jolla California, USA. [www.graphpad.com](http://www.graphpad.com)). Figures were created using Biorender (BioRender.com).

## Results

### Point mutation in *folE* and *tyrR* Knock out improves L-DOPA production in *E. coli* Nissle 1917

To evaluate whether L-DOPA production could take place in *E. coli* Nissle 1917 (EcN), the truncated version of *R. rattus* TH was cloned and expressed, yielding 40 mg/L of L-DOPA when 100 mg/L of tyrosine was supplemented. As hydroxylase activity in TH is limited by available MH4 cofactor, a previously described mutation<sup>[12]</sup> in *folE* gene (T198I), known to increase activity of this bottleneck enzyme was introduced. Results show a more than 2-fold increase in L-DOPA production compared to the WT EcN, harboring the same plasmid (Fig. 1A), when supplemented with 100 mg/L of L-Tyrosine. In order to confirm whether L-Tyrosine was limiting for L-DOPA production, cultures were supplemented with different concentrations of the amino acid (Fig. 1B). Supplementing 100 mg/L seem to be the only concentration able to increase L-DOPA production significantly. Approximately 40 mg/L of L-DOPA was produced without tyrosine supplementation, revealing that L-DOPA could be produced with no need of exogenous tyrosine. Overall, these results confirm the importance of increasing cofactor availability for optimal hydroxylation of L-Tyrosine into L-DOPA.

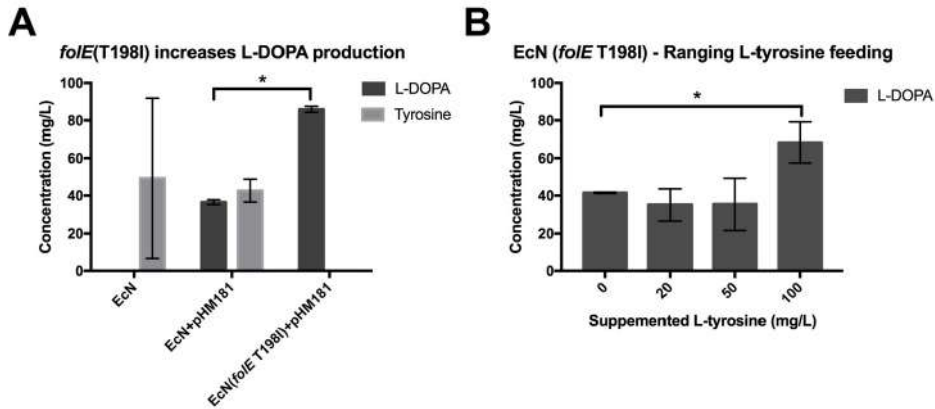


Figure 1. L-DOPA production in *E. coli* Nissle 1917 A) Effect of *folE* (T198I) on L-DOPA production, feeding 100 mg/L L-Tyrosine. B) L-DOPA production from EcN (*folE* T198I) supplemented different concentrations of the precursor L-tyrosine. Each column represents the mean of biological triplicates, error bars show standard deviation. T-student test was used on (A) and multiple comparison One-way ANOVA was used in (B) with CI of 95%. Significance at  $P < 0.05$  is shown with an asterisk.

Following the *folE* modification, increasing TH expression levels was evaluated by cloning 2 strong promoter variants from the Anderson promoter library<sup>[77]</sup> and 2 constitutive promoters developed in-house and validated *in vivo*<sup>[74]</sup>. In the same experiment, a *tyrR* knock out (KO) was also investigated with the different plasmid constructs. *tyrR* is a major repressor for various genes involved in the shikimate and aromatic amino acid biosynthetic pathway. L-DOPA production shows to be the highest for both genetic variants when using promoter number 7 developed in-house, being significantly superior to the previous system used. Furthermore, a drastic difference was observed when having the *tyrR* KO present as well.

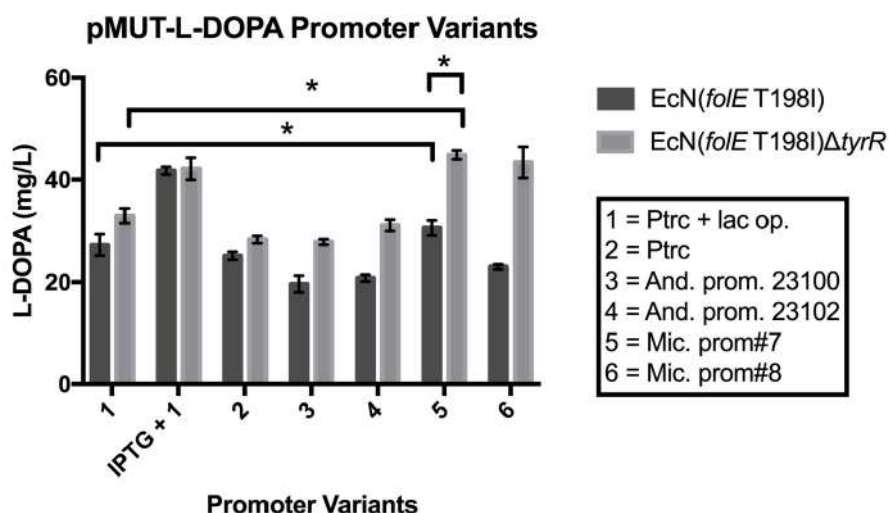


Figure 2. Increasing L-DOPA production by optimizing expression levels from pMUT1 based expression. Different promoters were tested for TH expression and compared using a EcN *folE* (T198I) strain either with (black bars) or without (gray bars) the tyrosine repressor (*tyrR*) on the genome. Each column represents the mean of biological triplicates, error bars show standard deviation. One-way ANOVA was used for Dunnett's multiple comparisons test against variant 1, with CI of 95%. Significance at  $P < 0.05$  is shown with an asterisk. Significance is only shown for the variant showing increased levels for both genotypes, variant 2 was not consider in the analysis. T-student test was used to compare both genotypes of variant 5, test was executed with CI of 95%.

#### Overexpression of enzymes involved in MH4 biosynthesis improves L-DOPA production

After witnessing improvement of L-DOPA production by increasing endogenous GTP cyclohydrolase I (GCHI, also called *FolE*) catalytic activity, we sought to evaluate whether overexpression of enzymes involved in MH4 biosynthesis (Fig. 9) could assist in increasing L-DOPA production further. PhhB (a pterin 4a-carbinolamine dehydratase, also called PCD) is important for regeneration of dihydromonapterin (MH2)<sup>[78]</sup>, which can be further reduced to MH4. Thus, the native *aroF* promoter driving *phhB* expression was replaced with the strong *trc* promoter (with no *lacI* binding sites), upstream *phhB* from *Chromobacterium violaceum*. This, change did not result in a significant increase, however, the *trc* promoter was maintained due to its robust strong expression. Next, genes encoding for known enzymes in the tetrahydromonapterin pathway were introduced downstream *phhB* in an operon. A significant improvement was observed when *folE* (T198I) was introduced compared to the previous version. Nevertheless, when *folM* was introduced resulted in more than a 2 fold increase (Fig. 3), suggesting that dihydromonapterin reductase could probably be an enzymatic bottleneck. Additional overexpression of *folX* decreased significantly L-DOPA production, showing no benefit of overexpressing that particular enzyme. These results show that several genes involved in MH4 biosynthesis plays a crucial role for optimizing TH based L-DOPA production.

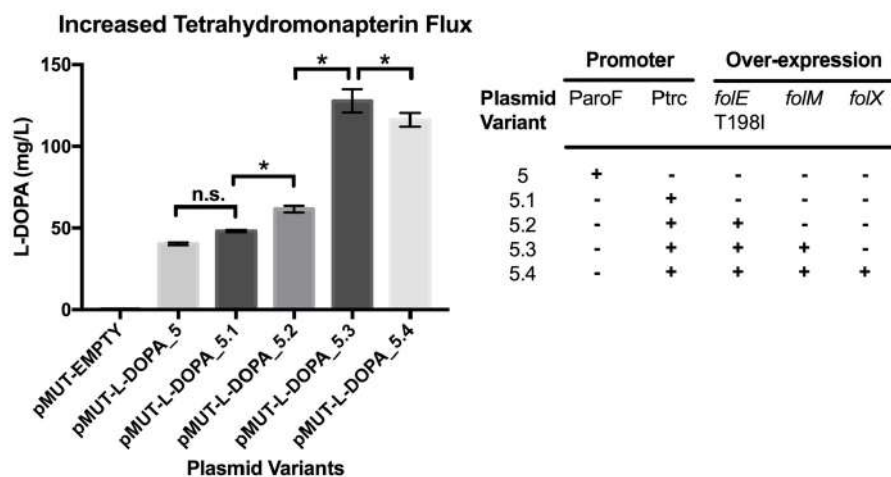


Figure 3. Increasing L-DOPA production by overexpressing tetrahydromonapterin biosynthetic genes. On the left, graph showing levels of L-DOPA production by EcN *folE* (T198I) $\Delta$ *tyrR* harboring different plasmid variants, which overexpress genes involved in tetrahydromonapterin biosynthesis, using. On the right, description of the different variants used. Each column represents the mean of biological triplicates, error bars show standard deviation. One-way ANOVA was used for Sidak's multiple comparisons test, with CI of 95%. Significance at  $P < 0.05$  is shown with an asterisk.

#### AMT based L-DOPA delivery *in vivo* and physiological read outs

To address the applicability of the AMT designed for L-DOPA delivery, *in vivo* experiments were conducted using mice as a model system. The experiment lasted 7 days with an initial gavage at day 0 and subsequent sampling of blood, urine and feces through the experiment, and gut content on the dissection day. A significant colonization difference among the different groups was observed only on day 1, where EcN\_L-DOPA had higher colonization compared to the other groups (EcN\_EMPTY ( $P=0.0006$ ), EcN\_EMPTY(carbidopa) ( $P=0.0022$ ) and EcN\_L-DOPA(carbidopa) ( $P=0.0035$ )). Two-way ANOVA). Colony forming units (CFU)s per gram of feces for both L-DOPA producing and empty control strains among the two different conditions, was approximately  $10^9 - 10^{10}$  until day 4. From that day a decrease in CFUs was observed for groups 2 - 4 (Fig. 4A). When comparing colonization of day 1 to the rest of days only EcN\_L-DOPA had a significant decrease from day 4 - 7 ( $P=0.0085$ ,  $P=0.0133$ ,  $P=0.0002$ ,  $P<0.0001$  respectively, Two-way ANOVA). CFU analysis of gut content, showed that the small intestine had the least amount of EcN ( $10^4 - 10^6$ ), while cecum and colon levels were very similar ( $10^8 - 10^{10}$ ) (Fig. 4B-D). Plasmid loss, assessed by comparing CFU counts of strep and strep+kan plates, showed differences on day 7 of the experiment for EcN\_L-DOPA in both treatment groups. It accounted for 60% loss in non-carbidopa treated and 80% in carbidopa treated groups approximately (Fig. 4E & F).

Unfortunately, as method developing for catecholamine quantification is ongoing to date, an adaptation of an LC-MS quantification method for serotonin was employed. This together with a strong matrix effect made the quantification of catecholamines and derivatives in biological samples difficult and unreliable. Nevertheless, increasing trends for tryptophan derived molecules (5-HTP &

5-HT) were observed for both urine ( $P=0.06$ ) and plasma samples ( $P=0.08$ ), belonging to mice gavaged with EcN\_L-DOPA from the carbidopa treated group (Fig. 5C-D) on day 7. Interestingly, a tendency to lose body weight was observed in animals given EcN\_L-DOPA (Fig. 5D). This tendency was more evident in the non-carbidopa treated groups, resulting in a significant decrease, when the percentage change of body weight between days -1 and 7 was compared among the two groups (Fig. 5B).

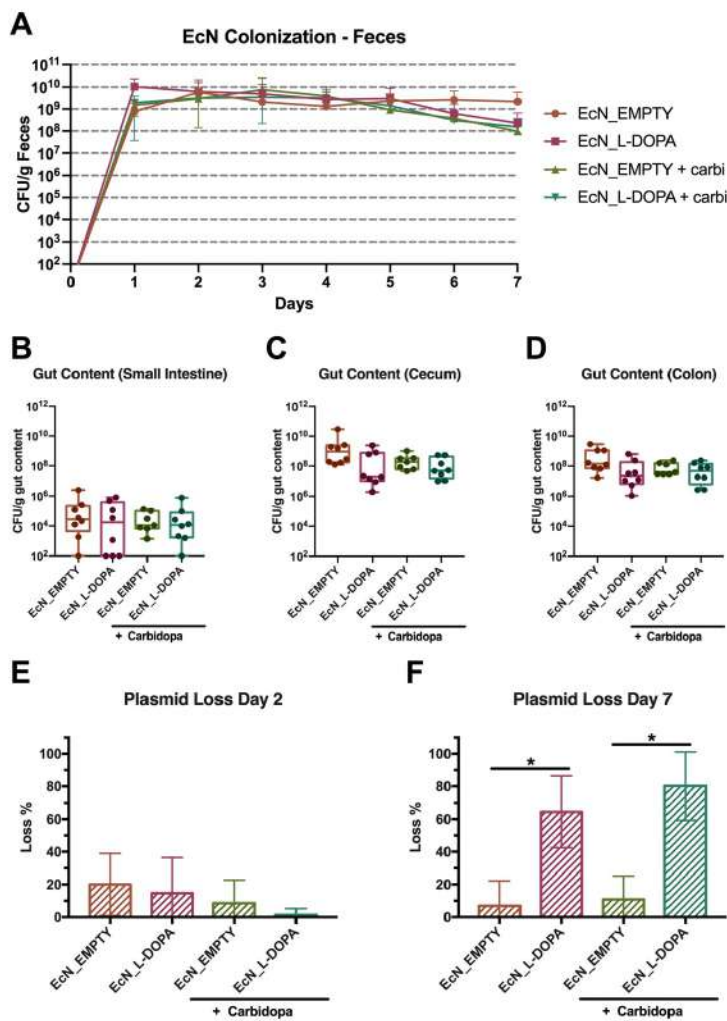


Figure 4. Colonization observations of L-DOPA producing AMT. A) Shows the amount of colony forming units per gram of feces throughout the experiment, gavage was performed on day 0 and animals were sacrificed on day 7. B, C & D) Show CFU per gram of gut content in the small intestine,

cecum and colon, respectively from samples taken on day 7. E & F) Shows plasmid loss of day 2 and 7 respectively, from the bacterial strains gavaged. Each data point represent the mean of 8 biological replicates. Ordinary One-way ANOVA was used to determine significant differences in gut content data. T-student test was used to compare plasmid loss between both strains in carbidopa and non-carbidopa treated mice. Both tests were executed with CI of 95% and significance  $P < 0.05$  is shown with an asterisk. EcN\_EMPTY contains an empty plasmid, and EcN\_L-DOPA contains pMUT-L-DOPA\_5.3.

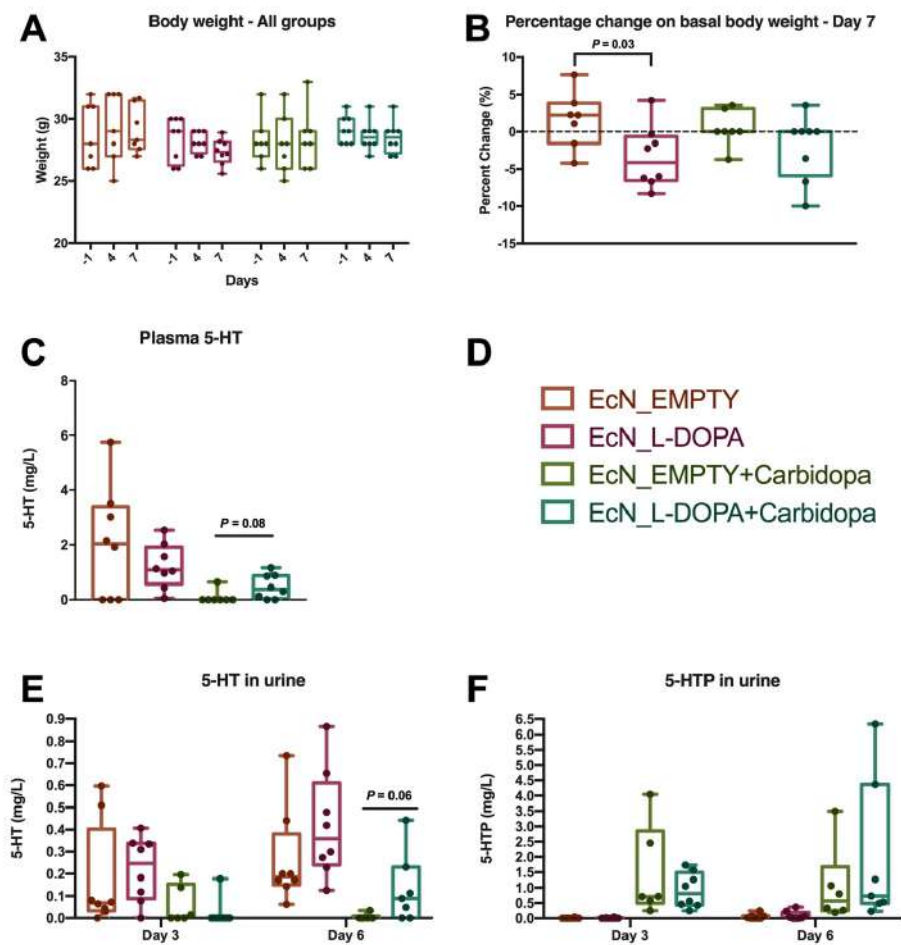


Figure 5. Biological readouts of L-DOPA AMT treatment. A) Shows body weight of mice groups for day 1 prior to gavage (-1), day 4 and 7 after gavage. B) Shows the percent change on body weight between days -1 and 7. C) Shows serotonin levels in plasma samples from day 7. D) Shows overview of groups and color codes for the different graphs. E & F) Serotonin and 5-HTP levels in urine samples from day 3 and 7. Each data point represent the mean of 8 biological replicates. T-student

test was used to compare both strains in carbidopa and non-carbidopa treated mice. T-student test was executed with CI of 95% and significance values are stated in the graph. EcN\_EMPTY contains an empty plasmid, and EcN\_L-DOPA contains pMUT-L-DOPA\_5.3.

#### L-DOPA producing *E. coli* Nissle inhibits *E. faecalis* by co-expression of bacteriocins

In order to assess a potential role of the L-DOPA AMT for modulating *E. faecalis* abundance, bacteriocins known to inhibit this specie were cloned downstream of the TH. Initial experiments demonstrated inhibition both using the supernatant and the bacteria itself (Supp. Fig. 4). A competition experiment carried out by inoculating and co-cultivating *E. faecalis* with the different bacteriocin-expressing EcN strains was performed to evaluate the inhibition potential and more importantly the impairment of L-DOPA metabolism by *E. faecalis*. Cultures only containing the given EcN strain (without *E. faecalis* inoculum) were also cultivated and designed hereafter as controls. As the EcN strains had constitutive expression of GFP, the relative fluorescence (GFP/OD<sub>600</sub>) was taken as a parameter to follow EcN growth in the competition cultures. This parameter was then normalized by relative fluorescence of the control cultures to assess whether the EcN strain was negatively affected (<1), not affected (1) or positively affected (1<) by the other bacterium. Significant differences were observed between EcN strains co-expressing bacteriocins and the control EcN with no co-expression of bacteriocin (Fig. 6A), the major differences were observed between 4 – 32 hours of the co-cultivation.

Metabolite profile of the competition cultures seem to also be affected by having EcN co-expressing bacteriocins. Overall, there were higher L-DOPA accumulation profiles in the cultures having EcN strains producing bacteriocins, a significant increase in L-DOPA was observed for all strains compared to the non-bacteriocin producing EcN, however, at different time points (Fig. 6B). The presence of dopamine was only observed in the competition culture having the non-bacteriocin producing EcN, it peaked at 24 hours and remained at low concentrations (Supp. Fig. 5E). L-Tyrosine present in the media appeared to be consumed at different rates, depending on the EcN strain. L-Tyrosine present in non-bacteriocin producing EcN cultures was depleted at 6 hours, contrary to this, depletion of L-Tyrosine in bacteriocin producing cultures was hampered to a significant extent by all bacteriocin producing strains for 24 hours, after which only EcN expressing H1 and EntA showed a difference (Fig. 6C). Tyramine, which is a decarboxylated L-Tyrosine, seemed to increase rapidly after 4 hours in the non-bacteriocin producing EcN culture, nonetheless, the rest of the EcN strains seemed to inhibit/delay this conversion (Fig. 6D). Further analysis uncovered a strong negative correlation between L-Tyrosine and tyramine, meaning that L-Tyrosine present in the media is being converted into tyramine (Supp. Fig. 4B). Additionally, control cultures, having no *E. faecalis* inoculum, show no drastic reduction of L-Tyrosine and no substantial production of tyramine (Supp. Fig. 4C), pointing towards *E. faecalis* as the sole responsible for this conversion. Lastly,

Table 1. Overview of bacteriocins tested in competition experiment.

Abbreviation	Accession	Name	Class	Producer organism
H1	BAC142	Hiracin JM79	Class II sec-dependent	<i>Enterococcus hirae</i> DCH5
F3	BAC150	Ubericin A	Class IIa	<i>Streptococcus uberis</i>
ENTA	BAC088	Enterocin A	Class IIa, IIc	<i>Enterococcus faecium</i> ( <i>Streptococcus faecium</i> )



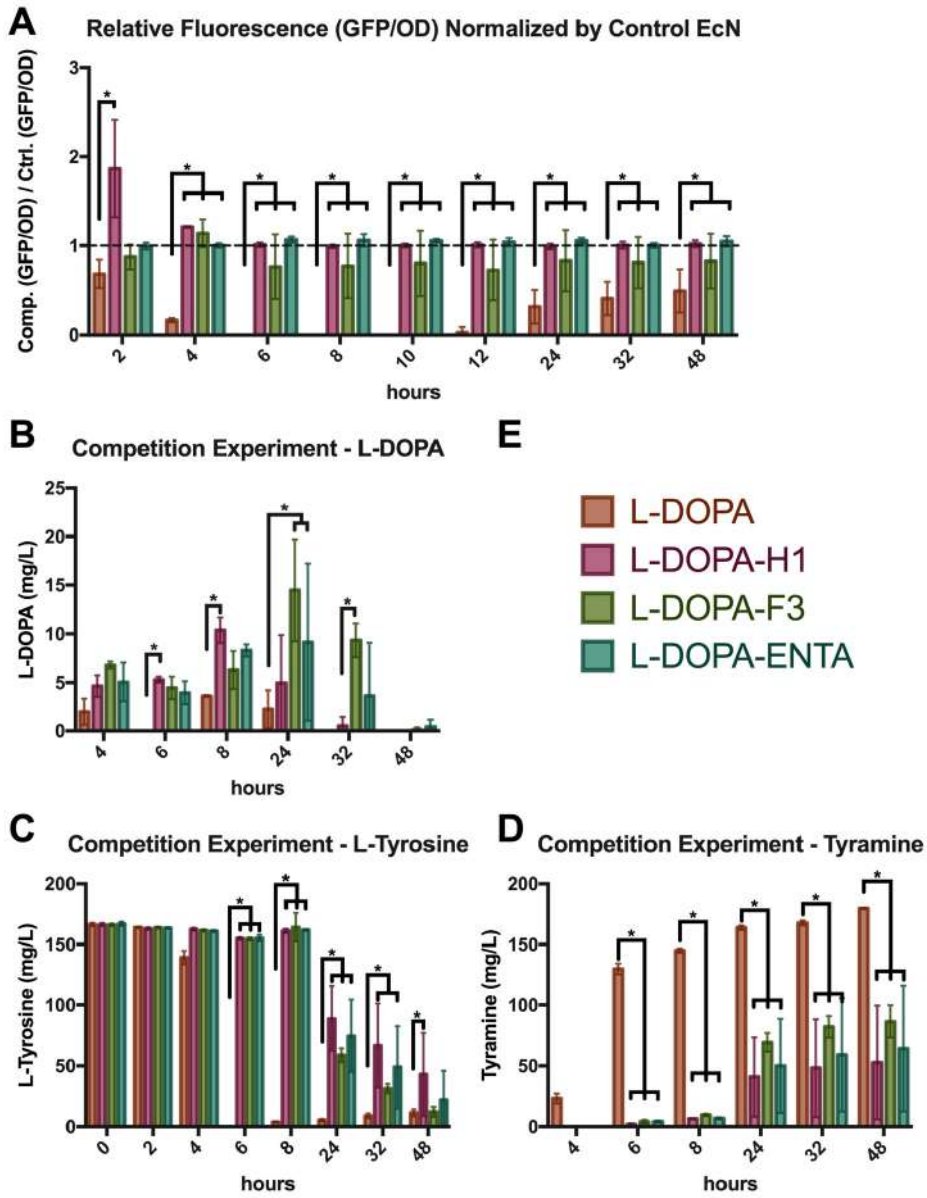


Figure 6. Competition growth experiment between *E. faecalis* and L-DOPA and bacteriocin co-expressing EcN strains. A) Shows the relative fluorescence (GFP/OD) of each competition (Comp.) normalized by a control (Ctrl.) culture with no *E. faecalis* present. Dash line represents the baseline

for the control cultures. B, C & D) HPLC quantification of L-DOPA, L-Tyrosine and Tyramine, respectively, from the competition cultures. Each data point represent the mean of 3 biological replicates. One-way ANOVA was used for Dunnett's multiple comparisons test against L-DOPA strain (no bacteriocin production), with CI of 95%. Significance at  $P < 0.05$  is shown with an asterisk. All graphs follow the same color coding seen in graph A.

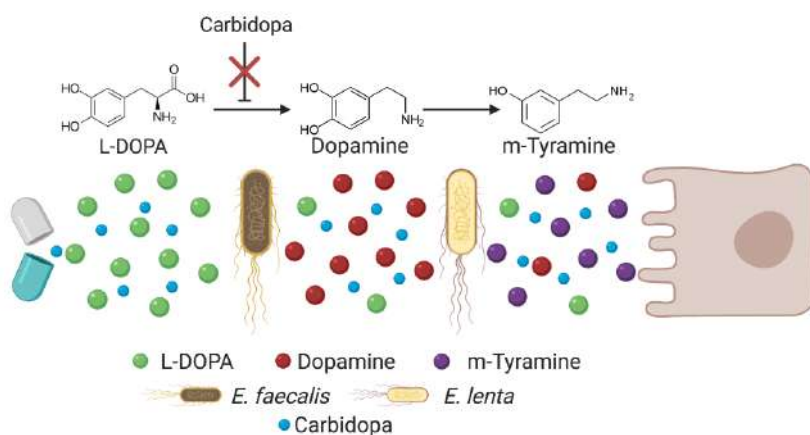


Figure 7. L-DOPA metabolism by gut microbiota. L-DOPA administered is metabolized into dopamine by *E. faecalis*, DA is later metabolized to tyramine by *E. lenta*. As a consequence, less L-DOPA is absorbed, decreasing treatment efficacy.

#### Dopamine production in EcN

In order to identify an appropriate and compatible decarboxylase that would convert L-DOPA into dopamine without major byproducts that would pose a risk for *in vivo* application of the AMT, a panel of DDCs, designated A-M (for confidential purposes related to IP) were transformed into EcN harboring pHM181, the initial L-DOPA producing plasmid. Only five DDCs (A, D, E, L and M) showed to be able to produce dopamine, however, to relative small amounts (0.9 – 14 mg/L) (Fig. 8A). Nevertheless, other decarboxylated by-products (tyramine, tryptamine, PEA) were observed with these DDCs, except DDC E that had no other detectable by-product. Interestingly, DDCs L and M were able to produce more than 120 mg/L of tyramine even though only 100 mg/L of L-Tyrosine was supplemented into the media, suggesting quite a dramatic preference for this substrate.

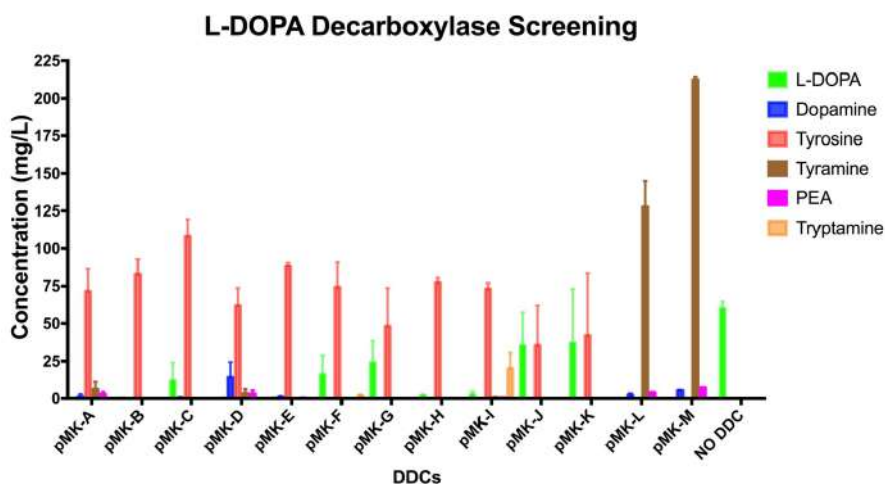


Figure 8. Dopamine production in *E. coli* Nissle 1917. Screening of decarboxylases for dopamine production was performed supplementing 100 mg/L of L-Tyrosine.

## Discussion

Increasing tetrahydropterin (MH4) biosynthesis as an approach for improving bacterial L-DOPA production

Conventional metabolic engineering approaches for improving L-Tyrosine and L-DOPA production in *E. coli* have included: 1) The removal of enzymatic steps driving precursor molecules away from the shikimate and/or the aromatic amino acid pathway, 2) upregulation of bottleneck enzymes, 3) development of feedback resistant enzymes, 4) improving enzymatic activity of the hydroxylase, and 5) the knock-out of pathway repressors (*tyrR*) and/or catabolic genes (*tyrA*). These approaches<sup>[1-12]</sup> have all resulted in a significant increase of L-DOPA production when using the native monooxygenase HpaB and the reductase HpaC in *E. coli*. However, HpaB has a broad substrate specificity<sup>[13]</sup>, posing a substantial challenge and risk, as AMTs are envisioned to colonize and deliver L-DOPA in a complex environment containing a diverse mix of molecules. Poor specificity towards L-Tyrosine could decrease L-DOPA production and lead to unspecific hydroxylation of molecules in the gut, having unknown biological consequences to the host that may decrease therapeutic potential of the AMT. To overcome this, a truncated tyrosine hydroxylase from rat<sup>[12]</sup>, which has higher specificity towards tyrosine, was used. Nevertheless, THs use tetrahydrobiopterin (BH4) as the proton donor, a cofactor not found in *E. coli*<sup>[12,14]</sup>. Introduction and overexpression of genes for producing BH4 from native pterin precursors have been attempted earlier<sup>[12,15,16]</sup>, however, heterologous expression of these genes could risk the stability of the AMT. In fact, deletions in *pts*, *spr* and *dpr* were commonly observed in an adaptive laboratory evolution experiment performed for optimizing this pathway<sup>[12]</sup>, suggesting fitness cost and lack of stability. Even though *E. coli* does not produce this cofactor, previous studies have demonstrated that TH can also utilize MH4<sup>[17]</sup>, a native pterin from *E. coli*, as a cofactor. As previously reported<sup>[12]</sup>, the incorporation of the amino acid change in position 198 of the GTP cyclohydrolase I (*folE*) showed a dramatic increase in L-DOPA production in *E. coli* Nissle. Further plasmid overexpression of this version of *folE*, and *folM* also increased production, suggesting that co-factor availability was limiting for L-DOPA production when using a

TH. Further experiments are still needed in order to determine the individual contribution of the different MH4 biosynthetic genes with regards to L-DOPA production.

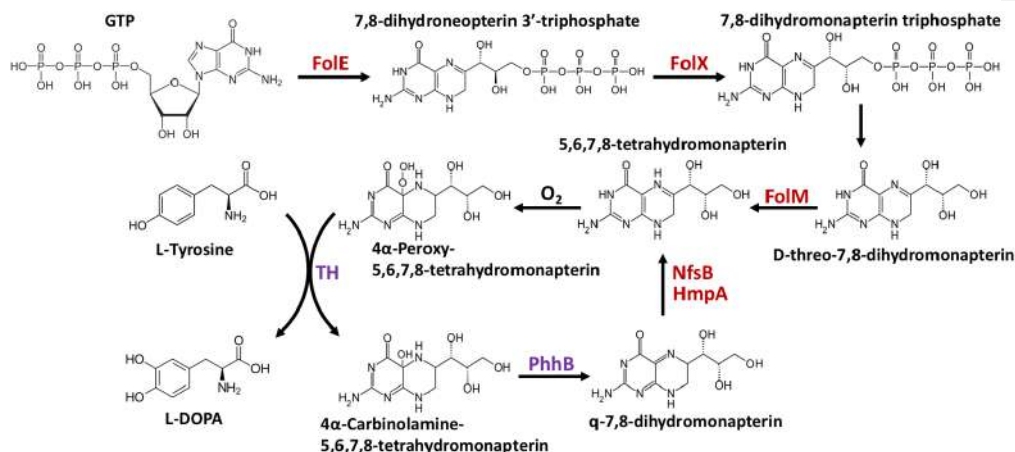


Figure 9. Tetrahydromonapterin biosynthetic and recycling pathway. Enzymes in red are native to *E. coli*, enzymes in purple are from other organisms.

#### Colonization stability and delivery efficacy of the L-DOPA AMT

*EcN* has been studied to be capable of colonization the human gut and remaining an active part of the intestinal flora for months<sup>[18]</sup>. This colonization capability together with inhibitory action towards known pathogens made *EcN* a great probiotic strain, that has been in the market for a century<sup>[19]</sup>. Streptomycin treatment in mice renders them susceptible for stable intestinal colonization, mainly due to the antibiotic clearance of native microbial commensal species<sup>[20]</sup>. In agreement with previous studies, a single gavage proved to be sufficient for stable colonization, based on fecal CFUs. Gut content, however, showed that CFUs counts in the small intestine were lower compared to the cecum and colon. The small intestine represents a quite different environment compared to the rest of the intestines. Here, gastric, pancreatic and hepatic fluids come together in order to finalize the breakdown of food and allow appropriate uptake of nutrients. This represents a challenging environment for most bacteria, in fact, short transit time, bile acids<sup>[21]</sup> and the low pH are known to limit microbial growth, accounting for a reduced abundance of bacteria in this location<sup>[22,23]</sup>. Additionally, *E. coli* species are known to colonize lower parts of the intestine to a greater extent, supporting the colonization profile seen in this study.

On the other hand, plasmid stability seems to be a major concern. Studies have demonstrated the importance of fitness cost with regards to pathway and plasmid stability<sup>[24]</sup>. Expressing an heterologous pathway or enzyme will most likely result in fitness cost, as a consequence of channeling cellular resources towards the production of the molecule of interest. Moreover, presence of genomic copies of *folE* and *folM* in the plasmid could be driving recombination events, leading to pathway disruption and plasmid rearrangements. Recombination has been observed to take place among horizontally transferred genetic material with as minimum 23 base pair homology in *E. coli*<sup>[33,34]</sup>. Even though complete plasmid loss should be minimal, due to the presence of the *hok-sok* stabilizing locus<sup>[35]</sup>, which ensures plasmid segregation through the expression of a toxin-antitoxin

system, it is possible that plasmid remnants after recombination still have this locus but have lost part of the pathway and antibiotic resistance gene, which are not needed in the given environment. Upregulation of the MH4 biosynthetic pathway could also be having a negative effect on plasmid stability. Pterins represent a group of diverse molecules that have a wide range of biological implications<sup>[25]</sup>, being involved in aerobic and anaerobic metabolism, detoxification of toxic compounds, global nutrient cycles and the utilization of non-canonical carbon and nitrogen sources. Even though the role of MH4 in *E. coli* is elusive, studies have shown that MH4 is mainly excreted from the cell<sup>[26]</sup> possibly binding receptor molecules and mediating cellular responses involved in biofilm formation<sup>[27]</sup>, osmotic response<sup>[28]</sup>, and signal transduction<sup>[29]</sup>. Furthermore, overproduction of this pterin *in vivo* could be having a profound effect in physiological traits, yet to be understood, causing stress and selection pressure against the overexpressed pathway. Even though expression of the TH resulted in reduced growth rate, further overexpression of MH4 biosynthetic genes had no effect (Supp. Fig. 2), indicating that TH expression could be limiting plasmid stability. Further sequencing experiments are still required to conclusively point out the cause of the poor *in vivo* stability seen in this study and whether this is also observed *in vitro*.

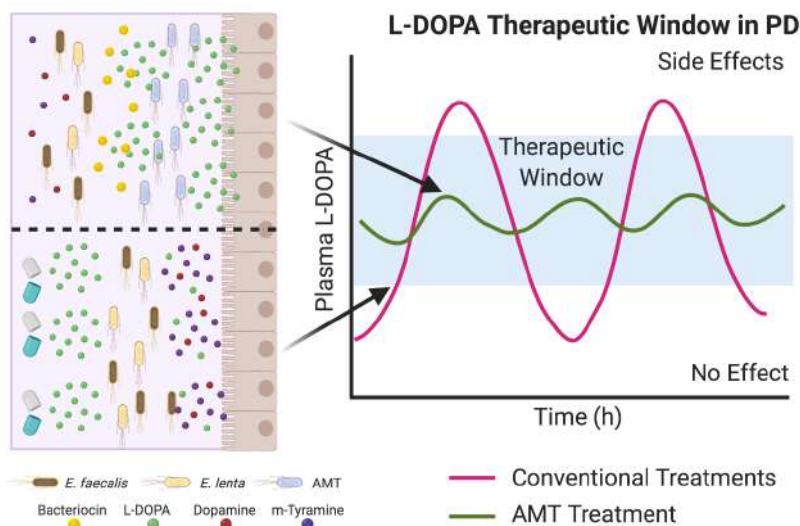


Figure 10. Therapeutic advantage of using an AMT approach for delivering L-DOPA in PD patients.

Dopaminergic and serotonergic neuronal systems exhibit a complex interplay of functions and regulations that affect several neurological and physiological traits<sup>[30]</sup>. Acute and local administration of L-DOPA has shown to increase levels of serotonin in the brain<sup>[31]</sup>. However, the common consensus seems to support that chronic administration of L-DOPA does not alter or decreases extracellular concentrations of 5-HT and 5-HTP, presumably by displacing these molecules from secretory vesicles and also by affecting extracellular levels of tryptophan<sup>[32,36-39]</sup>, the precursor molecule for serotonin. Even though quantification of L-DOPA and catecholamine metabolites was unsuccessful, 5-HT levels were found to be increased within carbidopa treated mice given the L-DOPA producing strain, however, these groups had overall lower serotonin levels as a result of carbidopa treatment<sup>[43]</sup>. This increase in 5-HT, hints towards a possible interaction between

dopaminergic and serotonergic systems in the gut environment, that could be comparable to the effect seen in the brain under acute exposure of L-DOPA<sup>[31]</sup>, resulting in the release of intestinal neurotransmitters<sup>[40,41]</sup>. Results of a recent patent application show a similar increase using an *hpaBC* expressing EcN strain for the production of L-DOPA *in vivo*<sup>[42]</sup>, highlighting L-DOPA delivery and recovery of some locomotor functions in MitoPark mouse model. In spite of the short duration of the experiment, body weight of EcN\_L-DOPA treated mice (with no administration of carbidopa) showed a significant decrease compared to the control. This effect could be attributed to common side effects of L-DOPA, that include anorexia and nausea<sup>[43]</sup>, further proving L-DOPA delivery by our bacterial approach. Nevertheless, L-DOPA could be having a more profound effect by its conversion into dopamine through the action endogenous DDCs. DA can act as a agonist for several receptors and has been shown to activate browning of adipose tissue<sup>[45]</sup>, increase energy expenditure and activity<sup>[46]</sup>. Further *in vivo* experiments are still necessary to properly validate L-DOPA delivery using the strategy presented in this study and its beneficial advantages over conventional treatments for Parkinson's disease.

#### L-DOPA producing EcN inhibits *E. faecalis* through bacteriocin production

Recently, accumulating evidence suggests that gut microbiota may be playing a deeper role than previously thought regarding the initiation, progression and treatment of PD<sup>[48]</sup>. For instance, motor deficits are exacerbated when gut microbiota is transplanted from a PD patient into a germ-free PD mouse model. However, treatment with antibiotics ameliorated this pathophysiology<sup>[47]</sup>, suggesting a possible causal effect on the disease. Additionally, patients undergoing PD treatment given L-DOPA have shown higher relative abundances of *Enterococcaceae*, Clostridiales family XI among others<sup>[49]</sup>. A recent study<sup>[50]</sup> uncovered an interspecies bacterial L-DOPA metabolism pathway, consisting of the tyrosine decarboxylase (*tyrDC*) from *E. faecalis* and a dopamine dehydroxylase (*Dadh*) from *Eggerthella lenta* A2 (Fig. 7). This pathway was shown to turn administered L-DOPA into m-tyramine in the gut, contributing to a reduced treatment efficacy. Long-term L-DOPA treated patients often display variable responses and loss of treatment efficacy<sup>[51]</sup>, drug-microbiota interactions presenting a plausible driving factor<sup>[52]</sup>. Our findings demonstrate the impairment of metabolic capabilities of *E. faecalis* towards dopamine and L-Tyrosine decarboxylation by the expression of bacteriocins. Thus, postulating a novel strategy for overcoming microbiota limitations of L-DOPA delivery. Even though the *TyrDC* from *E. faecalis* is known to convert L-DOPA into dopamine, its preferred substrate is tyrosine, explaining the dramatic decrease of tyrosine and increase in tyramine among the competition cultures. Remarkably, bacteriocin expressing EcN were able to drastically impair *E. faecalis* capacity to metabolize tyrosine and had overall higher levels of L-DOPA and a closer resemblance in relative abundance compared to control strains not exposed to competition against *E. faecalis*. Other bacteriocins and combinations reported to inhibit *E. faecalis* successfully<sup>[55]</sup> were also constructed, however, based on the constitutive nature of the expression system used, many resulted toxic for EcN. Frame shift mutations and stop codon insertions were often detected (data not shown) when trying to clone the bacteriocins. Overall, these findings supports the envisioned strategy to use EcN to limit L-DOPA metabolism in the gut by *E. faecalis*, and increase bioavailability of the drug for intestinal absorption. Nonetheless, further *in vivo* characterization of these inhibitory properties are necessary to determine its applicability.

EcN produces a clear dopamine profile using DDC E

Dopamine production in *E. coli* has already been reported<sup>[54]</sup>, however, in depth characterization of the enzymatic products have not been done before. Here we show that among all the DDCs tested, DDC E gave the cleanest byproduct profile, possibly due to high specificity towards L-DOPA and discrimination against similar molecules. In addition, it appears that decarboxylated products may be inhibiting further activity of TH, as no quantifiable L-DOPA was detected. In fact, dopamine is known to inhibit in a feedback manner TH, competing with BH4 and interacting with the regulatory domain<sup>[53]</sup>.

## Conclusion

Taken together, the findings of this study outline different engineering aspects that need to be considered for the development of an advanced microbiome therapeutic for L-DOPA delivery, effectively treating Parkinson's disease (Fig. 10). Besides the introduction of a truncated variant of the Rat tyrosine hydroxylase, for driving L-DOPA production and reducing side products, known to bacterial monooxygenase variants. Upregulation of the tetrahydromonapterin biosynthetic pathway was successfully engineered to overcome bottlenecks created by the lack of cofactor needed to drive TH activity. Although, experiencing technical limitations for proper detection of catecholamines, we show biological activity of our L-DOPA producing EcN strain in mice, mainly by detecting increased serotonin levels in plasma and urine, and a significant reduction of body weight. Additionally, to our knowledge, this is the first report where efficacy to inhibit *E. faecalis* metabolism of L-DOPA has been performed using an L-DOPA and bacteriocin producing EcN strain. Lastly, a clean dopamine production profile was achieved by employing the Rat's TH and DDC E, that could pave the road towards the development of a dopamine delivery AMT.

In all, AMTs represent a novel approach for treating diseases, giving a refine assessment of the factors involved when treating the pathology. Several major aspects have been overlooked for decades, as in the case of PD and the gut microbiome. Understanding these factors could be a game changer for people suffering of chronic diseases, improving their life quality substantially. This technology, if proven efficacious, could revolutionize treatments for several diseases, as AMTs could be easily adapted to respond to physiological cues of the disease, making the treatment as personalized as possible, surpassing in any way available treatments.

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## Supplemental materials

Supplemental table 1. Genetic parts used in this study

Promoter name	Sequence	Features
trc promoter	ttgacaattaatcatccggctcgataatg	IPTG-inducible, catabolite-repressed promoter
trc promoter with lac operator	ttgacaattaatcatccggctcgataatgtgtggaattg tgagcggataacaatttcacacaggagtaaaa	With lacI binding site
trc promoter without lac operator	ttgacaattaatcatccggctcgataatgtgtggaatt cacacaggagtaaaa	lacI binding site removed
BBa_J23100	ttgacggctagctcagtcctaggtacagtgtctagc	constitutive promoter. Strongest promoter from BioBricks library
Ba_J23102	ttgacagctagctcagtcctaggtactgtgtctagc	constitutive promoter. Second-strongest promoter from BioBricks library
MSKL7	tgcttgactcgctgttccctctacgtgtataattgg	constitutive promoter, optimized for in vivo application (ref: Novel High-Throughput Methods for Rapid Development of Cell Factories. PhD Thesis, MS Klausen, 2019). Second-strongest from library
MSKL8	tgcttgactcgctgttaccctacgtgtataattggc	constitutive promoter, optimized for in vivo application (ref: Novel High-Throughput Methods for Rapid Development of Cell Factories. PhD Thesis, MS Klausen, 2019). Strongest from library

Supplemental table 2. Overview of mice groups

Group	Strain Gavaged	Carbidopa	Streptomycin
1	EcN_Empty	No	5 ml/ml in water
2	EcN_L-DOPA	No	5 ml/ml in water
3	EcN_Empty	Yes	5 ml/ml in water
4	EcN_L-DOPA	Yes	5 ml/ml in water

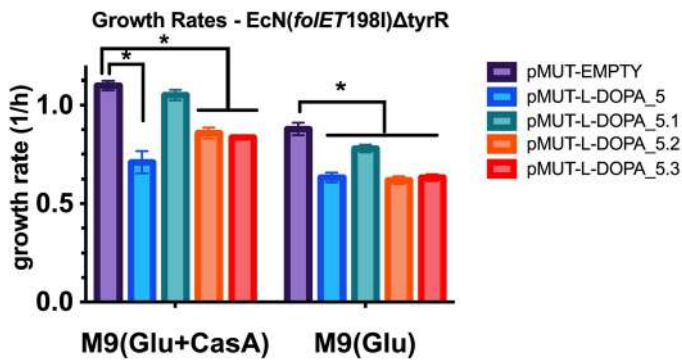
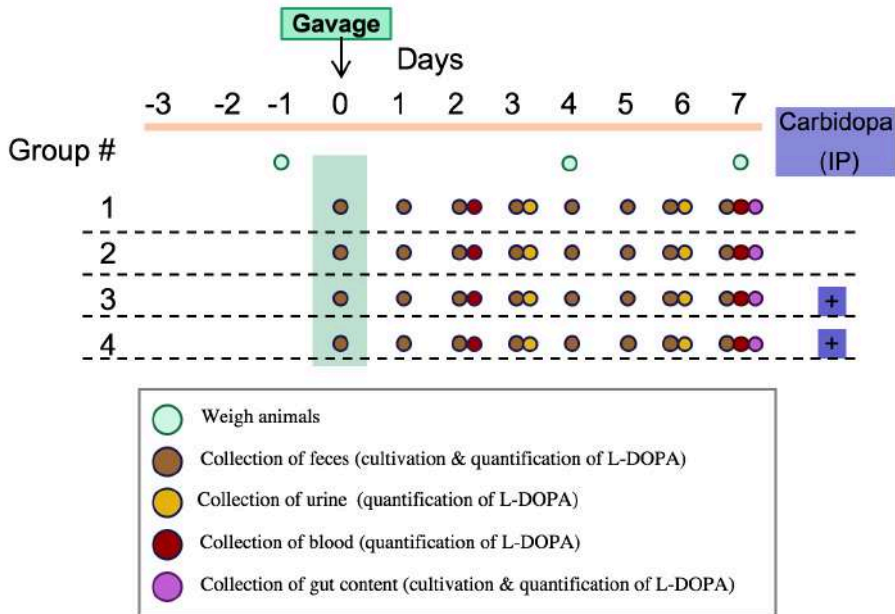
Supplemental table 3. M9 media composition

Stock Solution/ Compounds	Per 1 Liter	Comments
MQ H <sub>2</sub> O	Up to 1 L	Autoclaved
20% (w/v) glucose	20 ml for 0.4%	Filter sterilized
10X M9 salts	100 ml	House stock
2M MgSO <sub>4</sub>	1 ml	House stock
Trace elements	500 µl	Supp. Table 4
Wolfe's vitamin	1 ml	Reference 79
1M CaCl <sub>2</sub>	100 µl	House stock

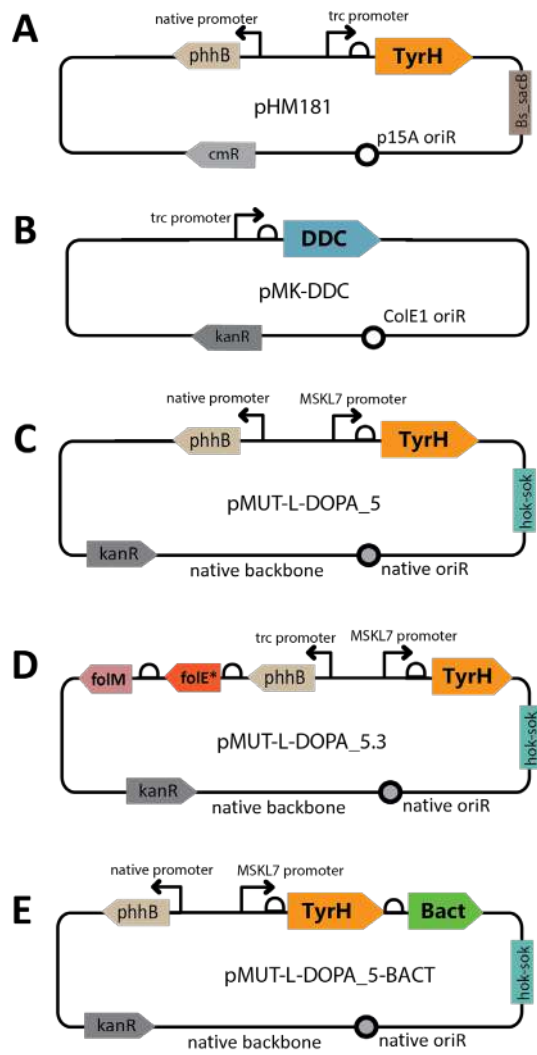
Supplemental table 4. Trace element solution

Compound	Amount per 1 L
Disodium EDTA	15 g
ZnSO <sub>4</sub> 7H <sub>2</sub> O	4.5 g
MnCl <sub>2</sub> 4H <sub>2</sub> O	0.7 g
CoCl <sub>2</sub> 6H <sub>2</sub> O	0.3 g
CuSO <sub>4</sub> 2H <sub>2</sub> O	0.2 g
Na <sub>2</sub> MoO <sub>4</sub> 2H <sub>2</sub> O	0.4 g
CaCl <sub>2</sub> 2H <sub>2</sub> O	4.5 g
FeSO <sub>4</sub> 7H <sub>2</sub> O	3 g
H <sub>3</sub> BO <sub>3</sub>	1 g
KI	0.1 g

Supplemental figures



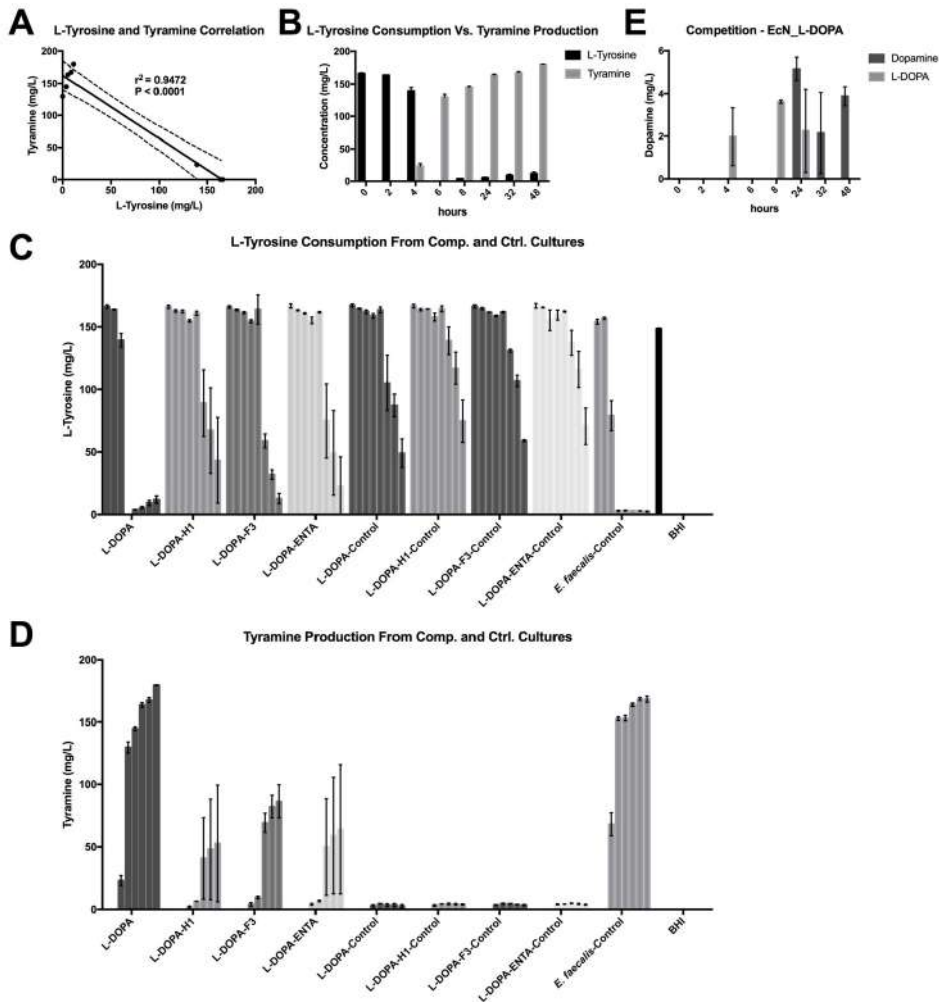




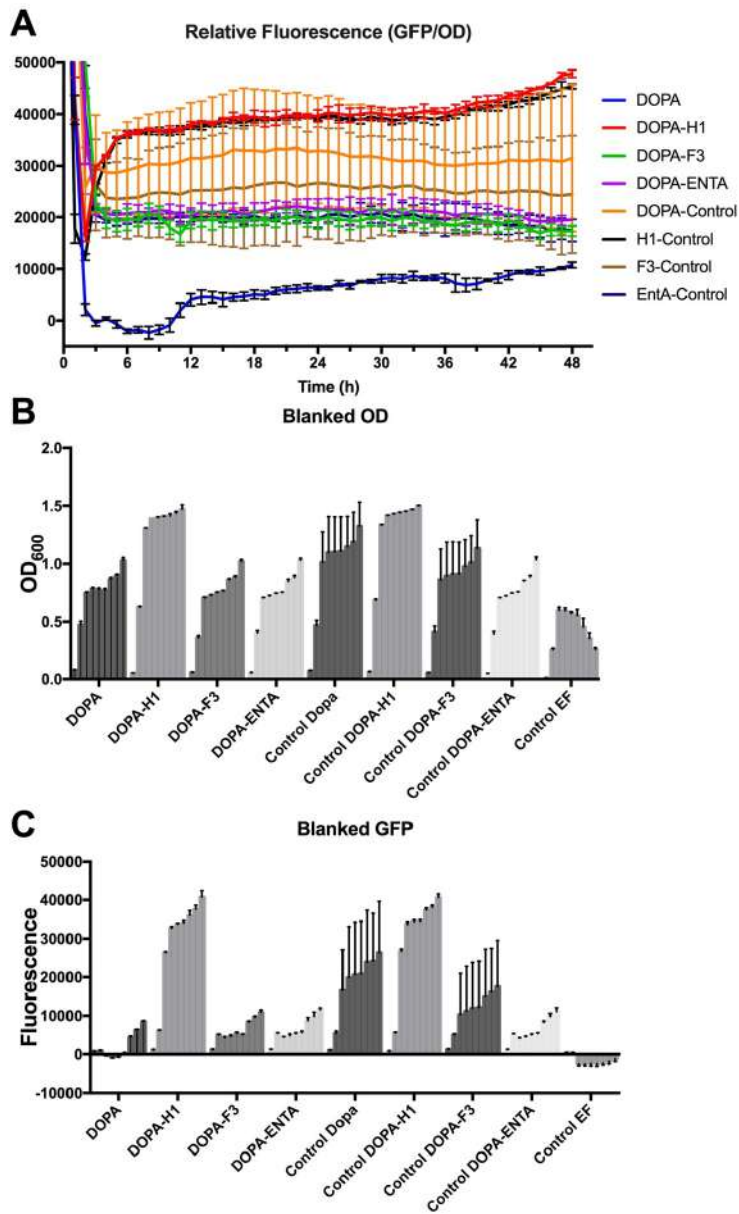
Supplemental figure 3. Schematic representation of plasmids constructed for this study.



Supplemental Figure 4. Inhibition halos of *E. faecalis*, as a result of L-DOPA producing EcN co-expressing bacteriocins. Left, overnight supernatants inhibiting *E. faecalis*. Middle, EcN strains inhibiting *E. faecalis*. Right, EcN control strains (no bacteriocin production) showing no inhibition.



Supplemental Figure 5. A) Shows L-Tyrosine and tyramine concentrations from competition experiment with a control strain not producing any bacteriocins. B) Shows correlation analysis between L-Tyrosine and tyramine concentrations through the competition experiment of the same control strain. C & D) Shows L-Tyrosine and tyramine concentrations of all cultures throughout the experiment, respectively. E) Shows L-DOPA and dopamine profiles of the non-bacteriocin expressing EcN\_L-DOPA strain.



Supplemental figure 6. Relative fluorescence (A), blanked OD<sub>600</sub> values (B) and blanked GFP values (C) from competition and control cultures.

## V. Concluding Remarks and Perspectives

The main objective of the thesis was to develop advanced microbiome therapeutics for treating disorders related to bile acids and neurodegeneration. Even though, the final AMT for the proposed disorders has not been achieved yet, extensive information has been gathered for improving the designs and optimizing production and therapeutic capabilities *in vivo* of such AMTs. In the following paragraphs the key conclusions and perspectives for each experimental chapter will be summarized.

Chapter II suggests that, contrary to previous reports, bile acids are not a native product of marine isolates. It is highly possible that as a consequence of inadequate use of control strains and analytical techniques, previous research reached the conclusion that marine bacteria could produce bile acids. We further support our claims by the identification of putative BSH genes and a cholate degrading pathway, which may account for the different bile acid profiles observed in other studies.

Chapter III explores different approaches for the identification of strategies for degrading and/or neutralizing secondary bile acids. Even though many aspects of the functional screening could be optimized, it seems unlikely that catabolic genes can be found in this manner. Results, however, gave a very interesting view of complex resistance mechanisms employed by bacterial species in order to withstand the toxic effect of bile acids. The utilization of specific compounds of interest as the sole carbon source for the isolation of bacterial species capable of degrading these molecules seemed to be a valid approach for identifying deoxycholic acid degraders. Nevertheless, proper validation with the corresponding biological replicates is still necessary to be certain of these results. However, if proven true and safe, these strains could have the potential to be employed as true probiotics for dealing with secondary bile acid toxicity *in vivo*. Lastly, we report an engineered strain of *E. coli* Nissle 1917 capable of sulfonating secondary bile acids, by expressing a human sulfotransferase. Novel metabolic engineering targets, consisting of native sulfatases and sulfatase maturing enzymes, are identified and tested for optimizing sulfonation of deoxycholic acid. *In vivo* validation of the efficacy of such strain is still required to properly evaluate its therapeutic potential.

Chapter IV demonstrates the use of a mammalian tyrosine hydroxylase for the production and delivery of L-DOPA *in vivo*. Tetrahydromapterin biosynthesis was identified to be a bottleneck for the production of L-DOPA, when using a TH. Experiments having EcN co-express bacteriocins, in order to limit *E. faecalis* metabolic effect with regards to L-DOPA, suggested a clear inhibiting in dopamine formation and tyrosine decarboxylation. Even though proper quantification of L-DOPA was not achieved, a significant change in body weight and a tendency to increase serotonin was observed in mice, suggesting biological activity of the L-DOPA AMT. Screening of a panel of decarboxylases led to the identification an enzyme capable of producing clear dopamine profiles with no quantifiable byproducts. Despite the initial indications, further investigations need to take place in order to validate L-DOPA delivery *in vivo* and the pharmacokinetic profiles achievable by an AMT approach. The effect on body weight could be a promising therapeutic effect that needs to be investigated further. Lastly, proper validation of the AMT in a Parkinson model would be necessary to assess its real efficacy for treating the disease.

In all, the development of an AMT is not trivial, there are numerous aspects to take into consideration not only from an engineering perspective but also from a medical one. The field requires and would be most benefited from interdisciplinary interactions along these lines. This thesis had very ambitious goals that unfortunately will need to be achieved in following investigations. Nevertheless, this work will serve as a foundation and reference for future efforts.