# The role of diet in host susceptibility to *Listeria monocytogenes* infection


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# Thesis Abstract

Currently the world is facing an epidemic increase in metabolic disorders linked to the Western-diet, a diet consisting of an increased intake of ready-to-eat food products rich in sugar and animal fat. Diet is a significant influencer of gastrointestinal function and the interplay between epithelial physiology, intestinal inflammatory state and commensal bacteria is fundamental to ensure gut homeostasis. Recent epidemiological reports emphasize that diet is a key lifestyle factor driving the rise of gastrointestinal inflammatory diseases. Disease pathology is likely to be linked to aberrant modulation of regulatory events associated with intestinal physiology, inflammatory regulation, nutrient availability in the lumen and microbiota composition and metabolism. Dietary-driven modulation of the environment in the gastrointestinal tract directly affects bacterial adaption in the gut by redirecting bacterial metabolism in response to nutrient availability. This is particularly critical in the context of infection with foodborne pathogens, where diet is beginning to be implicated as an external factor altering host susceptibility to infection.

Even though a Westernized-diet has been linked with the development of pathological inflammatory conditions, relatively little is known regarding the implications of such diets in the progression of infectious disease. For this reason, the focus of this research was to investigate the impact of short-term dietary interventions in host susceptibility to infection with an important foodborne pathogen, *Listeria monocytogenes*.

*L. monocytogenes* is the etiological agent of listeriosis, an invasive foodborne disease with a high fatality rate of 20-30%. By coordinating a complex regulatory gene network, *L. monocytogenes* is able to sense and adapt to a vast range on environmental stimuli, essential for survival in both saprophytic and gastrointestinal environments.

*L. monocytogenes* is of special concern in ready-to-eat meat products, with several listeriosis outbreaks traced to the consumption of such foodstuffs.

Here, we determine the impact of the consumption of a Western-diet upon susceptibility to *L. monocytogenes* in a murine disease model. We adopted a systems approach to identify the effects of a two-week dietary supplementation with either animal fat or L-carnitine, through analysing changes in host physiology and microbiota composition.

In the present study we evaluate the effects of a high-fat diet on modulation of the host physiological landscape and microbiota composition, parameters associated with the *L. monocytogenes* infectious process, both before and after oral infection. Our data indicate that a short-term increase in dietary fat intake results in an increased susceptibility to infection, related to significant alterations in host microbiota composition, epithelial cell and immune cell function in both the ileum and liver.

In addition, we analyse the role of L-carnitine in the *L. monocytogenes* transcriptomic profile in response to osmotic stress and determine the role of OpuC (part of *L. monocytogenes* osmotic stress response machinery) in the pathogens growth and virulence *in vitro*. Our results show that L-carnitine ameliorates the effects of the osmotic stress response on *L. monocytogenes* fitness and cellular homeostasis and induces virulence gene expression. Furthermore, we demonstrate that L-carnitine uptake trough OpuC is fundamental for *L. monocytogenes* growth and invasion of epithelial cells during the osmotic stress response under conditions mimicking the gut environment. Finally, we analysed the role of dietary supplementation with L-carnitine on the ability of *L. monocytogenes* to adapt to the high osmolarity environment of the gastrointestinal tract and establish oral infection in the murine model. The results

suggest that additional L-carnitine in the diet does not increase susceptibility to oral *L. monocytogenes* infection, though the OpuC system does play a significant role in the infectious process. Overall the work significantly enhances our understanding of how diet may influence host parameters which are significant for the progression of *L. monocytogenes* infection.

# Acronym List

**ANOVA**- Analysis of variance

**ABS**- ATP-binding cassette

**Agr**-Accessory gene regulator

**APC**- Antigen-Presenting Cells

**ATP**- Adenosine triphosphate

**BCFA**- Branched-Short-Chain Fatty Acids

**BHI**- Brain Heart Infusion

**Ccl2**- C-C motif chemokine 2

**c-di-AMP**- Diadenylate Cyclase

**CFU**- Colony forming units

**Cyp7a1**- Cholesterol 7 alpha-hydroxylase enzyme

**D**- Day

**DB-FFAP**- Chromatography column

**DMEM**- Dulbecco's modified Eagle's medium

**DMEMS**- Dulbecco's modified Eagle's medium supplemented with 3% NaCl

**DMEMSC**- Dulbecco's modified Eagle's medium supplemented with 3% NaCl and 1mM L-carnitine

**DMM**- Defined Minimal Media

**DS**- Defined Minimal Media supplemented with 3% NaCl

**DSC**- Defined Minimal Media supplemented with 3% NaCl and 1mM L-carnitine

**DTX**- Di-N-butyl phthalate in xylene

**E-cad**- E-cadherin

**EHEC**- Enterohaemorrhagic *E. coli*

**FBS**- Foetal Bovine Serum

**FDR**- False Discovery Rate

**FOS**- Fructooligosaccharides

**FOXP3**- Fork head box P3

**gC1qR**- Receptor for the globular component of complement C1q

**GC-FID**- Gas Chromatography Flame Ionisation Detection

**GLP1**- Glucagon-like peptide 1

**GOS**- Galactooligosaccharides

**GP96**- Endoplasmic reticulum chaperone

**GPR**- G-protein coupled receptors

**$H_2O$**- Water

**HF**- High-fat

**IBD**- Inflammatory Bowel Disease

**IBS**- Irritable Bowel Syndrome

**Ig**- Immunoglobulin

**IG**- Intragastric

**IL**- Interleukin

**IFN-γ**- Interferon-gamma

**Inl**- Internalin

**iNOS**- inducible Nitric Oxide Synthase

**IP**- Intraperitoneal

**IVIS**- *In vivo* Imaging System

**LB**- Lysogeny Broth

**LCFA**- Long-chain fatty acids

**LEE1**- Locus for enterocyte effacement-encoded regulator 1

**LF**- Low-fat

**Lgr5$^{+}$**- Leucine-rich repeat-containing G protein coupled receptor 5

**LLS**- Listeriolysin S

**LPS**- Lipopolysaccharides

**M 1/2-** Macrophage 1/2

**MCFA-** Medium-chain fatty acids

**MET**- Hepatocyte growth factor receptor

**mN-cad**- murine N-cadherin

**mrp2**- ATP-binding cassette, sub-family C, member 2

**muc 2**- Mucin-2

**Myd88-** Myeloid differentiation primary response 88

**NaCl**- Sodium Chloride

**NF-κB**- Nuclear-factor kappa B

**NS**- Non-significant

**ntcp**- $Na^{+}$-taurocholate co-transporting polypeptide

**OCTN**- Organic cation transporters

**OxyR**- Oxidative response regulon

**PAS**- Periodic Acid Schiff

**PBP-** Type-2 periplasmic binding fold-protein

**PBS-** Phosphate-Buffer Saline

**PCoA-** Principal Component of Analysis

**PiK3 ap1**- Phosphoinositide 3-kinase adapter protein 1

**PMN**- Polymorphonuclear leukocytes

**PrfA**- Positive regulatory factor A

**$pT_{reg}$**- Peripherally derived regulatory T-cells

**PTS-** Phosphotransferase Systems

**PUFA-** Polyunsaturated fatty acids

**qRT-PCR**- Reverse transcription quantitative Real-time polymerase chain reaction

**RAB10**- Ras-related protein

**RDP-** Ribosomal database project

**RegIII-γ**- Regenerating islet-derived protein III-gamma

**RNA**- Ribonucleic acid

**RNA-seq**- Sequencing of RNA transcripts

**RS3**- Type-3 resistant starch

**SCFA**- Short-chain fatty acids

**SEM-** Standard-deviation of the mean

**SigB-** σB-dependent promoter

**Spingo-** Species-level identification of metagenomics amplicons

**SRA**- Sequence read archive

**Stx**- Shiga toxin

**T3SS**- Type III secretion system

**TCR**- T-cell receptor

**TLR**- Toll-like receptor

**TMA-** Trimethylamine

**TMAO**- Trimethylamine-N-oxide

**TNF-α**- Tumour necrosis factor alpha

**TOS**- Transgalactostlated oligosaccharide

**$T_{reg}$**- Regulatory T-cells

**UCHIME**- Algorithm for detecting chimeric sequences

**UK**- United Kingdom

**USEARCH**- Sequence analysis tool

**Wnt-β-catenin**- Wingless/Integrated-beta -catenin

**WT**- Wild-type

**XOS-** Xylooligosaccharides

**ZO-** Zonula occludens

**γBB-** γ-butyro-betaine

# Chapter I

# The links between diet and infection:

# Impact of food matrix composition in host susceptibility to bacterial infection

## Literature Review

# Contents

# 1. Abstract

The growing metabolic disease epidemic has been linked to current dietary transitions towards an increased consumption of processed sugars as well as fat from animal sources. The relationship between epithelial physiology, the intestinal immune system and commensal bacteria is essential to preserve healthy gut homeostasis and determine host susceptibility to disease. Epidemiological studies highlight that diet is one of the most influential lifestyle factors contributing to the rise of gastrointestinal inflammatory diseases, reflecting the fact that diet is involved in many regulatory events associated with the intestinal landscape, microbiota composition and nutrient availability in the lumen. The modulation of the gastrointestinal environment directly affects the ability of bacteria to adapt, interfering with their metabolism in response to nutrient fluctuations. In the context of infection with foodborne pathogens this is particularly critical, as diet is associated with altered susceptibility to infection.

In this review we cover the role of diet in the host susceptibility to infection with foodborne pathogens, by evaluating the interplay between host dietary composition, gastrointestinal physiology and inflammatory regulation, and microbiota composition and metabolism.

# 2. Introduction

Populations worldwide have experienced a series of nutritional transitions which are associated with socio-economic development and improved food availability. Nutritional transition is a process of sequential modifications in the pattern of nutrition and consumption, which accompanies changes to the health profile of a population. Recent studies focused on the current dietary transition to a so-called Western-diet, which shows a trend towards the increased consumption of meat, fat, processed food, sugar, and salt, a phenomenon seen especially in developed countries (de Boer and Aiking, 2019; Juneja et al., 2019).

Currently the world is facing an epidemic incidence of chronic illnesses and health problems highly associated with diet, which represents a serious threat to public health (Cordain et al., 2005). Evidence suggests a link between nutritional transitions and chronic diseases of the gastrointestinal tract, including Crohn's disease, Irritable Bowel Syndrome (IBS) and ulcerative colitis, together with more systemic diseases such as obesity and type I and type II diabetes and related metabolic comorbidities, such as cardiovascular diseases and non-alcoholic fatty liver diseases (Ussar et al., 2015). Understanding the interplay between the risk factors, such as diet (caloric intake and dietary composition) and sedentary lifestyle (levels of activity and non-exercise-related energy expenditure), and their role in the increased incidence of many of these inflammatory and metabolic diseases has become a major priority in order to fight this epidemic trend (Bortolin et al., 2017).

The gastrointestinal environment represents a dynamic system where many factors are involved in several intestinal biological functions, having a crucial role in maintaining energy homeostasis (O'Neill and O'Driscoll, 2015). The intestinal epithelium permits

vital interactions with the external environment, allowing the absorption of nutrients and fluids while providing a protective barrier against toxins and microorganisms, while also maintaining a symbiotic relationship with a complex ecosystem consisting of thousands of microbial species, known as the intestinal microbiota (Heinken and Thiele, 2015). Nutrients that reach the upper gastrointestinal tract are digested by endogenous enzymes and become available as substrates for bacteria in the gut, therefore dietary changes are directly related to changes in the gut microbial environment. In fact, many studies have documented changes in the molecular ecology of gut microbial communities in response to dietary changes (Ding et al., 2010). Furthermore, the intestinal microbiota has been tightly associated with host metabolic regulation, especially as an influencer of the immune response (**Fig. 1**). Thus, dietary changes may alter the host's ability to resist infection by foodborne pathogens (Brown et al., 2012). In this review we discuss the role of dietary matrix composition as an extrinsic factor contributing to changes in the gastrointestinal environment with a consequent influence on host resistance against foodborne infectious diseases.

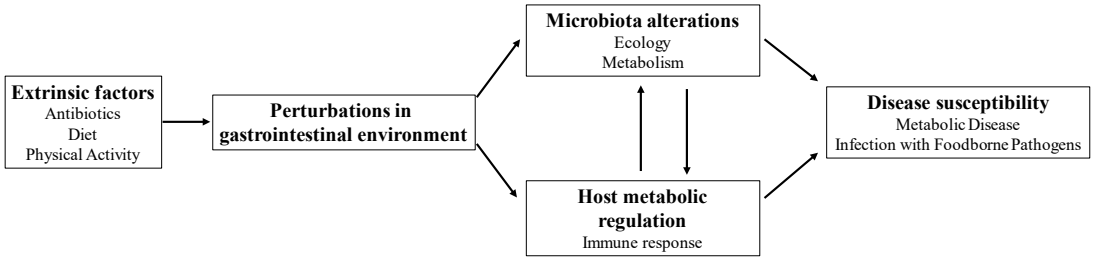


**Figure 1.** Linking nutritional transitions in Westernized-diet with increased incidence of metabolic disorders and susceptibility to infection with foodborne pathogens.

# 3. Dietary modulation of host physiology

## 3.1. The gut epithelium

With its remarkable surface area, epithelial cells have two particular physiological functions in the body, firstly as a barrier to external stimuli, and secondly as a selective transporter of nutrients, fluid and electrolytes. At a physiological level, it is possible to divide the adult intestinal epithelium into two structurally and functionally different areas, the crypt and the villus (**Fig. 2**). The base of the crypt is populated by both Paneth cells, responsible for the secretion of antimicrobial peptides and maintenance of stem cell niche environment, and intestinal stem cells, which differentiate into mature cells that will move upwards along the villus-crypt axis. The villi are mainly populated by three different types of mature cells: enterocytes, goblet cells and enteroendocrine cells. Enterocytes are the most abundant of the epithelial cells and their main role is nutrient absorption. Goblet cells are responsible for the synthesis and secretion of the glycoproteins mucins, which will form a mucosa layer over the mucosal surface, ensuring barrier function and facilitating the movement of the luminal content. Finally, enteroendocrine cells constitute the largest endocrine system in the body and are involved in the secretion of gut hormones that regulate major biological functions (Kong et al., 2018).

The organization of these cell types along the epithelium ensures the regulation of food intake, gastric emptying, gut motility and gut barrier formation among other major biological and physiological functions (Levitan et al., 2018).

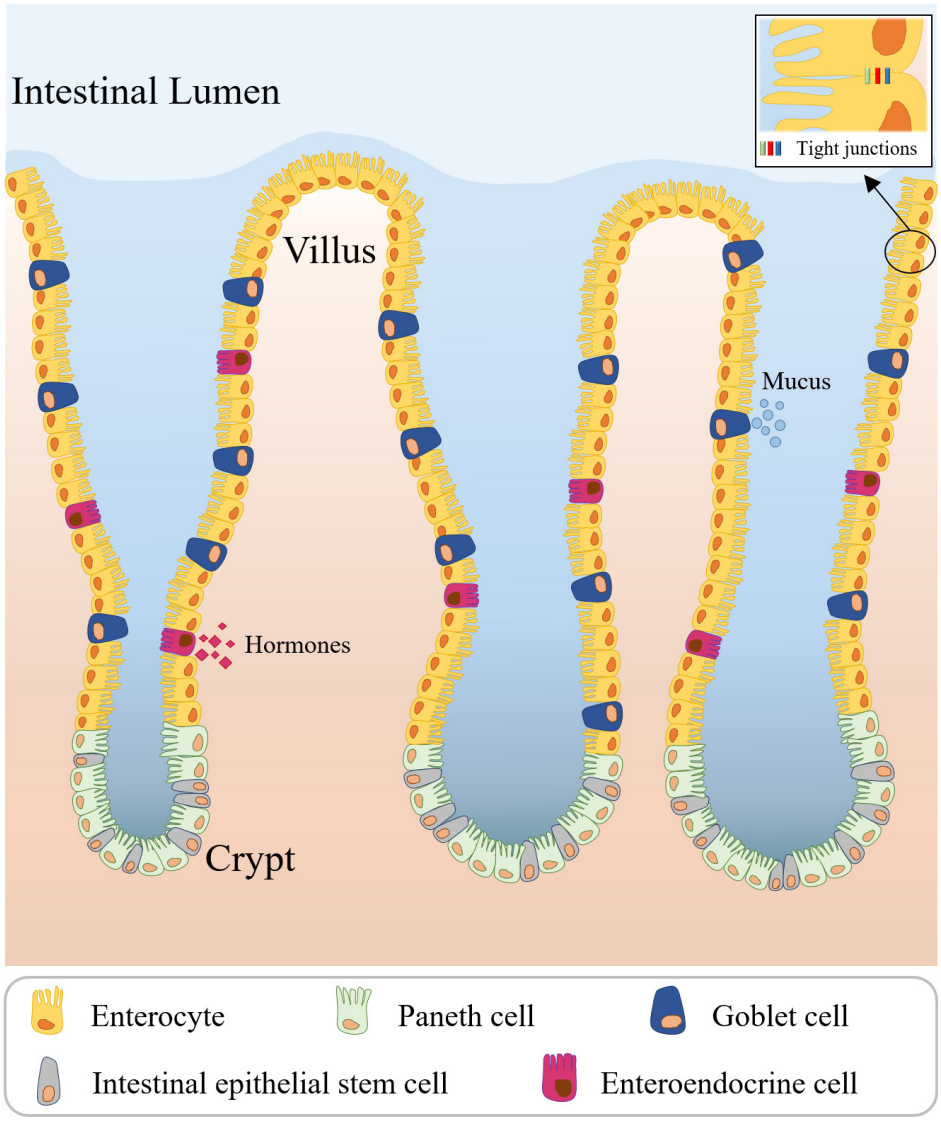


**Figure 2.** Schematic representation of the key epithelial cell types of the small intestine epithelium, highlighting their identity and spatial arrangement. Regeneration and maintenance of the intestinal epithelium is ensured by intestinal stem cells (located at the bottom of the crypt). These cells can proliferate or differentiate into mature cell types required for normal gut function.

Gut barrier function is ensured by epithelial tight-junction proteins between adjacent epithelial cells which maintain a barrier between apical and basolateral membrane domains. This is key in regulating the paracellular trafficking of macromolecules. This paracellular diffusion barrier is semi-permeable, being able to select based on both charge and size of the molecules (González-Mariscal et al., 2003). Furthermore, tight-junction proteins form a bidirectional signalling platform between adjacent cells and are involved in signal transduction into the cell interior to regulate migration, differentiation, proliferation and survival. The junctional protein complex includes transmembrane proteins such as claudins and occludin, adaptor proteins and cytoskeletal linkers, such as the zonula occludens (ZO) proteins ZO-1, ZO-2 and ZO-3 (Zihni et al., 2016).

The intestinal barrier prevents the permeation of pro-inflammatory molecules, antigens and foodborne pathogens, from the luminal environment into the circulatory system. Disruption of the intestinal tight-junction barrier leads to increased paracellular permeability, inducing an alteration of the mucosal immune system and inflammatory state, which consequently may act as a trigger for the development of intestinal and systemic diseases such as Inflammatory Bowel Disease (IBD) or type I diabetes. Intestinal barrier function and paracellular permeability is dynamically regulated by multiple extracellular stimuli and is directly associated with susceptibility to disease (González-Mariscal et al., 2014; Lee, 2015).

### 3.2. Role of nutritional modulation in gastrointestinal physiology and energy homeostasis

The development, maintenance and function of the epithelial barrier are strongly influenced by external nutrition. Nutrients have a major role in the physiological state

of intestinal epithelial cells and play a major homeostatic role which encompasses processes such as differentiation, proliferation and renewal (Kong et al., 2018).

Cell differentiation and proliferation is driven by the luminal content, and not systemic nutrients (Winesett et al., 1995). The quantity and type of nutrients consumed impacts these cellular processes and results in alterations in size and cellular make-up of the epithelial tissue (Dailey, 2014). Understanding the mechanisms behind the nutritional modulation of gastrointestinal physiology and metabolism is necessary to appreciate the extent at which external factors influence susceptibility to disease.

In general, studies demonstrate that the increase in the luminal content of any nutrient works as a stimulus for modulation of epithelial cell proliferation, villi length or crypt depth, while nutrient deprivation does the opposite (Dunel-Erb et al., 2001). These effects of luminal nutrients in epithelial physiology have been reported to be independent of systemic factors and body weight. Obese patients, showing increased villi height and deeper crypts, present a decrease in cell proliferation rate of colonic epithelial cells when under caloric restriction (Steinbach et al., 1994). The amount of luminal nutrients may therefore be a critical factor in determining the differences in epithelial morphology between obese and lean patients and consequently a driver of gastrointestinal heath.

### 3.2.1. Dietary fibre

Previous work revealed that a diet with increased fibre consumption results in alterations to mucosal morphometry (Wenk, 2001). Dietary non-starch polysaccharides and lignin, more specifically, oligofructose and long-chain inulin, have been linked with an increased height of the villi coupled with deeper crypts and a thicker mucous layer associated with an increased population of goblet cells

(Kleessen et al., 2003). Furthermore, dietary fibre has been linked to increased intestinal transit and delays in gastric emptying, which leads to the slower absorption of nutrients. These epithelial physiological alterations appear to be associated with a decrease in the ratio between villi height and crypts depth coupled with the increase in cell proliferation rate in the crypt, leading to a faster cell turnover in the small intestine (Lien et al., 2001; McDonald et al., 2001; Montagne et al., 2003).

### 3.2.2. Glucose intake

Evidence demonstrates that higher glucose intake induces general physiological changes at the level of the intestinal epithelium, such as the increase of proliferation of cells in the crypt. This is supported by changes at the level of cell metabolism, driving the energy production through the glycolysis pathway and consequently increasing the mitochondrial respiration capacity of these cells (Zhou et al., 2018). Favouring glycolysis is essential for biomass production by cells proliferating under elevated rates of cellular turnover (Vander Heiden et al., 2009).

Increased cell proliferation is important to maintain intestinal homeostasis, by keeping a healthy crypt depth/villus height ratio.

### 3.2.3. High-protein diet

Proteins are another nutritional component that has been previously reported to affect epithelial morphology and metabolism (Johnstone et al., 2011). Studies suggest that diets containing a high percentage of proteins from milk may have a potential to modulate immune homeostasis in the gastrointestinal tract. Rats fed with a protein rich diet showed increased expression of the gene encoding for TNF-α, while several genes related to innate immunity were down-regulated. Furthermore, other cellular metabolic functions also seem to be affected by such specific diet composition, with

the down-regulation of oxidative phosphorylation occurring simultaneously with the up-regulation of genes associated with cellular apoptosis, suggesting again an increase in cellular turnover rate (Mu et al., 2016). Moreover, increased dietary protein content has also been linked with increased intestinal transit, shown by a higher colonic content weight of animals fed a high-protein diet in relation to the control (Andriamihaja et al., 2010; Beaumont et al., 2017).

### 3.2.4. High-fat diet

Despite the elevated morbidity associated with high levels of lipid intake and deregulated lipid storage seen in obese patients, our knowledge of lipid-regulated mechanisms involved in epithelial physiology modulation is to some extent limited.

Models of high-fat (HF) induced obesity reveal an effect of fatty acids, more specifically palmoleic acid and omega-3-polyunsaturated fatty acids (omega-3 PUFAs) in modulating the induction of stem cell differentiation and proliferation through modulation of the Wnt-β-catenin signalling pathway (Efeyan et al., 2015). It has also been demonstrated that the intestinal absorption capacity can be adjusted to the fat content in the diet. Studies with mice suggest that a diet rich in triglycerides is able to induce intestinal cell proliferation, increases in villi height and enterocyte migration rate along the crypt-villus axis (Petit et al., 2007). Diets with an increased intake of fat from animal sources (60% of caloric intake from fat) are reported to reduce villi height and increased crypt depth coupled with higher number of stem cells expressing $Lgr5^{+}$ (proliferating stem cells induced by the Wnt-β-catenin signalling pathway), indicating an increased rate of self-renewal at the expense of differentiation (Beyaz et al., 2016). Another mouse model study revealed that a diet containing 46% of caloric intake from fat compromised the integrity of the intestinal barrier, with

down-regulation of tight-junction proteins such as claudin-1, followed by an increased level of endotoxins in the serum (Gulhane et al., 2016). Evidence also seems to indicate that later stages of increased dietary fat feeding are associated with increased goblet cell differentiation and mucous layer thickness (Plovier et al., 2016). Although the impact of most dietary lipids remains elusive, it can be seen that not only does high dietary fat trigger a coordinated response at the level of energy metabolism, nutrient metabolism and absorption and epithelial physiology but also that the intestine retains a remarkable mechanism for modulation of intestinal morphology influenced by the nutritional status.

A schematic of the potential behind dietary modulation of gastrointestinal physiology is presented bellow (**Fig. 3**).

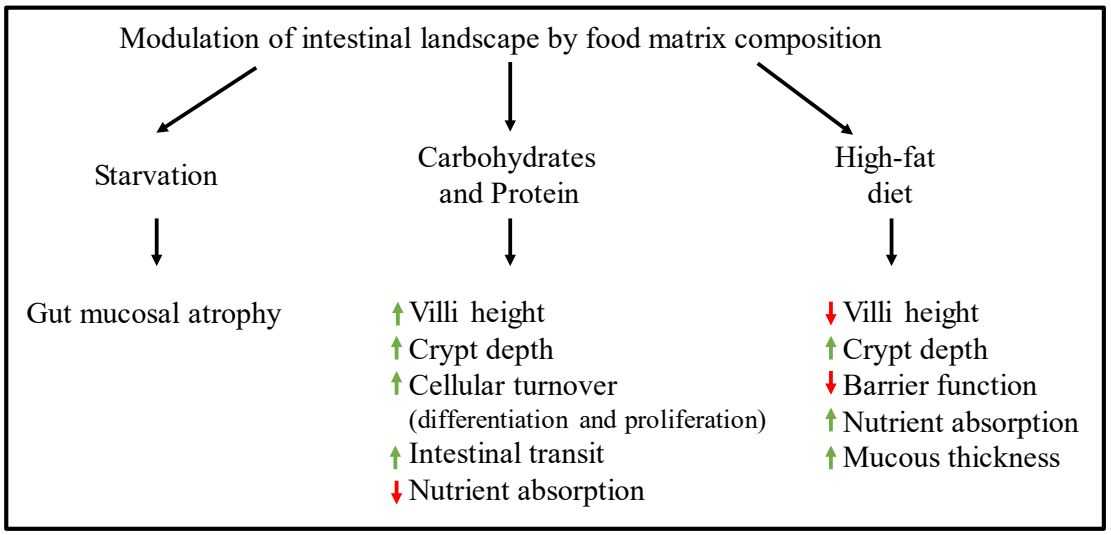


**Figure 3.** Impact of the host diet upon intestinal epithelial physiology. The availability and specificity of nutrients modulates key cellular mechanisms in the host, indicating a crucial link with the endocrine and immune system. The luminal content is clearly a driving force shaping gastrointestinal landscape, highlighting the host diet as a key factor in intestinal health and a necessary target for therapies against metabolic disorders and enteric disease.

# 4. Cross-talk between intestinal microbiota and host during health and disease

The intestinal microbiota refers to the microbial community of the human intestine, comprising thousands of different bacterial species (Bäckhed et al., 2005). In adults, the gut microbiota is typically dominated by Firmicutes and Bacteroidetes phyla, whereas Proteobacteria, Verrucomicrobia and Actinobacteria, are generally minor constituents (Eckburg et al., 2005). Changes in microbial diversity (number of unique species) or relative abundance (representation of an individual species as a percentage of the overall diversity) are generally associated with a disruption in the microbiota and are regarded as a potential risk for metabolic disorders.

The complex but dynamic microbial ecosystem has evolved to establish a symbiotic relationship with the host. (Amit-Romach et al., 2008; Round and Mazmanian, 2009). The microbiome (the collective genomes of the microorganisms in the gastrointestinal environment) has the potential to significantly influence host physiology in health and disease (Valdes et al., 2018). This well-established mutualistic relationship provides the host with metabolic and genetic attributes, such as the extraction of nutrients from otherwise indigestible compounds and involvement in the development of the intestinal architecture. Furthermore, the microbiota has been linked with the clearance of opportunistic pathogens by working as a barrier against colonization (O'Mahony et al., 2008).

Characterization of the baseline healthy microbiota would allow an understanding of the symbiotic relationship between the microbiota and their host in order to establish differences in the context of health and disease. Yet, determining what would be considered the “normal” microbiota within a population or an individual can be quite

challenging, due to the high complexity of the ecosystem and variation between and within individuals (Everard and Cani, 2013). Understanding factors influencing changes in microbiota composition and function in the gut will aid in the design of therapies that target host susceptibility to metabolic disorders and infectious disease.

### 4.1. Diet as a key factor for microbiota modulation

Nutrient availability is a modulating factor for commensal bacterial metabolism in the gastrointestinal tract. The host diet has a strong and direct impact on the nutrient pool available in the gut, therefore, changes in the diet can modulate microbial ecology. Diet not only induces alterations at the level of microbial diversity and composition, but also at the functional level, with reports showing changes in bacterial proteomic profile, with pathways involved in amino acid and bile acid metabolisms being particularly affected (Scott et al., 2013). Moreover, metabolic analysis identified a diet specific profile of the colon content, which included differences at hormonal and antimicrobial levels (Daniel et al., 2014).

Bile acids are also known for their critical role in microbiota composition modulation, due to their bacteriostatic activity. The amount of circulating bile acids signals the feedback mechanisms across the gut-liver axis (Vajro et al., 2013). Disruption of bile flow has been previously linked with decrease in barrier function and bacterial proliferation in the small intestine (Inagaki et al., 2006), with reports demonstrating that external bile acid administration manipulates the gut microbiota composition (Musso et al., 2010). Moreover, the ability of the microbiota to metabolize intestinal bile acids into their unconjugated forms is essential for metabolic homeostasis in the gastrointestinal tract (Jia et al., 2018).

Short-chain fatty acids (SCFA) such as butyrate, propionate and acetate, are involved in the modulation a range of tissue-specific mechanisms, including barrier function and immune modulation in the gastrointestinal tract (Chambers et al., 2018). Studies have shown that SCFA are able to modulate hormonal secretion involved in glucose and energy homeostasis, as SCFA receptors are co-localized in the epithelium with enteroendocrine cells that secrete key modulators of glucose metabolism, such as GLP-1 (Lin et al., 2012). SCFA availability in the intestinal lumen is determined by the composition of the microbial community, as they are primarily produced as microbial by-products of fermentation (Davis, 2018). SCFA play a critical role in the link between diet and microbial metabolism in the intestine.

Studies relating the interaction between the microbiota and host epithelia have revealed its critical role in modulation of gastrointestinal physiology. Recent studies have reported the discovery of a human microbiota-encoded long-chain N-acyl amide that interacts with G-protein-coupled receptors (GPRs) and, therefore, are able to regulate diverse cellular functions. GPRs are a family of membrane receptors in eukaryotes identified as key mediators of the host-microbiota dialogue and a common target for therapeutic approaches against metabolic disorders. Evidence seems to suggest that convergence between human signalling molecules and microbiota-produced primary metabolites are a common commensal mechanism of modulation of host cellular responses (Cohen et al., 2017).

### 4.1.1. Prebiotics

Increased dietary intake of prebiotics also appear to have a considerable impact upon the microbiota landscape and metabolism (**Fig. 4**). By definition, prebiotics correspond to nutrients able to induce specific changes in the composition or metabolic

activity of the microbiota, conferring benefits to the host health (Gibson et al., 2004). The most common prebiotics used include xylo-oligosaccharides (XOS), galacto-oligosaccharides (GOS) and fructans, including fructo-oligosaccharides (FOS) and inulin. A study has shown that increased dietary intake of FOS caused a considerable modulation of the microbiota, and also reported the alteration of 25 bacterial taxa positively correlated with the increased number of L-cells (enteroendocrine cells) (Cani et al., 2013). Furthermore, dietary FOS also seemed to be associated, in both mice and human studies, with the 100-fold increased abundancy of bacteria belonging to the genus *Akkermansia*, producers of propionate and acetate, bioactive compounds in the regulation of GPR43 (modulator of GLP-1 secretion) (Everard et al., 2013). Kleessen et al. (2001) showed that a mix of FOS and long-chain inulin increased the abundancy of the Clostridiales cluster XIVa (Firmicutes phylum), an effect not seen by FOS or inulin alone (Kleessen et al., 2001).

The increased intake of prebiotics and consequent alterations of microbiota also seem to be associated with the fermentation of non-digestible carbohydrates, affecting the gastrointestinal availability of bioactive metabolites, such as SCFA. Studies have identified an increase in bifidobacteria and *Collinsella aerofaciens* (belonging to the Firmicutes phylum) in humans, and an increase in *Roseburia* and *Eubacterium* in humanized rat models upon a dietary increase of FOS, coupled with a consequent increase in butyrate production (Ramirez-Farias et al., 2008; Van den Abbeele et al., 2011). Studies revealed the inter-individual variation that occur in response to prebiotic treatment approaches, indicating differences in the bacterial ability to metabolize prebiotics at both the species and strain-specific level (Roberfroid et al., 2010; Scott et al., 2013). It is therefore possible to hypothesize that the induction of beneficial physiological effects in the gastrointestinal tract could be individual-

specific and be based on a combination of both type of prebiotic and specific microbial strain and/or community administration.

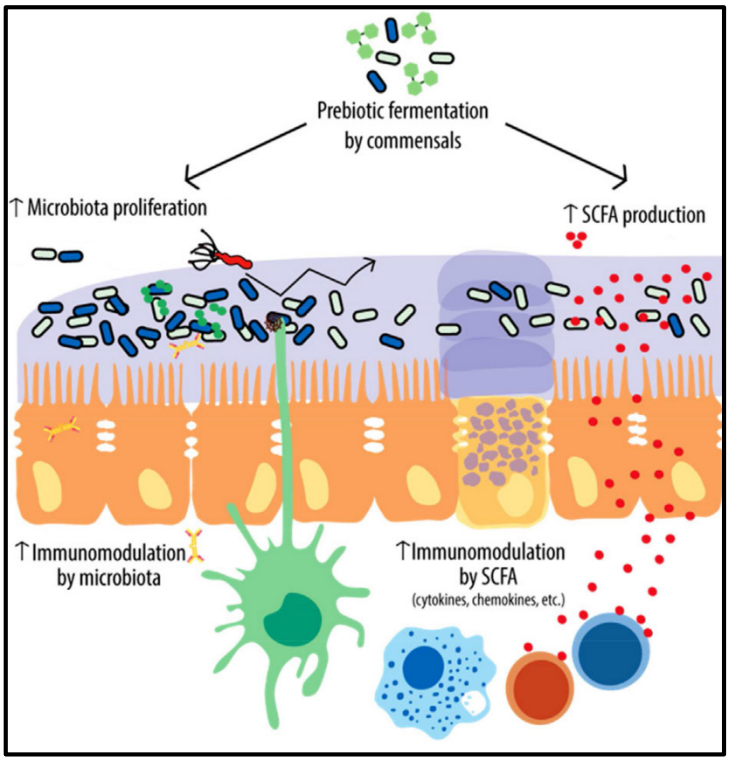


**Figure 4.** Impact of prebiotic dietary supplementation on the gastrointestinal homeostasis. Prebiotic-driven alterations of the gut environment and consequent changes in the host-microbiota dialogue and lumen content. Adapted from (Azagra-Boronat et al., 2019).

### 4.1.2. Dietary carbohydrates

Changing dietary carbohydrate intake (including resistant starches, non-starch carbohydrates, oligosaccharides or di- and mono-saccharides) has a strong influence on the gut microbiota and their metabolism (Brinkworth et al., 2009). A human dietary intervention study showed that a diet rich in type 3 resistant starch (RS3) or non-starch polysaccharides was able to drive bacterial population changes in just three to four days after diet introduction, but also reported that the same amount of time sufficed

for such changes to reverse once the control diet was reintroduced (Walker et al., 2010). In the same study, members of the Ruminococcaceae and Eubacteriaceae families (Clostridia class) had the highest increase in abundancy, when comparing the resistant starch diet with the reduced carbohydrate weight loss diet. A previous work revealed that the amylolytic ability of gut bacteria vary in their capacity to metabolise different types of starch, with significant differences between different types of starch (type 2 which consists of native granular starch and type 4 which is chemically processed indigestible starch) metabolization by distinct bacterial communities (Martínez et al., 2010). Another study has demonstrated that the degree of polymerization of fructose polymers may act as a significant factor influencing the dynamics of the microbial community (Kleessen et al., 2001). Others have demonstrated that reducing the dietary intake of total carbohydrate led to a decreased abundancy of members of the *Roseburia* genus (Lachnospiraceae family, Clostridia class), leading to a reduction of SCFA present in the stool of obese patients (Duncan et al., 2007). Evidence suggests that unabsorbed sugars also seem to drive microbiota metabolism in the colon, as increased lactulose and L-rhamnose consumption led to increased levels of SCFA in the plasma of humans, when compared to glucose (Vogt et al., 2004).

### 4.1.3. High-protein diet

Given the strong influence of diet in gut microbiota composition, recent studies have also focused on the impact of diets rich in animal-derived products in comparison with a vegetable-based diet, varying in total protein and carbohydrate intake. Clinical trials reveal that subjects consuming a vegetarian diet display a significant increase in microbiota richness and Bacteroidetes abundancy when compared to omnivores, however the composition of the whole bacterial community is surprisingly similar

between diets (Losasso et al., 2018; Wu et al., 2016). Although the composition of the microbiota is not greatly affected by the diet, the plasma metabolome of vegans differs markedly from omnivores (Wu et al., 2016). In this study, the major differences in the metabolome composition were attributed to increased levels of by-products of the metabolism of plant-derived products in the vegans, while omnivores presented increased levels of amino acids and lipids. Moreover, others have demonstrated that subjects consuming a vegetarian diet showed significantly lower stool pH, a result attributed to metabolic modulation of the resident bacteria, positively correlated with higher abundancy of *Escherichia coli* and *Enterobacteriacea* (Zimmer et al., 2011).

The high-protein turnover nature of the colon provides nitrogen and amino acids for proteolytic bacteria. Deamination represents the main pathway of amino acid metabolism in the colon, which leads to the production of SCFA and ammonia. Bacteria involved in this conversion include species of *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, *Clostridium* and *Enterobacterium* (Diether and Willing, 2019).

#### 4.1.4. High-fat diet

Alterations in commensal microbial communities induced by increased dietary fat content have been associated with an impairment of the barrier function that is reflected in increased levels of lipopolysaccharides (LPS) in the plasma (Cani et al., 2007). Mice fed an obesogenic HF diet showed a considerable decrease in the numbers of intestinal bifidobacteria, when compared to a low-fat diet (LF) (Hildebrandt et al., 2009). Supplementation of the HF diet with FOS restored the *Bifidobacterium spp.* counts and down-regulated the expression of pro-inflammatory cytokines, showing that the strong response of the microbiota can be reversible, demonstrating that the microbiota response to dietary changes is independent of the obesity condition.

Moreover, HF diet is also known to impact the special arrangement of the microbiota in the small intestinal-mucosa, with a 4-week period of dietary fat supplementation appearing to be associated with the colonization of the intervillous zone of the ileum, an area generally considered to be sterile (Tomas et al., 2016). The same study also revealed a significant impact of the HF diet on microbiota composition, characterized by an increased abundancy of Firmicutes, Proteobacteria and Verrucomicrobia, and a reduction in abundance of Bacteroidetes. Furthermore, HF diet lowered the bacterial abundance of the Lachnospiraceae and Ruminococcaceae families, in a study that showed a positive correlation with the production of SCFA.

### 4.2. Dietary-driven influence of the gut microbiome on the immune system

Epidemiological studies highlight diet as one of the most influential lifestyle factors contributing to the increment of inflammatory diseases, with microbiota metabolites receiving considerable attention in studies related to the development of the immune network and maintenance of a healthy gastrointestinal barrier (Quin and Gibson, 2019).

After birth, the development of the host adaptive immune response is mediated by microbial antigens. Indeed, the intestine of germ-free mice contains a lower number of Peyer's patches and decreased cell proliferation on the crypts, coupled with an impairment of the development of the immune system as indicated by smaller lymphoid follicles. These findings indicate the importance of the microbiota in shaping gastrointestinal development in early life (Simon et al., 2015). Mice subjected to a gut microbial transplant were able to develop normal immunity.

In the intestinal epithelium, the nexus between nutrient metabolism and the host immune system occurs at many levels, ranging from direct sensing of nutrients by

immune cells, to tightly regulated endocrine signalling. The host-microbiota dialogue is currently extended to the digestive, circulatory and nervous systems, with greater research efforts now focusing upon the links between the central nervous system and gastrointestinal tract (gut-brain axis) (Vajro et al., 2013). It is becoming evident that there is a need for a greater understanding of the intersection between gut microbial ecology, the endocrine system and the gastrointestinal immune system (Cani et al., 2013).

The intestinal microbiota is a key factor in the development of local and systemic immunity in the gastrointestinal tract, by interacting with the intestinal epithelial cells' extensive repertoire of innate immune receptors (Simon et al., 2015). Innate immunity, the non-specific branch of the immune system, does not require a previous interaction with an antigen and constitutes the first line of defence against foreign agents. The innate immunity fraction of the gastrointestinal tract includes barriers such as the mucosa layer, phagocytic cells, including polymorphonuclear leukocytes and macrophages, anti-inflammatory cells and antimicrobial cytokines and chemokines (Thaiss et al., 2016). The microbiota itself plays a critical role in gastrointestinal immune stimulation and a good example of such a role is the observation of commensal-driven stimulation of the MyD88 adaptor involved in toll-like receptor (TLR)-mediated signals. Such responses are known to induce RegIII-γ expression in Paneth cells, an anti-microbial lectin that targets Gram-positive bacteria (Brandl et al., 2007). Among commensal bacteria, *Bacteroides thetaiotaomicron* was reported as an inducer of RegIII-γ expression, while the opposite effect was mediated by *Bifidobaterium longum* (Sonnenburg et al., 2006).

Immunostimulants can trigger two types of innate immune responses, inflammatory responses or phagocytosis, through the recruitment of macrophages or neutrophils

(Ochando et al., 2019). Epithelial interactions with microbes or microbial peptides have been linked to inflammation and consequently associated with breaches in barrier function (Beutler, 2009).

Unwanted inflammatory responses are regulated by forkhead box P3 (FOXP3)-expressing $CD4^+$ regulatory T ($T_{reg}$) cells, playing a crucial role in maintenance of immune homeostasis (Tanoue et al., 2016). Among the different subpopulations of the $T_{reg}$ cells, peripherally-derived $T_{reg}$ cells ($pT_{reg}$) prevent inflammatory responses against ingested food and commensal bacteria.

Both diet and microbiota have been known to be involved in the regulation of $T_{reg}$ development and functional maturation. In the presence of microbial antigens and metabolites, T-cell receptors (TCRs) are activated to induce $T_{reg}$ differentiation, proliferation and maturation in the colon. An example is the activation of G protein-coupled receptor 15 (GPR15) in response to SCFA taken up by colonic epithelial cells or diffused through the epithelium, inducing $pT_{reg}$ colonic accumulation (Kim et al., 2013). SCFA have also been reported to promote Treg induction and proliferation through induction of GPR43 (Smith et al., 2013).

The predominant bacterial species in the microbiota known to induce proliferation of colonic $pT_{reg}$ cells belong to the Clostridia clusters IV, XIVa and XVIII. *Bacteroides* species have also been associated with $T_{reg}$ maturation through by inducing IL-10 production by colonic $T_{reg}$ cells (Round and Mazmanian, 2010).

In addition to acting as an energy source for the host, SCFA provide a great example of how nutrient metabolism by the microbiota and host diet combine to modulate immune responses. The link between SCFA, host energy metabolism and gut microbiota has been extensively compiled in previous reviews (den Besten et al., 2013;

McNabney and Henagan, 2017). As an end-product of microbial fermentation, butyrate has been reported to reduce cytokine production profiles, in the colon. This includes reduction of IFN-γ and TNF-α production by T helper cells, and suppression of consequent inflammatory responses (Bird et al., 1998; Macfarlane et al., 2013).

Furthermore, increased levels of butyrate have been correlated with improved barrier function by facilitating the assembly of tight-junction proteins, which, in turn, limits the exposure of the epithelial mucosa to luminal microbes, preventing generation of inflammatory responses (Vanhoutvin et al., 2009). Among others, it is important to highlight the role of bacteria belonging to the clostridial clusters as significant butyrate producers, such as bacteria related to *Eubacterium rectale* and *Roseburia* spp., and members of the XIVa clostridia cluster (Firmicutes phylum) (Aminov et al., 2006; Flint and Louis, 2009).

Put together, these studies suggest a possible correlation between improved barrier function and increased gastrointestinal butyrate, associated with an increased abundancy of *Eubacterium rectale* and *Roseburia* spp, suggesting a direct link between bacterial metabolism and improved epithelial permeability.

Acetate and propionate have been described to regulate systemic homeostasis through an influence in the intestine-brain-liver axis (Jakobsdottir et al., 2013). Another study has recently highlighted the importance of bifidobacteria in the production of acetate, involved in homeostatic maintenance of the gut barrier and in preventing infection with enteropathogens such as *E. coli* O157:H7 (Fukuda et al., 2011). Moreover, increased concentration of propionate was linked with decreased IL-6 production in colonic organ cultures. In the same study, butyrate, acetate and propionate supressed LPS-induced production of TNF-α and IL-8 and inhibited NF-κB activity (Tedelind et

al., 2007). These data demonstrate the crucial role of dietary composition in driving microbial-derived SCFA production, likely to be associated with dietary-induced modulation of the microbial community, which affects enteric immunity.

The world is facing an epidemic increase in obesity cases, which is a critical health challenge (Chang et al., 2019). This is suggested to be linked with the current dietary transition towards a new Western-diet, characterized by increased amounts of ready-to eat processed foods rich in fat, in association with a shift to a pro-inflammatory state in the gastrointestinal tract (Lyons et al., 2016). The pro-inflammatory phenotype associated with increased dietary fat content is usually associated with infiltration and proliferation of immune cells in the intestinal lamina propria (Xu et al., 2003). Moreover, HF diet has been linked to a decreased number of anti-inflammatory $T_{reg}$ cells and macrophages (Feuerer et al., 2009). A low-grade inflammation is then established, since the immune cells remain in the tissue long after they are needed. Interestingly, reports point out that the precise composition of the HF diet stimulates specific responses in inflammation and adipose morphology, highlighting the concept that dietary fat composition confers differential functionality (Lyons et al., 2016).

The link between dietary lipids and immunomodulation has been extensively studied, with the PUFAs class being the most described (Quin and Gibson, 2019). Exploring the relationship between increased dietary fat content and host immunity and microbiota has led to the conclusion that different fatty acid structures trigger different inflammatory responses and promote different microbial communities. Macrophages are a key component of metabolic inflammation. Among the different populations of macrophages M1 macrophages are associated with pro-inflammatory responses whilst M2 macrophages are involved in anti-inflammatory responses (Lumeng et al., 2007). Omega-6 PUFAs and saturated long-chain fatty acids (LCFAs) such as palmitic acid,

are proposed to induce pro-inflammatory responses through the induction of M1 macrophage adhesion and activation (Carlsson et al., 2015). In contrast, dietary omega-3 PUFAs are commonly involved in the activation of M2 macrophages, resulting in the up-regulation of IL-10 (an anti-inflammatory response) and down-regulation of the inflammatory response, through down-regulation of TNF-α, IL1-β and IL-6 (Montero et al., 2015). Moreover, a diet containing 60% of fat from animal sources induced the expression of the pro-inflammatory cytokines IL-1β, TNF-α and IL-17a and also the induction of oxidative and endoplasmic reticulum stress in the colon, in a study suggesting the current Western-diet as a driver of intestinal pro-inflammation (Gulhane et al., 2016).

Increased dietary fat content from animal sources significantly reduced SCFA production in the ileum (Brinkworth et al., 2009), a phenomenon associated with dietary-driven impairment of gut barrier function and linked with bacterial translocation from the lumen into the host tissues (Nøhr et al., 2016 ).

The high potential of dietary lipids to modulate both innate and adaptive immunity makes it an important factor in regulating inflammatory responses in the context of metabolic disorders and enteric diseases.

### 4.3. Role of diet and microbiota in host health and disease

Metabolic disorders have been characterized by an increase in production of inflammatory cytokines by T-cells together with alterations to epithelial physiology (Kim et al., 2012; Musso et al., 2010). The link between IBD and the gut microbiota has been extensively studied, with reports suggesting that commensal bacteria play a key role in the regulation of the gut inflammatory response (Round and Mazmanian, 2009). IBD patients reportedly displayed imbalances in the gut microbiota

composition, characterized mainly by the enrichment of bacteria belonging to the Enterobacteriaceae family (Proteobacteria phylum), while exhibiting a reduction in abundancy of bacteria belonging to both Bacteroidetes and Firmicutes phyla (Nishikawa et al., 2009). A reduction in bacterial diversity was also observed in IBD patients, appearing as a recurrent factor linked to inflammation through activation of innate immune TLRs (Andoh et al., 2007).

Patients with IBS also exhibit lower bacterial diversity in the gut, coupled with bacterial overgrowth in the ileum and an increased Firmicutes/Bacteroides ratio (Rajilić–Stojanović et al., 2011). Moreover, the microbiota of IBS patients is also correlated with induction of low-grade mucosal inflammation (Brint et al., 2010).

Obesity, type II diabetes and metabolic syndrome have also been associated with microbial induced low-grade inflammation, with the impairment of barrier function and consequent increased levels of LPS (endotoxemia) in the plasma appearing as a potential mechanism underpinning induction of inflammation (Cani et al., 2007; Cani et al., 2008; Pendyala et al., 2012). Therapeutic approaches targeting microbiota modulation through diet could be the most promising approach to reduce endotoxemia, reducing the systemic inflammation seen in patients with metabolic syndromes.

With recent studies showing that metabolic diseases clearly have a gastrointestinal origin, the understanding of physiological mechanisms linking diet, gut microbiota and intestinal health are a major priority in order to develop new strategies to intervene in these disease states (Cândido et al., 2018). Despite the fact that probiotic therapies could be a key strategy to improve intestinal permeability and modulate gut inflammation, results still suggest a considerable inter-individual variation (Jones et al., 2018). Recently, faecal microbiome transplantations have been reported to be more

promising as a therapeutic approach, when compared to probiotic treatments. Here, the reconstruction of the microbiota niche after antibiotic treatment was more successful in both mice and humans (Suez et al., 2018). Furthermore, the ability of probiotics to elicit changes in adult patients with an established microbiota, are generally reported as more transient, which limits the current application of probiotic modulation. The variability of results seems to be associated with the unique combination of both host and microbiome, limiting the universalization of the proposed treatments (Zmora et al., 2018). A recent study has demonstrated that microbiota faecal transplantation could temporarily improve insulin resistance in obese patients, but with a considerable individual variability (Kootte et al., 2017). This result was associated with the fact that each patient had a specific microbiota profile interfering with the success of the treatment. With the advancement of metabolomics techniques, the future strategies should be based on the symbiotic mixture of both host diet and strain-specific modulation of intestinal landscape.

# 5. Overview of *in vivo* studies relating dietary composition and bacterial infection

Metabolic diseases do not represent the only threat to gastrointestinal health. Infections with foodborne pathogens represent a major concern for public health and food safety, with a huge impact on the global economy.

To establish disease, pathogens have to overcome diverse host response mechanisms in the gut, including the intestinal barrier and the host innate immune response. Furthermore, commensal bacteria also play a crucial role in keeping pathogens from establishing infection, in a mechanism known as intestinal colonization resistance, through direct mechanisms, including nutrient competition and pathogen inhibiting metabolite production, or through indirect mechanisms mediated by the induction of the mucosal immune response (Lawley and Walker, 2013). Microbiota mediation of colonization resistance against infectious agents has been recently reviewed (Keith and Pamer, 2019; Libertucci and Young, 2019).

In a natural environment, bacteria are subject to rapid changes in nutrient accessibility and face a continuous need for metabolic adaptation. Bacterial survival and growth depend on the ability to adjust their metabolism according to the environmental changes. In pathogenic bacteria, adaptation to an environmental condition often includes activating the transcription of stress response and/or virulence factors (Thomas and Wigneshweraraj, 2015). Many virulence genes are therefore regulated by environmental and nutritional signals. This is thought to be an evolutionary feature in order to optimize utilization of energy resources and allow bacterial survival under energy limiting conditions (Somerville and Proctor, 2009). Carbon source availability in the environment is a factor known to regulate virulence gene expression, via carbon

catabolite repression. When presented with a preferred carbon source, bacteria repress genes associated with the metabolism of secondary sources in order to conserve energy. In the absence of preferred carbon sources, bacteria activate the transcription of alternative metabolic pathways, which may lead to expression of virulence factors (Nelson et al., 2013; Poncet et al., 2009). Evidence suggests that the link between nutrient metabolism and virulence is tightly regulated to control niche-specific virulence in response to particular environmental nutritional content (Connolly et al., 2015).

Diet is a key risk factor in the gastrointestinal environment for the development of enteric disease. Global nutritional transition towards the so-called Western-diet, characterized by an increased dietary fat content has been linked to alterations in gut physiology and homeostasis, together with immune modulations. Moreover, the Western-diet has been associated with changes in the abundancy of microbial populations in the gut. However, the effects of diet on host susceptibility to infection with foodborne pathogens is unclear. Of particular interest are the mechanisms involved in dietary modulation of microbiota-driven colonization resistance, alterations of inflammatory regulation and host resistance to infection and alterations to host-pathogen interaction by modulation of intestinal physiology.

Studying host-pathogen interactions and considering the link between nutrition, modulation of bacterial pathogenicity and host susceptibility to infection is of great relevance for future interventions.

Presently, data on central obesity is limited, however, studies have indicated that obese patients are more susceptible to skin and respiratory infection, when compared to lean patients (Hamer et al., 2019). Surprisingly, obesity appears to be protective against

viral and fungal infections. To our knowledge, no epidemiological reports mention the associated risk with gastrointestinal infection in obese patients, demonstrating a potential knowledge gap in the epidemiological relationship between gastrointestinal infectious disease and obesity.

### *5.1. Listeria*

*Listeria monocytogenes* is a Gram-positive foodborne pathogen known to cause listeriosis, an invasive and potentially fatal infection in susceptible animals and humans. As a facultative intracellular pathogen, *L. monocytogenes* is able to grow in a variety of mammalian cell types but is also well adapted to saprophytic conditions, in which decaying vegetation is its primary nutrient source. Infection, survival and proliferation in the host requires a tight and coordinated regulation of virulence gene expression (Radoshevich and Cossart, 2018). Interpreting the complex arsenal of environmental adaptation mechanisms is fundamental to understanding how *L. monocytogenes* can process and sense environmental signals to ensure expression of virulence factors under appropriate conditions.

Listeriosis is still considered relatively rare and mostly confined to susceptible individuals, however, its mortality rate is relatively high among foodborne diseases, with reports mentioning between 20 and 30% (Silk et al., 2012). Listeriosis has been linked to multiple types of food products, especially raw and ready-to-eat processed foods. Notably, dairy products including milk and soft cheeses, deli meats and pates, and smoked or cooked ready-to-eat seafood are the most commonly implicated in clinical cases of *L. monocytogenes* infection (Churchill et al., 2019).

Previous studies have considered the role of the microbiota in host resistance to infection with *L. monocytogenes*. Antibiotic-mediated damage of the intestinal

microbiota prior to oral infection with *L. monocytogenes* increased bacterial carriage in the intestinal lumen and increased mice morbidity, associated with high bacterial loads in liver and spleen of antibiotic-treated mice (Becattini et al., 2017). These results indicate that host susceptibility to infection with *L. monocytogenes* increases upon antibiotic-treatment most likely through a reduction in microbiota-mediated colonization resistance. Indeed, the same study demonstrates that a small *L. monocytogenes* oral inoculum ($10^2$ CFU, compared to the standard $10^9$ in murine models) was still able to cause systemic infection after streptomycin-treatment, demonstrating that the intestinal microbiota is essential in murine resistance to *L. monocytogenes* oral infection (Becattini et al., 2017). Moreover, *ex vivo* studies demonstrated that the small intestinal content of conventional mice has a positive effect in reducing *L. monocytogenes* CFUs. These effects were not seen through transfer of intestinal contents of antibiotic-treated mice. This is evidence that the microbiota composition and their products of metabolism are an essential line of defence against infection (Becattini et al., 2017). A study relating host susceptibility to infection with *L. monocytogenes* with specific bacterial taxa has identified a group of Clostridiales significantly associated with host protection. More precisely administration of *Clostridium. saccharogumia*, *Clostridium ramosum*, *Clostridium hathewayi*, and *Blautia producta* prevented a systemic infection with *L. monocytogenes* in germ-free mice (Becattini et al., 2017).

Data exploring the extent of the interaction between this foodborne pathogen and the microbiota revealed that *L. monocytogenes* produces a toxin, listeriolysin S (LLS), with bactericidal activity against *Alloprevotella*, *Allobaculum* and *Streptococcus* (Quereda et al., 2017). LLS is a haemolytic cytotoxic factor that is involved in *L. monocytogenes* survival in polymorphonuclear neutrophils and virulence in the

intestine in a murine model (Cotter et al., 2008). The authors propose that this represents a strategy by the pathogen for the manipulation of the host microbiota in order to favour infection (Quereda et al., 2017).

*Lactobacillus* strains have been previously highlighted for their potential as probiotic treatments against *L. monocytogenes* infections. *Lactobacillus sakei* inoculation of germ-free mice resulted in increased survival of mice in the probiotic-treated group after 6-days post-infection (Bambirra et al., 2007). Mono-association of *Lactobacillus delbrueckii* was able to induce *L. monocytogenes* clearance in spleen and liver by inducing IFN-γ, TNF-α and IL-10 in the mucosal immune system of germ-free mice (dos Santos et al., 2011; Vieira et al., 2008). Moreover, treatment with *Lactobacillus casei* in combination with *Lactobacillus paracasei* significantly influenced the *L. monocytogenes* transcriptome, inducing expression of genes associated with carbon and nitrogen metabolism, in particular genes associated with propanediol and ethanolamine catabolism (Archambaud et al., 2012). In addition to influencing gene expression in the pathogen co-colonization of germ-free mice with the above mentioned *Lactobacillus* strains and *L. monocytogenes* was associated with induction of cytokine production suggesting the potential of these strains as modulators of the immune response in the context of infection. Another study has revealed that Abp118 bacteriocin production by *Lactobacillus salivarius UCC118* has a protective effect against *L. monocytogenes* infection in mice suggesting a specific mechanism by which some probiotic strains may protect against the pathogen (Corr et al., 2007). Whilst further work is necessary, these studies suggest that the microbiota of vulnerable patients could be modulated to reduce host susceptibility to infection and that probiotic therapies may be a viable approach against *L. monocytogenes*. Interestingly, a report demonstrates the efficacy of a phage treatment against *L. monocytogenes* oral infection

in mice, highlighted by a decrease in the pathogen ability to establish systemic infection (Mai et al., 2010). The results obtained with ListShield, a commercially available mixture of six naturally occurring bacteriophages, were comparable with antibiotic-treatment, highlighting the potential of bacteriophage as a treatment against foodborne infections.

Different non-digestible carbohydrates have completely different effects on modulation of host susceptibility to infection with *L. monocytogenes*, distinctly affecting both colonization and translocation of this pathogen. In a study comparing dietary supplementation of different carbohydrates in guinea pigs orally infected with *Listeria*, animals fed with XOS and GOS presented lower ileal bacterial loads coupled by lower bacterial translocation to liver and spleen, suggesting an impairment of *Listeria* survival in the gastrointestinal tract (Ebersbach et al., 2010). Inulin and pectin, on the other hand, facilitated bacterial translocation to both liver and spleen. Moreover, in the same study, the authors also show a strain-specific response of the pathogen to the different non-digestible carbohydrates, and hypothesize, based on *in vitro* studies, that this phenomenon could be associated with variations in the fermentative ability of different *Listeria* strains. Different results were seen by Buddington and colleagues, in a study showing that a 6-week dietary supplementation of either inulin or oligofructose improved the host ability to clear *L. monocytogenes* inoculated via the intraperitoneal route (IP), in a mouse model of infection (Buddington et al., 2002).

Recently GOS has been reported to enhance IgA and IgG concentrations in the small intestine and blood respectively, a mechanism involved in its protective potential against gastrointestinal *L. monocytogenes* and subsequent colonization of the liver and spleen (Sangwan et al., 2015).

The source of dietary protein was also found to have an impact on the ability of *L. monocytogenes* to infect the liver and spleen. Mice with a dietary supplementation of soy protein presented a lower bacterial load in spleen and liver, following oral infection, in comparison to mice supplemented with dietary milk casein. The results suggest that dietary protein is associated with inflammation and local gut responses in the context of infection with *L. monocytogenes* (Kuda et al., 2012).

Dietary fat is also known to influence host susceptibility to infection with *L. monocytogenes*. Previous *in vitro* studies have demonstrated that BHI medium supplementation with fatty acids commonly present in milk decrease *L. monocytogenes* survival (Wang and Johnson, 1992) and reduce the pathogens invasion of epithelial cells (Petrone et al., 1998). These results were later explained by the down-regulation of PrfA-activated genes by medium- and long-chain fatty acids (Sternkopf Lillebæk et al., 2017). The PrfA transcriptional regulator in *Listeria* controls the expression of key virulence genes during host cell invasion (Phelps et al., 2018). Unsaturated long-chain fatty acids, such as palmitoleic acid and γ-linolenic acid, exert an antimicrobial effect in *L. monocytogenes* in rich medium (BHI) and reduce expression of PrfA-activated virulence genes such as *plcA*, *plcB*, *hly* and *inlA* (Sternkopf Lillebæk et al., 2017).

Sprong et al. showed that a diet supplemented with sweet butter milk in rats protected the host against colonization with *L. monocytogenes.* Butter milk is rich in bovine milk fat globules, containing a considerable range of antimicrobial peptides, such as mucin and lactadherin. Based on *in vitro* studies this group hypothesized that the diet could inhibit bacterial adhesion to the intestinal mucosa (Sprong et al., 2012).

Another study focused on immune modulation by diet and consequent alterations in response to a secondary infection with *L. monocytogenes*. The authors fed mice diets rich in either olive oil, sunflower oil, high-oleic sunflower oil or fish oil and compared to a LF control for 8 weeks. Results revealed that dietary supplementation with fish oils, rich in omega-3 PUFAs, were the only supplement improving the host immune response against a secondary infection with *L. monocytogenes*, a result associated with dietary modulation by down-regulation of IL-12 expression, coupled with an induction of TNF-α and IFN-γ (Cruz-Chamorro et al., 2011). Similar results were also seen in another study comparing the impact of mice fed a diet supplemented with fish oil (omega-3 PUFAs), soybean oil (omega-6 PUFAs) or lard (low-PUFAs content) for 4 weeks. The study resulted in significantly lowered production of both IL-12p40 in mice fed with fish oil or soybean oil, in relation to lard-fed animals. Moreover, the fish oil-fed group had significantly lower IFN-γ in the spleens in the early phase of systemic infection with *L. monocytogenes* (Fritsche et al., 1999). IL-12 is a particularly important cytokine in the induction of T-cell differentiation (Sun et al., 2015). Even though this study does not correlate the impact of these dietary lipids on host immune modulation with susceptibility to gastrointestinal infection with *L. monocytogenes*, it still demonstrates impairment of the host immune response against the pathogen.

Fish oil was reported to impair *L. monocytogenes* clearance and survival of mice infected via IP, when compared to a lard fed group, after 4-weeks feeding (Fritsche et al., 1997). The same group demonstrated the impact of dietary lipid composition in the host susceptibility to infection. Fish oil impaired the development of the immunological memory response against *L. monocytogenes*, when compared to lard-fed mice. Dietary supplementation with fish oil significantly impairs the host ability to develop an antigen-specific memory response against *L. monocytogenes* during a

primary infection, increasing host susceptibility to a secondary infection (Irons et al., 2003). The evidence supporting the fish oil-induced impairment of the memory immune response against *L. monocytogenes* remains inconclusive however, as a study has demonstrated that increased fish oil intake did not reduce the number of *Listeria*-specific memory/effector T-cells after an intravenous infection (Irons et al., 2005).

Disparity of results reported in different studies could be associated either with differences in concentrations of nutrient supplementations tested, or with infection routes. Neither of the studies mentioned involve oral infection with *L. monocytogenes*, and it is important to highlight that the gastrointestinal route of infection could provide a better understanding of the dynamics behind the host-pathogen interaction following natural infection with the pathogen.

### *5.2. Salmonella*

*Salmonella* is a Gram-negative facultative intracellular anaerobe pathogen, belonging to the Enterobacteriaceae family, that causes both enteric and systemic salmonellosis disease in humans. In the intestine, *Salmonella* invades non-phagocytic epithelial cells, through the injection of effector proteins by type III secretion system (T3SS), encoded by pathogenicity island 1 (Cirillo et al., 1998). This foodborne pathogen is able to establish an asymptomatic carrier stage, characterized by the increased replication of the bacteria in presence of an active adaptive immunity (Lawley et al., 2006).

Salmonellosis is mainly associated with the consumption of swine and poultry meat or contaminated eggs, causing a significant economic impact in these industries (Van Immerseel et al., 2003). This pathogen is prevalent in raw ingredients, as it can survive under harsh, dry conditions for long periods of time. The survival of this bacterium is

not only dependant on the water activity of the environment or food but also on other factors such as matrix composition and storage temperature (Finn et al., 2013). Outbreaks associated with *Salmonella* constitute a significant health threat and a serious medical challenge around the world, especially due to the emerging drug resistance seen in *Salmonella* strains (Karp et al., 2018).

In the context of infection with *Salmonella*, antibiotic treatments and associated impairment of the microbiota and intestinal barrier function represent a major threat to limiting disease incidence of the pathogen (Brummer et al., 2010).

The role of SCFA metabolism in the gut represents a critical factor influencing the pathogenesis of salmonellosis, with evidence suggesting that deletion of *ydiQRSTD* (encoding a pathway for β-oxidation of fatty acids in the presence of nitrate) in *Salmonella typhimurium* impairs the ability of the pathogen to metabolize butyrate, severely attenuating epithelial invasion and intestinal inflammation (Bronner et al., 2018).

Probiotic strains have been revealed to be a promising strategy against *S. typhimurium* infection, with *Lactobacillus* or *Bifidobacterium* strains showing promise for limiting the infection (Isolauri et al., 2002). A mixture of three *Lactobacillus acidophilus* strains (*LAP5*, *LAF1* and *LAH7*) was used in a mouse infection model, to evaluate their ability to inhibit *S. typhimurium* oral infection, with results indicating a lower bacterial translocation to both liver and spleen in the probiotic treated mice (Lin et al., 2007). Moreover, an interesting study has combined the antibiotic ofloxacin and the probiotic strain *Lactobacillus fermentum* ME-3 as a treatment against *S. typhimurium*, showing that this strategy was able to eliminate the presence of the pathogen in the blood, ileum and liver in a murine model (Truusalu et al., 2008).

In a mouse model of infection evidence suggests that the probiotic *Bifidobacterium lactis* (HNO19) impairs host inoculation with *S. typhimurium*, with animals presenting a lower pathogen translocation to visceral tissues, such as spleen and liver (Shu et al., 2000). Similar results were seen in a study with *Bifidobacterium longum* Bb46, where probiotic treated mice challenged with a *S. typhimurium* intragastric inoculation showed evidence of a protective effect, with higher murine survival and decreased levels of IFN-γ in and IgG2a in the serum (Silva et al., 2004).

Despite the promising effects of probiotic strains, the output is still not good enough to replace antibiotic utilization and some studies suggest that combining probiotics with specific dietary components may constitute a better approach to infection with *S. typhimurium*. An intervention consisting of both a probiotic, *Bifidobacterium breve* (strain Yakult), and a prebiotic, the non-digestible carbohydrate transgalactostlated oligosaccharide (TOS), revealed this to be a very promising strategy to clear both primary and secondary infection with *S. typhimurium*, in an antibiotic-induced murine infection model. In this study, the metabolic activity of the microbiota is presented as a significant source of SCFA which was key in lowering the intestinal pH and a major physiological barrier against *S. typhimurium* (Asahara et al., 2001).

Fatty acids have been extensively used as food additives of poultry and swine as a strategy to replace antibiotics as growth promoters and in improving resistance to *Salmonella*. Chickens given a dietary supplement consisting of encapsulated SCFA were challenged with *Salmonella*, with results demonstrating that acetic and formic acid increased pathogen load in internal organs, while propionic acid had no effect in bacterial colonization when compared to the placebo control (Van Immerseel et al., 2004). Animals fed with the butyrate dietary supplement, on the other hand, presented a lower pathogen burden in the cecum, but not in internal organs, supporting the idea

that butyrate is protective against the pathogen survival in the gastrointestinal tract. Another study demonstrated that dietary supplementation with sodium butyrate showed a significant impact on the late phase of infection with *Salmonella* in chickens, with a lower bacterial load detected in the liver and cecum (Fernandez-Rubio et al., 2009). In piglets, dietary supplementation with either formic or propionic acid led to a reduction of *Salmonella* carriage in the small intestine and reduced bacterial faecal shedding which, consequently, led to a reduction in the number of secondary infected piglets, supporting the utilization of SCFA as a way to minimize bacterial spread in the pig industry (Taube et al., 2009).

A more recent study has focused on the role of LCFAs in their ability to clear *Salmonella* infection. The work demonstrated that free external LCFAs act as environmental signal in the intestine and down-regulate virulence gene expression upon oral infection in mice (Gulhane et al., 2016).

Dietary supplementation with carbohydrates also showed promising effects in improving host resistance to infection with *Salmonella*. The role of β-1,4 mannobiose in the modulation of the immune system in response to infection with *Salmonella* has been extensively studied. β-1,4-mannobiose is a non-digestible disaccharide involved in IgA stimulation and is known to induce the abundance of *Lactobacillus* and *Bifidobacterium* in the gut and to stimulate the production of butyrate by the microbiota (Kovacs-Nolan et al., 2013). β-1,4 mannobiose dietary supplementation has been shown to reduce bacterial loads in visceral organs and reduced the pathogen carriage in the cecum and faecal shedding in chickens (Agunos et al., 2007; Ibuki et al., 2010).

Lactulose has also been used in the pig industry as an immune modulatory supplement associated with enhanced IgG and IgA production. This non-digestible carbohydrate has been shown to modulate the porcine B-cell mediated immune response in the context of S. *typhimurium* infection (Naqid et al., 2015).

Maltodextrin, a polysaccharide commonly used as an additive in the food industry, has been demonstrated to disrupt the mucus layer in the intestinal epithelium, de-regulating host-microbiota dialogue and increasing host susceptibility to infection with *Salmonella* in a mice infection model. In animals fed with a maltodextrin dietary supplement, oral infection with this pathogen led to increased bacterial loads in the cecum, together with higher levels of endosomes in lamina propria cells, co-localized with *Salmonella* containing vesicles. These results suggest that micronutrients may also have a strong influence in the intestinal barrier function and in the intestinal landscape, proven to be a key environmental factor involved in the host susceptibility to infection with *Salmonella* (Nickerson et al., 2014).

Another study demonstrated in a mouse model of infection that dietary supplementation with FOS and XOS were associated with increased colonization of internal organs by *Salmonella*, when compared to a control diet. Apple pectin dietary supplementation presented higher bacterial counts in the ileum and higher bacterial shedding, suggesting that different prebiotics present a clear difference in their ability to modulate host susceptibility to infection (Petersen et al., 2009).

Dietary rice bran, a relevant food staple worldwide, known as a rich source of non-digestible polysaccharides and polyphenols and fatty acids, previously associated with microbiota and immunological modulation in mice, was tested as a dietary supplement in its ability to inhibit mice colonization with *Salmonella*. One-week supplementation

with up to 20% of rice bran decreased bacterial faecal shedding and serum concentration of pro-inflammatory cytokines (Kumar et al., 2012).

The role of dietary fat in *Salmonella* infection is poorly studied. One study demonstrated that the immunomodulatory effect of fish oil dietary supplementation did not interfere with the pathogen ability to cause infection in Wistar rats, with decreased serum levels of IFN-γ presenting no differences in bacterial systemic infection, when compared with the corn oil control (Snel et al., 2010).

It is evident that the intestinal environment has a critical role in the host response to infection, and that, in the context of *Salmonella*, both probiotics and prebiotics represent possible alternatives to antibiotic utilization. It is noteworthy that, as studies suggest, future therapies based upon synergistic combinations of bacterial strains and nutrients could improve the results seen in the eradication of *Salmonella* from both poultry and pig industries, potentially reducing antibiotic use in the food industry.

A previous study focused on microbiota composition and *S. typhimurium* infection in mouse models identified a group of fifteen commensal bacterial strains able to provide colonization resistance against this pathogen (Brugiroux et al., 2016). These fifteen strains represent the five most abundant phyla in the intestinal microbiota and include bacteria belonging to the Verrucomicrobiaceae, Bacteroidaceae and Clostridiales families. Inoculation of germ-free mice with this selection of strains achieved colonization resistance to the same level as conventional mice.

### *5.3.* Enteropathogenic *Escherichia coli*

*Escherichia coli* is a Gram-negative facultative anaerobic bacteria considered as a commensal bacterium of most animals. However, some strains have evolved to become pathogenic by acquiring virulence factors or pathogenicity islands through

plasmids, transposons or bacteriophages (Clermont et al., 2000). Some *E. coli* strains, can therefore, cause a wide variety of intestinal and extra-intestinal diseases, such as diarrhoea, urinary tract infections, septicaemia, and neonatal meningitis. Among pathogenic *E. coli* strains, the Shiga toxin producer enterohaemorrhagic *E. coli* (EHEC) causes haemorrhagic colitis and haemolytic uremic syndrome in humans (Lim et al., 2010). As non-invasive pathogens, EHEC are able to adhere to epithelial cells, causing lesions characterized by the localized destruction of microvilli (Nakanishi et al., 2009). Usually, EHEC outbreaks are associated with contaminated meat products, with the most recent studies revealing association with fresh vegetables, such as sprouts and leak (Yang et al., 2017).

Nutrient availability and consequent competition with commensal bacterial for carbon sources is fundamental to niche maintenance in the gastrointestinal tract. In the context of EHEC infection, the lumen content is clearly of great importance in the regulation of virulence gene expression in the intestine. Sugar metabolic pathways, have been proven to contribute to the growth of the pathogen on mucus, improving the pathogens fitness during colonization (Chang et al., 2004). EHEC virulence gene expression, in an environment of nutrient competition in the gastrointestinal tract, is very much dependent on nutrient availability as the pathogen can switch between glycolytic metabolism during initial colonization, to gluconeogenic metabolism to facilitate long term establishment of this niche (Fabich et al., 2008). Fucose is a sugar considered to be a preferred carbon source for EHEC, as it can act as a signalling molecule in the intestine, with increased concentrations being able to induce expression of genes in the LEE1 pathogenicity island (Pacheco et al., 2012).

Stress adaptation in the gastrointestinal environment may also play a critical role in the pathogen survival, which in turn can result in a more severe infection. A study has

shown that a lactose-rich diet affects the osmotic-stress response, by inducing up-regulation of OxyR-dependent proteins. This study demonstrates a diet-induced osmotic stress response associated with an increased severity of infection confirmed in a germ-free murine model of infection (Rothe et al., 2012).

Besides metabolic modulation by luminal nutrients, the interaction between EHEC and the microbiota also shapes the landscape in which the pathogen survives in the host. The microbial composition of the gut significantly affects the class of molecules available, governing *E. coli* virulence gene expression and survival in the intestine, with quorum sensing mechanisms appearing to be especially relevant (Pifer and Sperandio, 2014). Interestingly, reports have suggested that butyrate (but not acetate or propionate) is capable of inducing virulence gene expression and bacterial motility *in vitro*, highlighting overlap between central metabolism and regulation of virulence gene expression (Tobe et al., 2006). The mechanisms by which the microbiota impedes EHEC infection still remain unclear However, a previous study has demonstrated the role of *Bacteroides thetaiotaomicron* metabolism of host glycan to produce fucose. Increased fucose availability in the intestine can induce virulence gene expression of EHECs providing a potential to facilitate robust colonization and infection (Pacheco et al., 2012). However, to our knowledge, a correlation between increased abundancy of *B. thetaiotaomicron* and increased host susceptibility to EHEC infection, still remains unknown.

The link between host diet and infection, has been considered in previous studies as a possible justification for inter-patient variability in the context of infection, with some investigations evaluating the impact of dietary modulation on microbiota composition and metabolism. Microbiota-driven increase in levels of butyrate has been linked to promotion of *E. coli* O157:H7 infection potentially by increasing virulence gene

expression, by inducing up-regulation of Shiga toxin (Stx) production and increasing severity of murine infection (Zumbrun et al., 2013).

Recent studies have implicated the role of carbohydrates in EHEC virulence and host colonization. Mice fed a diet with increased galactomannan polysaccharide (considered as a high-fibre diet) presented higher mortality relatively to mice fed low-fibre diet. Moreover, animals presented increased EHEC faecal shedding, coupled with increased body-weight loss (Zumbrun et al., 2013).

The impact of a high-fat/high-sugar diet was also considered in the context of *E. coli* infection in mice, with results showing a disruption of gastrointestinal barrier function, a decreased mucus layer and increased TNF-α secretion. These changes were accompanied by a shift in microbiota composition with a particular overgrowth of *Bacteroides* and *Prevotella*, all of which correlated with enhanced *E. coli* gut colonization. This study highlights the impact of a Western-diet in its ability to disrupt gut homeostasis and facilitate *E. coli* colonization (Martinez-Medina et al., 2014).

These findings may have a particular impact on the food industry. In order to study dietary effects further in a more translational model some dietary interventions have been done in alternative models, using pigs as an example, in an attempt to validate future strategies.

In a pig model, a diet with increased sucrose and glutamine resulted in a decrease in intestinal permeability associated with increased expression of tight-junction proteins, a reduced inflammatory response (decreased expression of IL-1β, IL-6 and IL-10) and lower *E. coli* intestinal colonization, when compared to the control diet (Ewaschuk et al., 2011).

Another study has demonstrated the role of dietary supplements derived from plant extracts, consisting of capsicum oleoresin, garlic botanical, or turmeric oleoresin, in the host response to a pathogenic strain of *E. coli* in an infection model using weaning pigs. The study examined gastrointestinal physiology, inflammation and colonization with the pathogen (Almeida et al., 2013). Dietary supplementation with plant extracts was able to lower the pro-inflammatory response by lowering both TNF-α release and recruitment of macrophages and neutrophils and increasing villi height and improving the overall response to infection with *E. coli*, appearing to be beneficial to pig health in the context on infection with this pathogen.

Interestingly, an approach that combined both diet and a probiotic supplementation, demonstrated the impact of protein dietary supplementation and a non-pathogenic *E. coli* probiotic strain on the performance of piglets challenged with a pathogenic *E. coli* strain. The low-protein diet coupled with the probiotic supplementation resulted in a better performance in the pigs in terms of body weight gain and lower ileal counts of the pathogenic strains, when compared to the high-protein diet coupled with both antibiotic or probiotic treatment (Bhandari et al., 2010). Other results have indicated the protective effect of casein glycomacropeptide, a glycoprotein involved in microbiota modulation. Dietary supplementation of casein glycomacropeptide was associated with modulation of the colonic lumen content as well as increased abundancy of *Lactobacillus* strains associated with a decreased adherence of non-commensal *E. coli* (Gustavo Hermes et al., 2013). This apparent contradiction in the role of dietary protein in the susceptibility of pigs to infection could be explained by variations in the nature of the different types of protein and consequent alterations in its modulatory effects. This places further emphasis on the need to compare different sources of protein.

These studies support the hypothesis that dietary factors can be crucial in reducing the severity of *E. coli* gastrointestinal infections by modulatory effects in the host intestinal barrier and pro-inflammatory response.

### *5.4. Campylobacter*

*Campylobacter jejuni* is a rod-shaped Gram-negative bacterium, with optimal growth under microaerophilic conditions. Able to infect a vast range of hosts, including animals and humans, *C. jejuni* infection is usually associated with dairy products, ready-to eat chicken meat products and municipal water (Moffatt et al., 2016). Even though *C. jejuni* is considered a commensal is some hosts, in humans it is associated with campylobacteriosis, a disease characterized by episodes of fever and bloody diarrhoea, with acute illness generally associated with epithelial invasion (Skarp et al., 2017). The prevalence of *C. jejuni* can be associated with its ability to survive in a broad range of environments, a feature which has evolved to ensure survival within the host gastrointestinal tract (Ellström et al., 2014). Moreover, previous *in vitro* studies have revealed a link between virulence gene expression and metabolism of de-conjugated bile acids (sodium deoxycholate) present in the gut, suggesting that the gut environment may have a role in regulation of virulence gene expression in *C. jejuni* (Malik-Kale et al., 2008).

Studies have identified limitations with the animal models of infection. Difficulties in using the mouse model as an adequate model are mostly due to the fact that the establishment of campylobacteriosis is strongly dependent on the host microbiota composition. The native microbiota of conventional mice is usually competent in conferring colonization resistance against *Campylobacter* which prevents infection (Masanta et al., 2013).

A study which focused on dietary-driven microbiota establishment in germ-free mice, by a high-fat/high-sugar diet in comparison with a control diet, has revealed that mice fed with the Westernized-diet had developed markers more similar to what would be considered a "humanized" microbiota and were more susceptible to infection with *C. jejuni.* The work highlighted the role of the microbiota in favouring *Campylobacter* infection and suggested that this is a more realistic murine infection model for modelling human infections (Bereswill et al., 2011). Another study demonstrated that microbiota modulation driven by inflammation eliminated murine colonization resistance against this pathogen (Haag et al., 2012a). Increased abundancy in commensal *E. coli* strains as a result of intestinal inflammation was highlighted as a risk factor, leading to hypotheses surrounding the interplay between the metabolism of both bacteria in the gut. The microbiota ecosystem modulates itself upon infection with *C. jejuni*, with a study demonstrating long-term effects of infection in the gut microbial community structure of human subjects. Specifically, infection led to overgrowth of *Bacteroides*, *Escherichia* and *Streptococcus* strains, contrasting with a decreased abundancy of Clostridiales and Lachnospiraceae, in relation to an uninfected control group (Dicksved et al., 2014).

Very little is known about the metabolic mechanisms underlying the impact of an individual microbiota fingerprint in host susceptibility to infection with *C. jejuni*. However, some probiotic strains were tested in regards to their ability to reduce susceptibility to *Campylobacter* carriage in chickens. A study used a multispecies probiotic treatment consisting of *L. salivarius*, *Lactobacillus reuteri*, *Enterococcus faecium*, and *Pediococcus acidilactici*, with results proving to be effective in the reduction of cecum colonization by *C. jejuni* in the probiotic treated group in relation to the placebo control (Ghareeb et al., 2012). Others have focused on the impact of

human-derived probiotic strains upon carriage of the pathogen in chickens. For instance, a combination of *L. paracasei* J.R., *Lactobacillus rhamnosus* 15b, *Lactobacillus lactis* Y, and *L. lactis* FOa, reduced bacterial load in the duodenum and cecum in the later growth phase of the chickens (Cean et al., 2015). Even though the results of probiotic treatment seem promising, further research is necessary to assess the mechanisms behind the interaction between the host microbiota and the pathogen, especially in the relationship between infection with *C. jejuni* and the intestinal physiology and immune response (Haag et al., 2012b).

Alterations in the feed composition have been reported to promote gastrointestinal clearance of *C. jejuni* in chickens. A study examining dietary supplementation with a variety of commercially available food additives consisting of organic or fatty acids (including SCFA mixtures or butyrate only), a commercial monoglyceride mixture, plant extracts or a prebiotic-like product, has demonstrated a considerable degree of variation among the birds, accompanied by an age-dependent effect. The study also suggests that the treatment consisting of butyrate coated microbeads and the prebiotic-like treatment consisting of *Saccharomyces cerevisiae* and the media on which it was grown were the only approaches able to lower the *Campylobacter* shedding below the threshold regarded as safe in the broiler industry (Chemaly et al., 2015).

The role of dietary fibre supplementation has been considered in the context of *Campylobacter* infection in both chickens and pigs. A dietary increase of water-soluble non-starch polysaccharides supplemented with degrading enzymes, more specifically xylanase and glucanase, was proven to have a protective effect against cecum *C. jejuni* colonization in chickens and was associated with an increase in concentration of total SCFA in the cecum (Molnár et al., 2015). Another study has associated a fructan-rich diet with decreased *C. jejuni* faecal shedding in pigs proving

to be associated with abundancy of Bifidobacteria and *E. coli* in the commensal bacterial community (Jensen et al., 2011).

Modification of the feed composition by dietary supplementation with fatty acids has also been tested to influence susceptibility to *C. jejuni carriage* in chickens. A mixture of medium-chain fatty acids (MCFAs) has been demonstrated to increase by 200-fold the bacterial load necessary to cause infection in the animals fed with the dietary supplement in relation to the untreated group indicating protection against infection in this case (Molatová et al., 2011; van Gerwe et al., 2010). Supporting these results, dietary supplementation with caprylic acid has been demonstrated to drive a decrease in cecum *C. jejuni* load (de los Santos et al., 2009).

The role of dietary modulation in the metabolism of *C. jejuni* in the host, as well as its impact in host physiology and microbiota community in the context of infection with this pathogen still raises a lot of questions, especially due to the lack of optimized infection models. However, the studies presented above open ways to hypothesize about the critical role of dietary components, with especial attention to MCFAs, in the control of *C. jejuni* prevalence in chicken husbandry. Dietary interventions to prevent carriage of *Campylobacter* species in chickens may therefore provide a strategic approach to limit transmission of campylobacteriosis to humans.

# 6. Conclusions

Diet is a relevant lifestyle factor interfering with the host gastrointestinal environment, directly influencing the intestinal landscape, including epithelial physiology, barrier function and microbiota composition. Moreover, diet is recognized to regulate bacterial metabolism which is correlated with changes in the metabolites available in the lumen. This is of particular concern in the context of infection with foodborne pathogens, as nutrient availability in the gut is key in regulating virulence gene expression in some pathogens. Modulation of the intestinal environment may also favour pathogen proliferation and colonization, either through alterations in the commensal bacterial ecosystem, impairment of colonization resistance, or through alterations in barrier function, which favour bacterial translocation.

With the increased need for replacement of antibiotics in the treatment against foodborne pathogens validations of alternative approaches, including dietary modulation to prevent or limit infection, are becoming a priority.

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# Chapter II

# Short-term consumption of a high-fat diet increases host susceptibility to *Listeria monocytogenes* infection

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**Authors Contributions**

Vanessa Las Heras did all analytical work, designed all experiments and analysed all the data. Vanessa Las Heras wrote the paper with significant input from Cormac Gahan and input from all authors. Adam Clooney, Fergal Ryan and Raul Rubio carried out bioinformatics analyses with significant work in figure editing, further analysis and configuration from Vanessa Las Heras. Pat Casey and Cara Hueston designed and performed the infection studies with significant input from Vanessa Las Heras. Vanessa Las Heras carried out the histological analyses, with significant input from Jorge Pinheiro and Silvia Melgar. Justine Rudkin, Silvia Melgar, Paul Cotter, Colin Hill, Cormac Gahan devised and co-ordinated the study and analysed the data. The final manuscript was read and approved by all authors.

# Contents

# 1. Abstract

A Westernized-diet comprising a high caloric intake from animal fats is known to influence the development of pathological inflammatory conditions. However, there has been relatively little focus upon the implications of such diets for the progression of infectious disease. Here we investigated the influence of a high-fat (HF) diet upon parameters that influence *Listeria monocytogenes* infection in mice.

We determined that short-term administration of a HF diet increases the number of goblet cells, a known binding site for the pathogen in the gut, and also induces profound changes to the microbiota and promotes a pro-inflammatory gene expression profile in the host. Host physiological changes were concordant with significantly increased susceptibility to oral *L. monocytogenes* infection in mice fed a HF diet relative to low-fat (LF) or chow-fed animals. Prior to Listeria infection short-term consumption of HF diet elevated levels of Firmicutes including *Coprococcus*, *Butyricicoccus*, *Turicibacter* and Clostridium XIVa species. During active infection with *L. monocytogenes* microbiota changes were further exacerbated but host inflammatory responses were significantly down-regulated relative to Listeria-infected LF or chow-fed groups, suggestive of a profound tampering of the host response influenced by infection in the context of a HF diet. The effects of diet were seen beyond the gut, as a HF diet also increased the sensitivity of mice to systemic infection and altered gene expression profiles in the liver.

We adopted a systems approach to identify the effects of HF diet upon *L. monocytogenes* infection through analysis of host responses and microbiota changes (both pre- and post- infection). Overall the results indicate that short-term consumption of a Westernized-diet has the capacity to significantly alter host

susceptibility to *L. monocytogenes* infection concomitant with changes to the host physiological landscape. The findings suggest that diet should be a consideration when developing models that reflect human infectious disease.

# 2. Introduction

Increased consumption of a Westernized-diet, comprising high caloric intake from fats and reduced consumption of fermentable fibre, has been linked to the current pandemic of chronic inflammatory conditions such as obesity, type II diabetes, inflammatory bowel disease and allergic asthma (Statovci et al., 2017). A diet rich in animal-derived fats can reduce gastrointestinal barrier function, influence microbiota composition and alter gastrointestinal and systemic inflammatory responses (Fritsche et al., 1999; Shanahan et al., 2017; Statovci et al., 2017). Such profound physiological responses in the host are likely to underpin pathological changes, particularly at mucosal surfaces (Statovci et al., 2017). However, the potential for a high-fat (HF), Westernized-diet to influence the progression of infectious disease has received relatively little attention. We proposed to investigate this phenomenon using *Listeria monocytogenes,* a foodborne pathogen that causes a serious invasive disease (listeriosis) in susceptible hosts, which is increasingly associated with large outbreaks and has a high mortality rate.

*L. monocytogenes* has been extensively investigated as a model intracellular pathogen to uncover the biological mechanisms involved in host cell invasion, intracellular parasitism and resultant host immunity (Radoshevich and Cossart, 2018). The majority of such studies have utilised cell culture models or systemic murine infection. However, an increasing number of studies have begun to focus upon the gastrointestinal phase of infection. It is clear that the pathogen can sense and respond to both local physico-chemical signals (Archambaud et al., 2012; Gahan and Hill, 2014; Toledo-Arana et al., 2009) and the presence of autochthonous organisms (Archambaud et al., 2012) in the gastrointestinal environment, and environmental

adaptation is likely to influence the ability of the pathogen to survive and transiently replicate in the intestine (Gahan and Hill, 2014; Toledo-Arana et al., 2009). Subsequent invasion of the host is through interaction between microbial Internalin A (InlA) and host E-cadherin (E-cad), a process which is most efficient in the vicinity of goblet cells where E-cad is more likely to be accessible (Nikitas et al., 2011). In addition, there is a role for the microbiota in providing a barrier to infection and in modifying the host immune response to the pathogen locally (Archambaud et al., 2012; Becattini et al., 2017; Corr et al., 2007; Lecuit et al., 2007).

We employed a systems approach to study the effects of a HF diet upon a number of parameters associated with the infectious process both before and after infection with *L. monocytogenes*. In particular, we examined both microbiota and host physiological changes influenced by diet both immediately prior to infection and also during the peak period of active infection. Our findings indicate that a relatively short-term change in diet, to a Westernized-HF-diet, increases susceptibility to oral infection with *L. monocytogenes* concomitant with a significantly altered physiological landscape in both the gastrointestinal tract and the liver. Furthermore, our findings show that diet influences the systemic phase of infection alone suggesting a profound systems-wide alteration to host physiology that alters susceptibility to infection.

# 3. Methods

## 3.1. Animal dietary intervention and infection

Seven-week-old female C57BL/6 mice (ENVIGO, UK), n=10, were housed in a controlled environment with free access to food and water. The mice were fed a control chow diet (Teklad Global 2018S Rodent Diet, ENVIGO, UK), a low-fat diet (DIO series diets D12450H, Research Diets, Inc., USA) or a high-fat (DIO series diets D12451, Research Diets, Inc., USA) for 13 days (**Table 1**). Thereafter the animals were either infected through oral intragastric inoculation (IG) or intraperitoneal infection (IP). Overnight culture of the murinized strain, *L. monocytogenes* EGDe InlA$^{m}$, was centrifuged (7000 g for 5 min), washed twice with PBS and ressuspended in PBS. A 200 µl inoculum comprised 3.2 x $10^9$ CFU for the IG infection and 9 x $10^4$ for IP infection. The progression of infection followed over a three-day period (Monk et al., 2010), with mice maintained on their specific diets for that period. Mice were euthanized and the internal organs aseptically removed and homogenized using stomacher bags and PBS. For CFU per organ enumeration, dilutions were plated on BHI (brain heart infusion) agar plates. Following IG infection, on days 14 (day 1 post-infection) and 16 (day 3 post-infection) the faecal pellets were collected and plated for CFU to determine shedding of *L. monocytogenes*.

## 3.2. RNA extraction and qRT-PCR analysis

A sample of the liver and the ileum were collected for the analysis of the host regulatory response to both diet and infection (transcriptome analysis). The samples were stabilized with RNAlater™ (Sigma) and stored at -80°C until total RNA extraction (RNeasy Plus Universal Mini Kit, Qiagen). The total RNA extracted was DNase treated (TURBO DNA-free™ Kit, Ambion) and the transcriptomic analysis

was done using RT-PCR (Transcriptor Reverse Transcriptase, Roche). qPCR protocol was carried out using LightCycler® 480 Probes Master (Roche™) with the Universal Probe Library from Roche. Primers are outlined in **Table S1**. The amplification setup used was 45 runs in 384 well plates with the MonoColor hydrolysis probe detection format.

### 3.3. Faecal samples and microbiota profiling

For analysis of mouse gut microbiota based on 16S rRNA gene amplicon sequencing, faecal samples were collected during dietary adaptation prior to infection (on days 0, 6 and 13), and after infection (on days 15 and 16). Pellets collected were used for microbiota analysis using 16S rDNA sequencing (DNA extraction using QIAmp fast DNA stool mini Kit, Qiagen). The quality of raw sequences was visualised with FastQC. This was followed by quality filtering using trimmomatic (Bolger et al., 2014). Briefly the first twenty and last twenty bases were trimmed and then a sliding window was applied (window size 4 with minimum quality 15) along with a minimum sequencing length of 250 bases. Subsequently, sequences were filtered via USEARCH with a maximum e score of 1 (Edgar, 2010) and following this, open reference Operational Taxonomic Unit (OTU) clustering was performed in Qiime against the Ribosomal Database Project (RDP) database v11.4 (Cole et al., 2014). For sequences failing to be clustered against the reference database de novo clustering was applied (Edgar, 2013). Chimeric sequences were removed with ChimeraSlayer with the Gold database and UCHIME (Edgar et al., 2011). The resulting OTU sequences were classified, phylum to genus, using Mothur (Schloss et al., 2009) and the RDP database v11.4 with any assignments with a bootstrap value of less than 80% labelled unclassified. Species classification was carried out using SPINGO (v1.3) against the RDP database (v11.4) with default parameters (similarity score of 0.05 and a bootstrap

cut-off of 0.8) (Allard et al., 2015). All downstream analysis was performed in R version 3.4.3. Alpha- and Beta-diversity was calculated using the R package phyloseq (McMurdie and Holmes, 2013). Differences in Alpha-diversity were assessed using the Mann-Whitney test. Differential abundant analysis was performed using DESeq2 (Love et al., 2014). The Adonis function in the vegan library was used to assess group level differences in the microbiota.

### 3.4. Tissue staining and Microscopy

Ileal samples were collected for histology analysis and the identity of sample groups was blinded to the investigator. The tissue sample was stored in 4% paraformaldehyde for 24h at room temperature and dehydrated with 70% ethanol for 72h at 4ºC, prior to paraffin embedding (dehydration and permeation in molten wax in the histokinette in a 21h overnight cycle; wax blocking of the samples using the console system TissueTek for 2h). The wax molds containing the embedded tissue were cut using the Leica RM2135 rotary Microtome. For goblet cell analysis ileal paraffin sections of 5 µm were stained with Alcian Blue and Periodic Acid Schiff (PAS) and counterstained with Schiff Reagent and Nuclear Fast Red Solution. Sections were mounted in DPX mounting reagent (Sigma) and imaged using the Olympus BX51 microscope (Olympus DP71 camera), with a 20x objective. Image analysis was performed using ImageJ. For histological scoring paraffin-embedded ileal sections (5 µm) were stained with haematoxylin and eosin according to standard procedures. The sections were blindly scored using a light microscope (Olympus BX51, Olympus, Germany). The histology score was adapted from Drolia et al. (Drolia et al., 2018) with some modifications. The ileal samples were scored on a scale of 0-3 for 2 parameters: infiltration of inflammatory cells (mostly mononuclear cells) to the villi and infiltration of mono- and polymorphonuclear cells to the crypts, yielding a maximum

score of 6. In our model, polymorphonuclear cells were mainly located at the bottom of the crypts. The gradient of the inflammatory cell infiltration was based on: 3 = highly increased; 2 = moderately increased, 1 = mildly increased and 0 = normal.

### 3.5. Statistical analysis

Statistical analyses conducted with Prism 5 (Graph-Pad Software). Mann-Whitney test was used to compare the means of 2 groups. One-way ANOVA with a Dunnett's Multiple Comparison Test was used for pair-wise comparison of means from more than 2 groups in relation to the control, or with Tukey's post-hoc test for comparison of means relative to the mean of a control group.

If the P-value falls above 0.05, the mean differences were considered statistically non-significant (NS). For statistically significant differences: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

### 3.6. Ethics statement

All the animal procedures were carried out in agreement with the guidelines of the European Commission for the handling of laboratory animals (directive 2010/63/EU), under authorisations issued by the Health Products Regulatory Authority (HPRA, Ireland) for the use of animals for scientific purposes and approved by the Animal Experimentation Ethics Committee of University College Cork.

### 3.7. Data availability

Raw 16S rRNA reads have been made available on the SRA under accession number SRP139708.

# 4. Results and Discussion

## 4.1. High-fat diet increases susceptibility to oral *L. monocytogenes* infection

We established a study design (**Fig. 1**) in which C57Bl/6J mice were fed either a HF diet (45% of the total caloric intake from fat), a matched low-fat (LF) diet (10% of the total caloric intake from fat) or regular chow (18% of the total caloric intake from fat) (**Table 1**). Feeding was for two weeks in order to avoid alterations in systemic fat deposition (obesity) and metabolism associated with longer term feeding in this model (Joyce et al., 2014). Indeed, murine body weights were comparable across the different groups after switching diets for 2 weeks (**Fig. 2**). At Day 13 mice were infected perorally with a strain of *L. monocytogenes* (designated EGDe InlA$^{m}$) in which the InlA protein has been altered to enhance interaction with murine E-cad, thereby increasing the efficacy of the model as a measure of invasive disease (Monk et al., 2010; Wollert et al., 2007). It is known that wild-type *L. monocytogenes* InlA interacts poorly with murine E-cad (Radoshevich and Cossart, 2018), most likely translocates passively at Peyer's patches in non-permissive models (Pron et al., 1998) and is incapable of significant invasive disease in normal mice. We, and others, recognise the limitations of both models (Becattini et al., 2017) and appreciate that the altered InlA expressed in *L. monocytogenes* EGDe InlA$^{m}$ in addition to enhancing interaction with E-cad may also interact with murine N-cadherin (mN-cad) (Tsai et al., 2013). However, the murine model is also reflective of InlA-E-cad independent pathways used by *L. monocytogenes* to invade and translocate across the intestine (Drolia et al., 2018) and this invasive mechanism is likely to be relevant to human infection (Gelbicova et al., 2015; Holch et al., 2013).

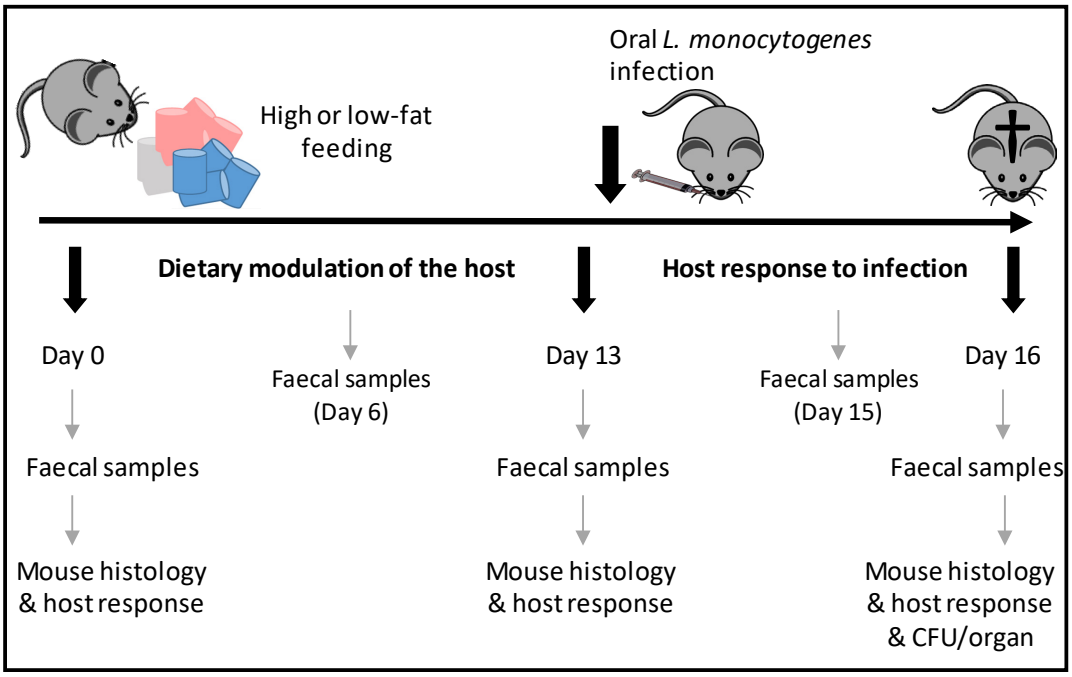



**Figure 1.** Effect of HF Westernized-diet on *Listeria monocytogenes* infection in mice. Study overview. C57BL/6 mice (n=10) were fed with a low-fat diet (10% fat), chow (18% fat) and high-fat diet (45% fat) for 13 days, orally infected with 5 x $10^9$ CFU of *L. monocytogenes* EGDe InlA$^m$ and infection determined at 72h post-infection (Day 16). Sampling points for faecal microbiota analysis during dietary modulation of the host and during infection are indicated. Animals were euthanized and the total number of *L. monocytogenes* EGDe InlA$^m$ CFU per organ were determined by plating homogenized organs. The phase from D0 to D13 represents the influence of diet upon the host and microbiota whereas D13 to D16 represents a 3-day infection with *L. monocytogenes*.

**Table 1.** Composition of experimental diets.

| | **Low-fat diet** | | **High-fat diet** | |
|---|---|---|---|---|
| | **g%** | **kcal%** | **g%** | **kcal%** |
| **Protein** | 19.2 | 20 | 24 | 20 |
| **Carbohydrate** | 67.3 | 70 | 41 | 35 |
| **Fat** | 4.3 | 10 | 24 | 45 |
| **Total** | **100** | | **100** | |
| **kcal/g** | **3.85** | | **4.73** | |
| **Ingredient** | **g** | **kcal** | **g** | **kcal** |
| Casein, 30 Mesh | 200 | 800 | 200 | 800 |
| L-Cystine | 3 | 12 | 3 | 12 |
| Corn Starch | 452.2 | 1808.8 | 72.8 | 291 |
| Maltodextrin 10 | 75 | 300 | 100 | 400 |
| Sucrose | 172.8 | 691.2 | 172.8 | 691.2 |
| Cellulose, BW200 | 50 | 0 | 50 | 0 |
| Soybean Oil | 25 | 225 | 25 | 225 |
| Lard | 20 | 180 | 177.5 | 1598 |
| Mineral Mix S10026 | 10 | 0 | 10 | 0 |
| DiCalcium Phosphate | 13 | 0 | 13 | 0 |
| Calcium Carbonate | 5.5 | 0 | 5.5 | 0 |
| Potassium Citrate, 1 H2O | 16.5 | 0 | 16.5 | 0 |
| Vitamin Mix V10001 | 10 | 40 | 10 | 40 |
| Choline Bitartrate | 2 | 0 | 2 | 0 |
| **Total** | 1055.05 | 4057 | 858.15 | 4057 |

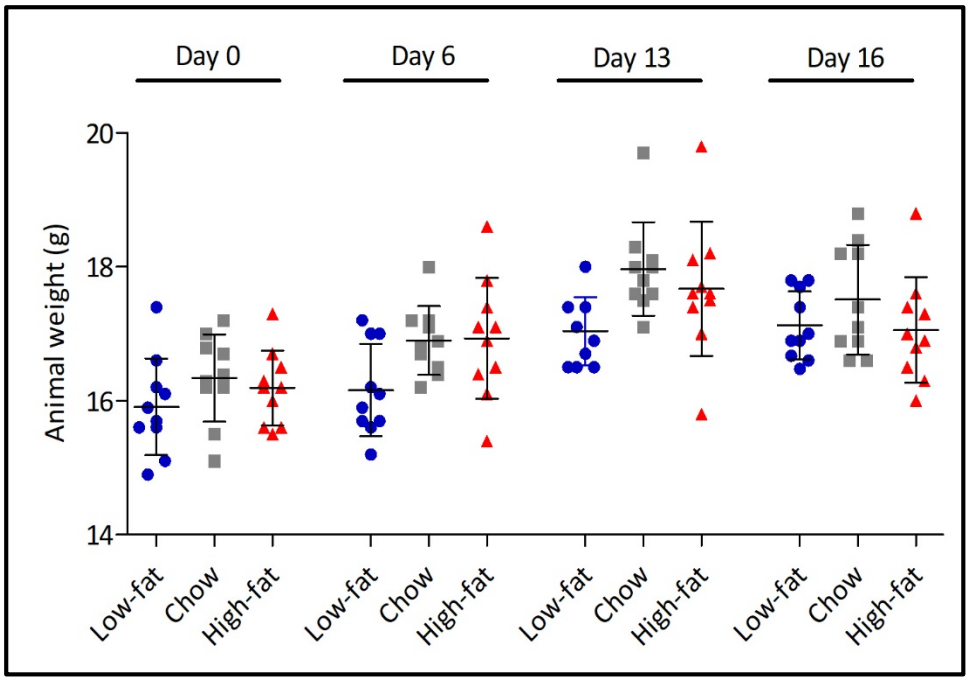



**Figure 2.** Variation of animal weight over time with dietary change. Statistical analysis was conducted using One-way ANOVA and Tukey's Multiple Comparison Test. Error bars represent ±SEM.

Feeding of HF diet for 2 weeks significantly increased susceptibility to oral *L. monocytogenes* EGDe InlA$^{m}$ infection compared to LF or chow-fed animals, as indicated by increased levels of the pathogen in internal organs and caecum at day 3 post-infection (**Fig. 3**). Faecal levels of the pathogen are indicated in **Fig. S1**. Repeat experiments utilising an engineered bioluminescent strain of *L. monocytogenes* EGDe InlA$^{m}$ showed similar results (**Fig. S1**). The data indicate a robust influence of diet upon susceptibility to oral *L. monocytogenes* EGDe InlA$^{m}$ infection in a murine model.

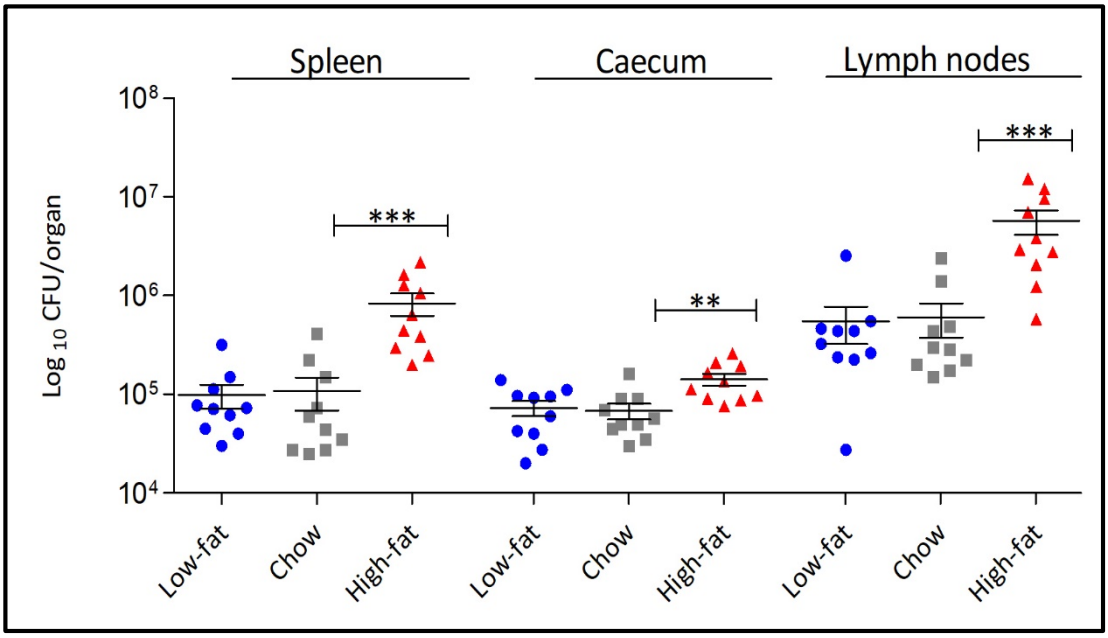



**Figure 3.** Increased dietary fat increases host susceptibility to oral infection with *L. monocytogenes* EGDe InlA$^{m}$. Listeria burden in the spleen, cecum and mesenteric lymph nodes of C57BL/6 mice fed with diets varying in percentage of fat content (n=10, standard deviation from the mean, statistical analysis was conducted using One-way ANOVA and Dunnett's Multiple Comparison Test in relation to chow diet) ** $P < 0.01$, *** $P < 0.001$. Error bars represent ±SEM.

### 4.2. Dietary modulation of host physiology prior to infection

Diet is known to influence the physiology of the host, and significant research has focused upon the changes that are associated with the onset of obesity in mice fed a HF diet (Cani et al., 2012). In contrast, relatively few studies have investigated the gastrointestinal or systemic changes that occur prior to the onset of obesity in this model (Araújo et al., 2017). We therefore investigated the influence of different diets upon the physiological response of mice following 2 weeks of dietary intervention,

immediately prior to oral infection with *L. monocytogenes*. This represents an index of the immediate environment into which Listeria is introduced and must establish early infection. We particularly focused upon parameters that are known to play a role in the pathogenesis of *L. monocytogenes*.

Blinded histological analysis indicated a significant increase in intestinal goblet cell numbers in mice fed a HF diet relative to both LF and chow-fed groups (**Fig. 4**). Our data suggest an early response of the gut to HF diet feeding that involves generation of goblet cells, and supports previous studies showing elevated goblet cell numbers associated with the onset of obesity albeit at a much later stage of HF dietary feeding (Plovier et al., 2016). As goblet cells are a preferential site of invasion by *L. monocytogenes,* including *L. monocytogenes* strains expressing murinized InlA (the model used in this study) (Nikitas et al., 2011; Tsai et al., 2013), the elevated goblet cell numbers seen in our system are likely to, at least in part, enable enhanced infection.

Transcriptional analysis of a range of relevant host markers was used to determine regulatory changes in the host that occurred between day 0 (the initiation of dietary intervention) and day 13 (just prior to *L. monocytogenes* infection). The host transcriptional profile was similar when comparing animals fed a commonly used mouse chow and animals fed the LF diet. However, HF dietary feeding significantly altered the pattern of gene transcription in mice (**Fig. 5**).

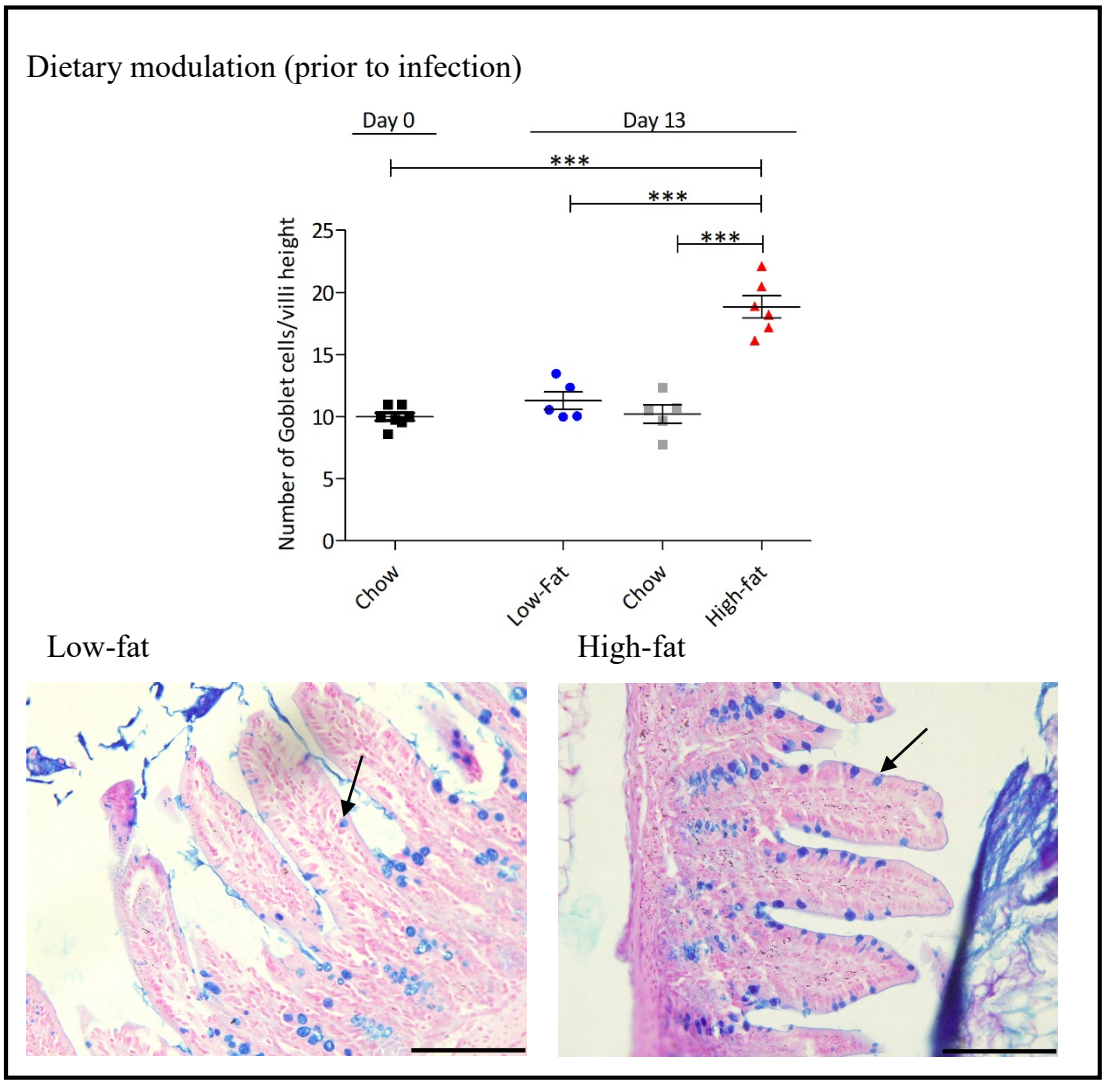



**Figure 4.** Diet influences the host physiological landscape prior to infection (D0 to D13). Number of goblet cells (D13) present on one villus was quantified and divided by the villus length (groups were blinded). Representative histological images demonstrating goblet cell density (left representative of low-fat (LF) group and right representative of high-fat (HF) group on day D13) (bar 200 µm). Ileal paraffin sections of 5 µm were stained with Alcian Blue and Periodic Acid Schiff (PAS) and counterstained with Schiff Reagent and Nuclear Fast Red Solution. Arrows indicate examples of goblet cells. Statistical analysis was conducted using One-way ANOVA and Dunnett's Multiple Comparison Test in relation to D0. *** $P < 0.001$.

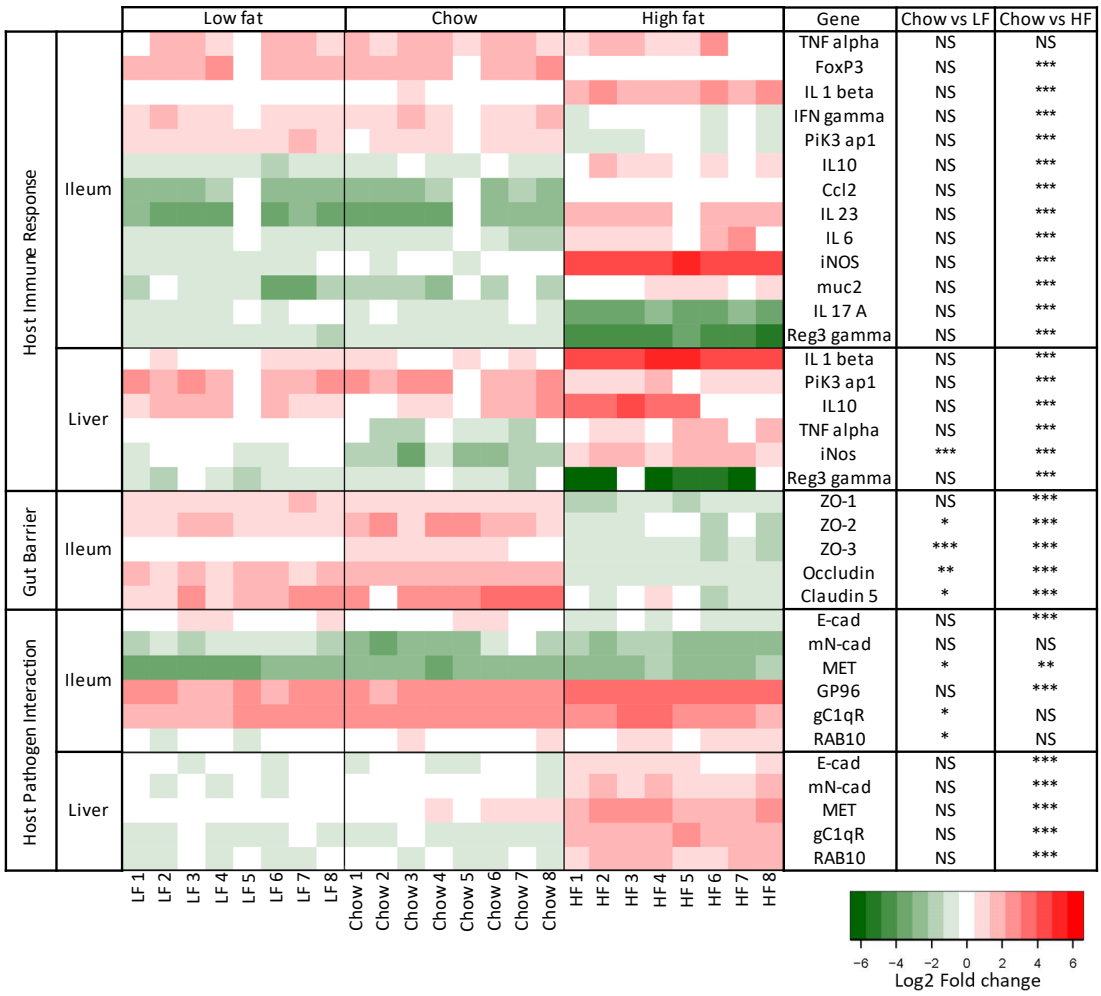



**Figure 5.** Diet modulates gene expression prior to infection (D0 to D13). Murine gene expression profile in response to increased dietary fat content by qRT-PCR. Represented as log2 of the fold change between the condition (D13 low-fat (LF) or D13 high-fat (HF)) and the control (D0). Statistical analysis was conducted using One-way ANOVA and Dunnett's Multiple Comparison Test.

Generally, mice fed a HF diet demonstrated elevated inflammatory gene expression relative to the LF and chow-fed mice (**Fig. 5**). The data are consistent with the concept that a HF diet promotes a pro-inflammatory state in the host and are supportive of longer-term studies examining host inflammation following the onset of obesity (Araújo et al., 2017; Rocha et al., 2016). In particular, the expression of IL-23, a key mediator of the inflammatory response in the gut, is upregulated in the ileum of the animals fed the HF diet. We also determined increased expression of genes encoding IL-1β in the ileum and liver, iNOS in the ileum and TNF-α in the liver (Nakane et al., 1988; Remer et al., 2001) all of which are associated with the onset of obesity. Whilst TNF-α is essential for anti-listerial resistance (Nakane et al., 1988) the role of both IL-1β and iNOS during Listeria infection is less pronounced and subject to some debate (Glaccum et al., 1997; Hirsch et al., 1996; Remer et al., 2001). Ileal expression of genes encoding anti-listerial cytokines IFN-γ (Dunn and North, 1991) and IL-17a (Hamada et al., 2008) was significantly reduced in HF mice prior to infection. Notably, reduced expression of RegIII-γ was more pronounced in HF diet fed animals in comparison to LF or chow-fed mice. RegIII-γ is an anti-bacterial lectin that is anti-listerial (Brandl et al., 2007; Loonen et al., 2014) and also plays a role in microbial homeostasis in the gut through targeting Gram-positive commensals (Vaishnava et al., 2011). The expression of RegIII-γ and other antibacterial peptides are known to be influenced by diet and are subject to control by the microbiota (Everard et al., 2013).

Overall transcription of genes encoding tight-junction proteins in the ileum was reduced in HF fed animals relative to the other groups (**Fig. 5**). Expression of tight-junction proteins can be used as an assessment of barrier function and has previously been shown to be reduced in obese mice (Gulhane et al., 2016; Hamilton et al., 2015). Short-term studies in rodents demonstrate a reduction of claudin-7 levels following 4

weeks of HF diet (Tomas et al., 2016), but no changes in ZO-1 at 3 days (Panasevich et al., 2016) or one-week (Hamilton et al., 2015) of HF dietary feeding, suggesting time dependent alterations which we see at 2 weeks of dietary intervention. Finally, expression of genes encoding mN-cad (Nikitas et al., 2011) and MET (Niemann et al., 2007), known binding sites for *L. monocytogenes* invasion factors InlA and InlB in our murine model of infection, were not altered in the ileum of HF fed mice but were significantly increased in the livers of these animals when compared to LF or chow-fed groups (**Fig. 5**). The gene encoding E-cad was down-regulated in the ileum in concert with other epithelial junction proteins. Overall, we demonstrate significant alterations to the gastrointestinal environment through short-term HF feeding just prior to infection in our model system with many changes potentially relevant to the pathogenesis of *L. monocytogenes*.

### 4.3. Short-term high-fat diet alters the gut microbiota

As the gastrointestinal microbiota is known to provide a barrier to *L. monocytogenes* infection (Becattini et al., 2017) and also influences local barrier function (Hamilton et al., 2015) and immune homeostasis (Cani et al., 2009; Claesson et al., 2012; Statovci et al., 2017), we investigated alterations of the microbiota in our model at day 13 (prior to infection) with a focus upon differences between groups fed HF and matched LF diets. The extent of similarity between microbial communities was visualized through Unweighted UniFrac PCoA of Operational Taxonomic Units (OTUs), grouped at 97% sequence identity. The β-diversity metrics support a clear dietary driven separation (along PC2) between the HF (in red) and LF (in blue) fed mice on day 13 (**Fig. 6a**). α-diversity metrics are represented in **Fig. S2**. Relative to animals on a LF diet, the HF group had an increased representation of bacteria belonging to the Firmicutes phylum and a decrease in the Bacteroidetes (**Fig. 6b**, **Fig. S3**). This shift in the

Firmicutes/Bacteroidetes ratio is associated with low-grade inflammation, reduction in barrier function and glucose intolerance in the context of a diet rich in animal fat (Rabot et al., 2016).

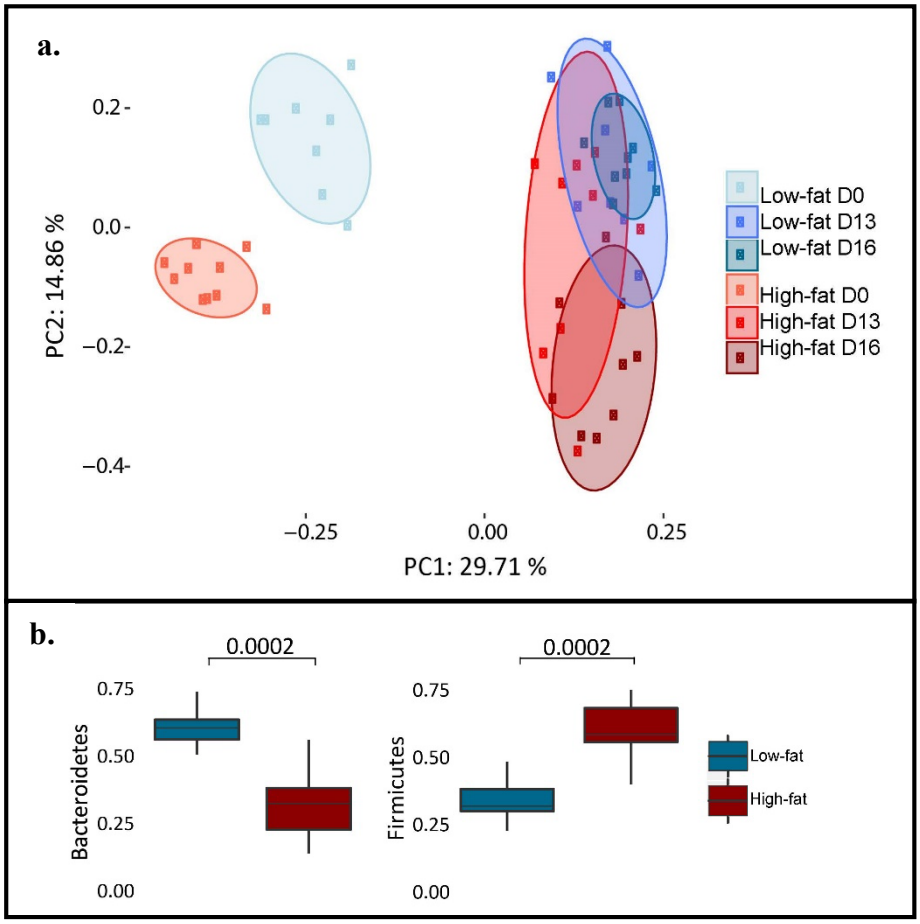



**Figure 6.** Dietary modulation of microbiota composition. **a.** Comparison of the changes in bacterial communities during controlled feeding. Unweighted UniFrac PCoA faecal microbiota distances between groups of mice fed different diets (blue representing low-fat (LF); red representing high-fat (HF)) prior to infection (time points D0, D6, D13). P-values were measured using an Adonis test (p-value=$1e^{-5}$, R squared (proportion of variance explained) is 0.48). **b.** Changes in bacterial abundance during dietary shifts (D0 to D13) for the most abundant phyla, Bacteroidetes and Firmicutes. Statistical analysis was conducted using Mann-Whitney nonparametric test. Results represent mean ± SEM.

**Figure 7** shows a significant increase in the abundance of Bacteroidaceae and Rikenellaceae in the LF fed group and a significant increase in abundance of Ruminococcaceae and Lachnospiraceae in the HF fed mice. These patterns of family-level alterations to the microbiota have previously been reported for healthy individuals and patients with disorders associated with obesity, respectively (Eid et al., 2017; Lin et al., 2016). The data demonstrate significant diet-related alterations to gut microbiota composition upon short-term feeding prior to Listeria infection. Therefore, in our experimental model *L. monocytogenes* is introduced into a gut environment in which there has been a considerable taxonomic shift influenced by diet and previously associated with reduction in barrier function.

To further investigate any potential links between microbiota changes and the host response to dietary feeding we correlated changes in host gene expression with abundance of individual members of the faecal microbiota at the genus level (**Fig. 8**).

*Butyricicoccus,* Clostridium XIVa*, Streptococcus* and *Mucispirillum* were more abundant in mice fed a HF diet and their abundance correlated with induction of genes encoding host inflammatory responses (**Fig. 8**). Associations between these genera and inflammatory conditions have been reported previously (Claesson et al., 2012; Jiang et al., 2015). *Parabacteroides* and *Bacteroides* were relatively more abundant in LF-fed mice and have previously been reported to be involved in the maintenance of immune homeostasis in the gut and maintenance of intestinal barrier integrity (Kverka et al., 2011; Lathrop et al., 2011). Herein we identified genera (e.g. Clostridium XI, Clostridium XIVa, *Enterococcus* spp.) which are influenced by HF dietary changes and are correlated with expression of genes encoding receptors for *L. monocytogenes* as well as genes associated with inflammation in the ileum and liver (**Fig. 8**).

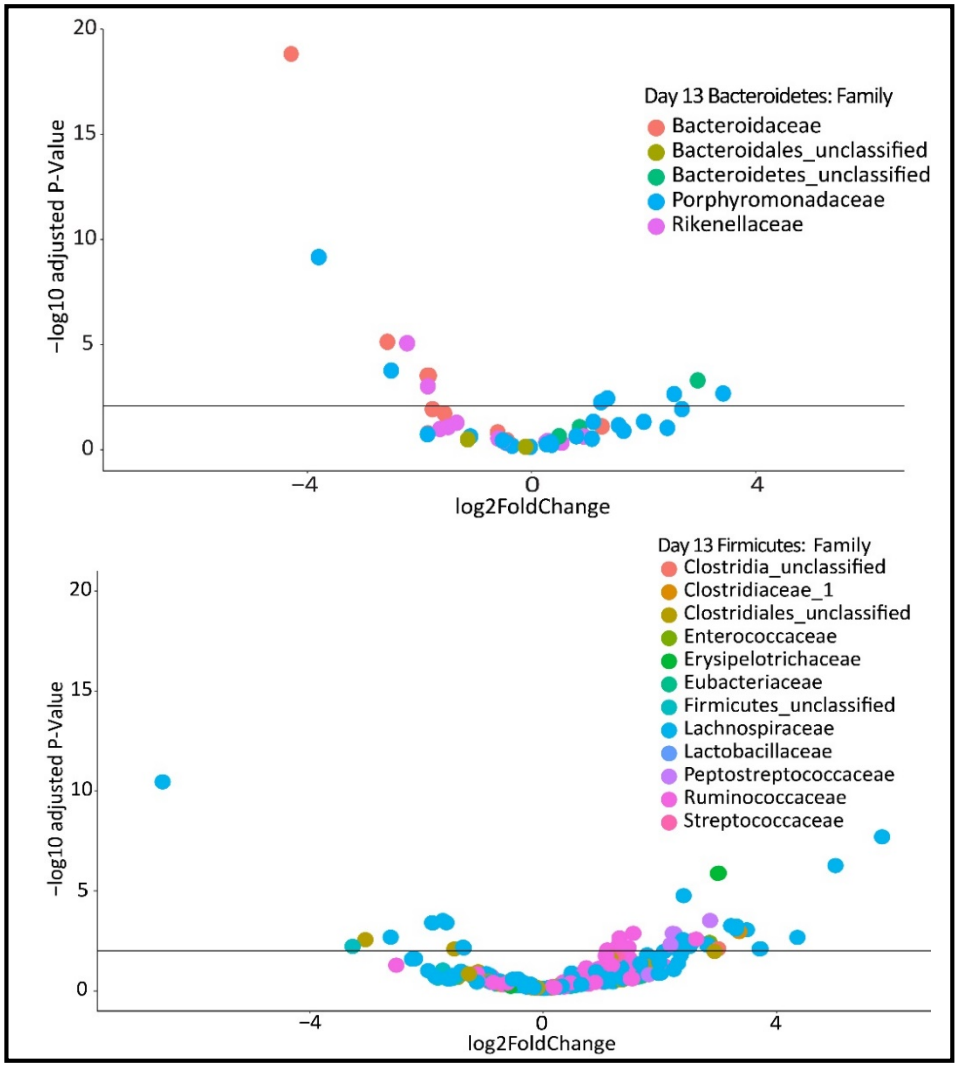



**Figure 7.** Changes in bacterial abundance between low-fat (LF) and high-fat (HF) diets at the family level for the most abundant phyla. A volcano plot showing the fold change between HF and LF diets at D13. Each point represents an Operational Taxonomic Unit (OTU). The x-axis represents the log2 of the fold-change while the y-axis is the negative log10 of DESeq2 p-values adjusted for multiple testing using the False Discovery Rate method. Points to the right of the plot with positive log2 fold-change values represent bacterial taxa with increased abundance in the mice on the HF diet relative to the mice on the LF diet and those with negative log2 fold-change values represent bacterial taxa with increased abundance in the LF diet relative to the HF diet. The horizontal black line represents the cut off for statistical significance, an adjusted P-value of 0.05.

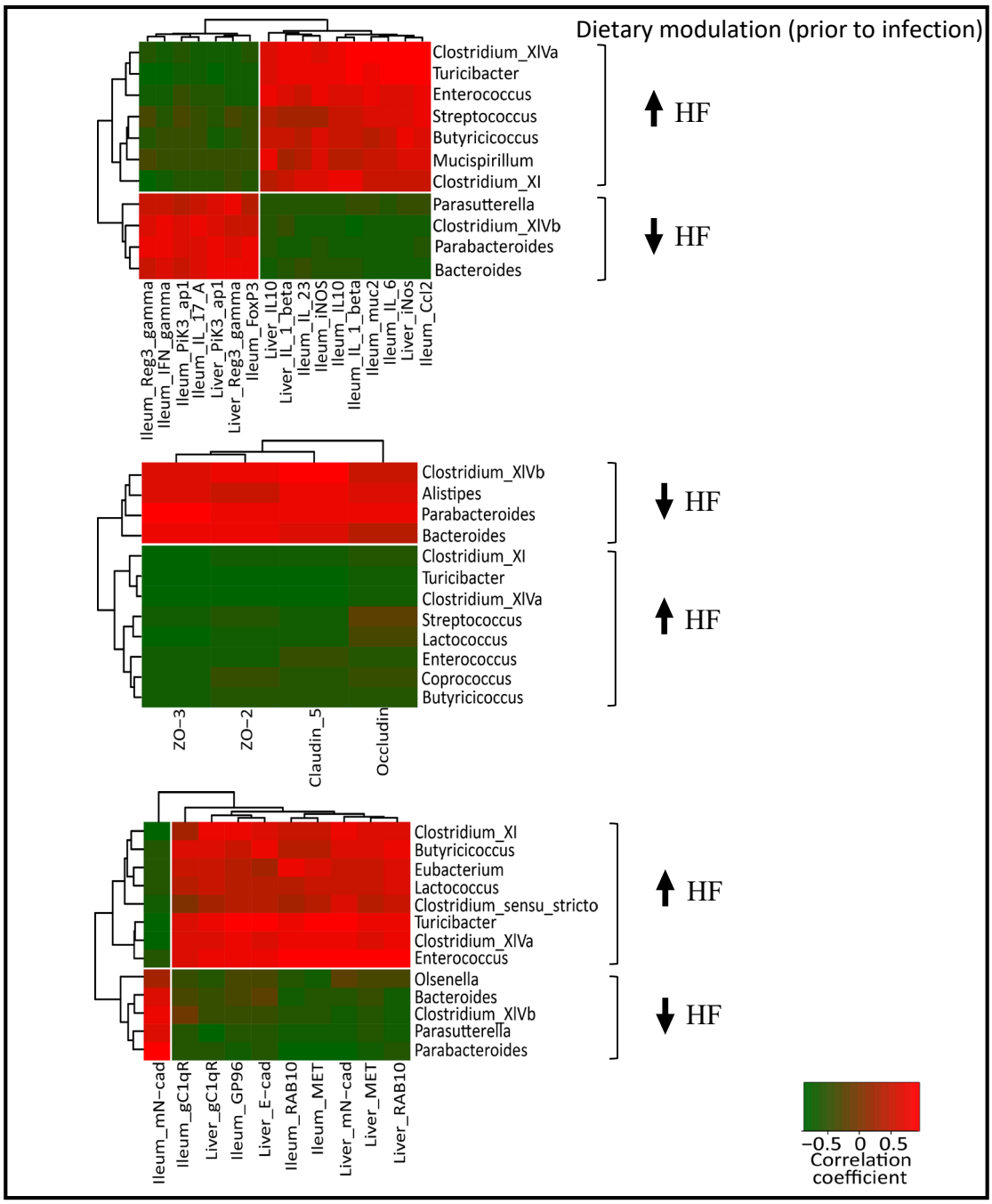



**Figure 8.** A correlation between the host regulatory response and the microbiota at the Genus level on D13. Spearman correlation, between the diet-dependent relative abundance of bacterial genera (arrows represent abundance in the mice on the HF diet relative to the mice on the LF diet) and the fold change for genes in both ileum and liver. Results shown separately for genes associated with host-immunity, tight-junction proteins and host-pathogen interaction. Represented are only significant hits, $P<0.05$.

### 4.4. High-fat diet alters the physiological response to *L. monocytogenes* infection

We subsequently determined the physiological response to oral *L. monocytogenes* EGDe $InlA^m$ infection at 3-days post-infection in the context of HF diet. Goblet cell numbers post-infection remained elevated in the ileum of HF diet-fed animals relative to LF or chow-fed animals (**Fig. 9**).

Gene expression profiling of target genes was used to compare gene expression post-infection with the time-point immediately prior to infection (day 13). Analysis revealed a reduction in expression of genes encoding inflammatory markers in both ileum and liver of HF diet-fed animals when compared to mice fed a LF diet or chow (**Fig. 10**). This is supported by histological analysis of ileal tissue which indicated reduced immune cell infiltration in response to infection in mice fed a HF diet (**Fig. 11**). These are unexpected findings as these mice have a higher infectious load in local tissues relative to LF or chow-fed animals. Furthermore, in our model we observed that Listeria infection in the context of HF diet feeding also resulted in a further reduction in expression of genes encoding tight-junction proteins suggestive of a further impairment of barrier function (**Fig. 10**). Our findings are potentially reflective of very recent studies demonstrating that *L. monocytogenes* crosses the intestinal epithelial barrier by inducing significant mislocalization and reduction of expression of Occludin, Claudin-1, and E-cad, through the induction of TNF-α and IL-6 (Drolia et al., 2018). We appreciate that a shutdown of gene expression may, in some manner, be a consequence of higher numbers of the pathogen in the tissue, but to our knowledge a dose-response correlating immune stimulation with increasing infectious load of *L. monocytogenes* has not been examined previously.

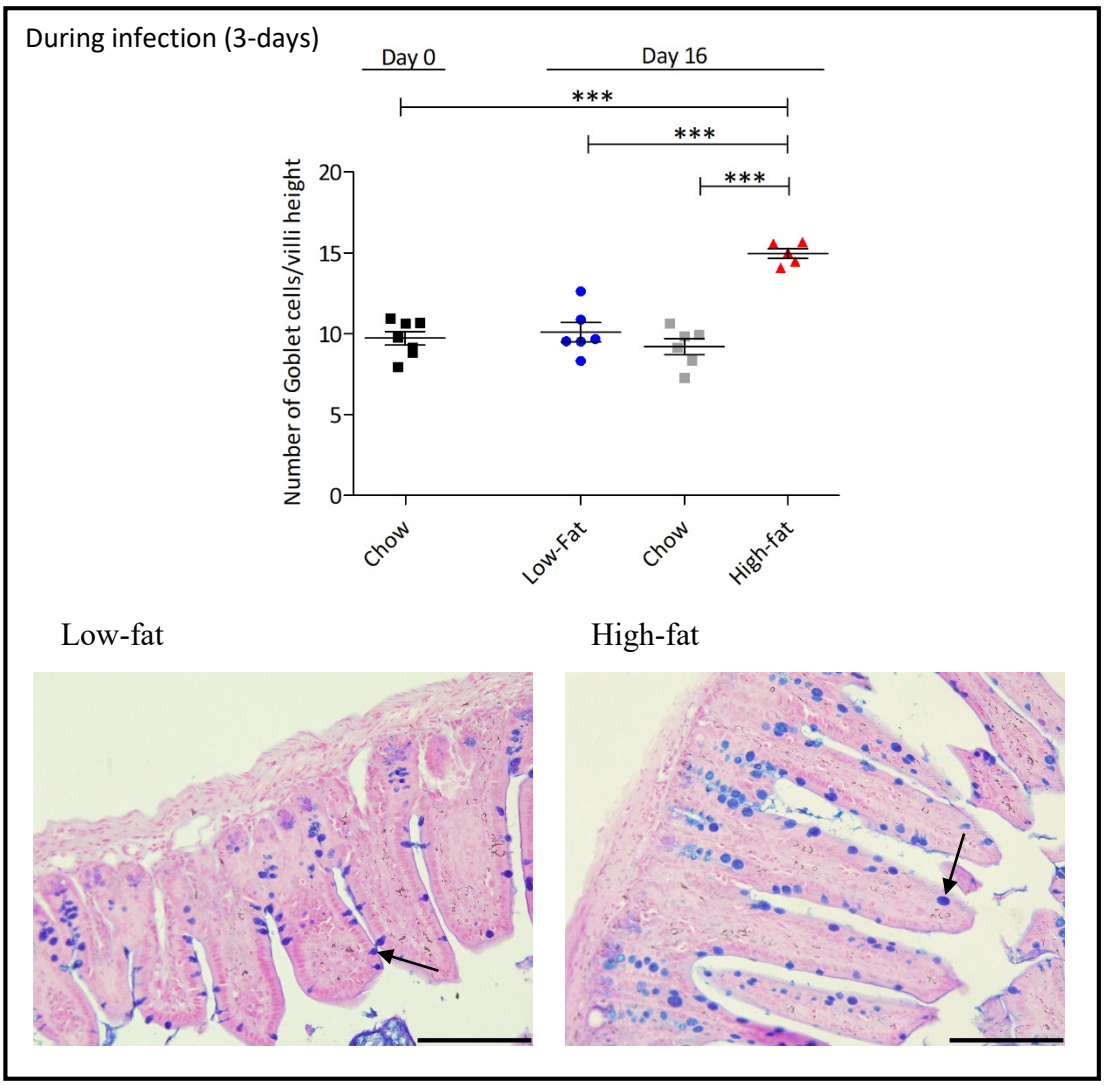



**Figure 9.** Number of goblet cells (D16) present on one villus was quantified and divided by the villus length (groups were blinded). Representative histological images demonstrating goblet cell density (left representative of LF group and right representative of HF group on day D16). Ileal paraffin sections of 5 µm were stained with Alcian Blue and Periodic Acid Schiff (PAS) and counterstained with Schiff Reagent and Nuclear Fast Red Solution. Arrows indicate examples of goblet cells. Statistical analysis was conducted using One-way ANOVA and Dunnett's Multiple Comparison Test in relation to D0 (results represent mean ± SEM). *** $P < 0.001$.

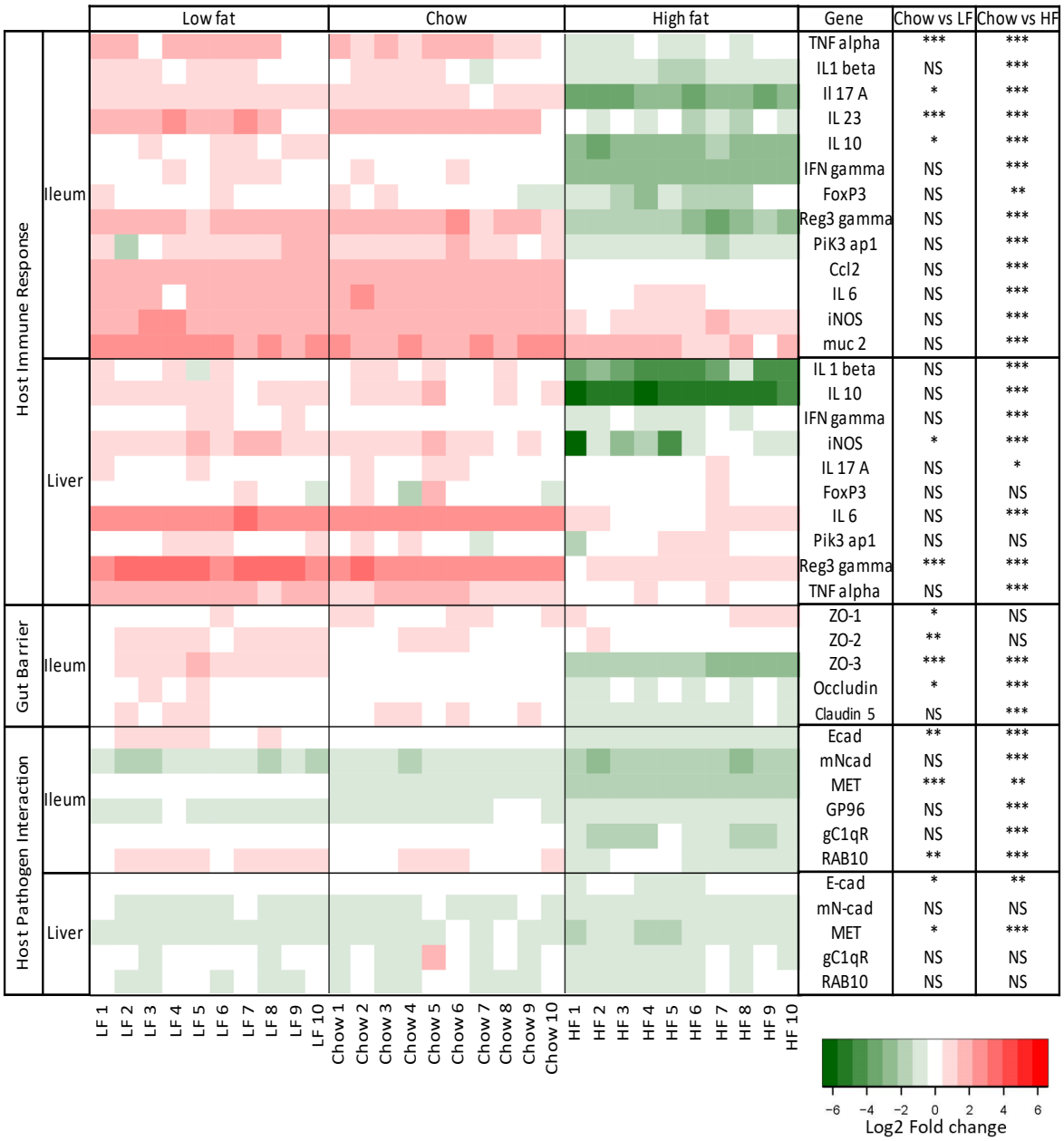



**Figure 10.** Effects of diet on host gene expression post-infection using qRT-PCR. Gene expression relative to D13 (pre-infection), within the same dietary group, in both ileum and liver on day 16 of dietary feeding (3-days post-infection). Represented as log2 of the fold change between the condition and the control (same diet D13). Statistical analysis was conducted using One-way ANOVA and Dunnett's Multiple Comparison Test.

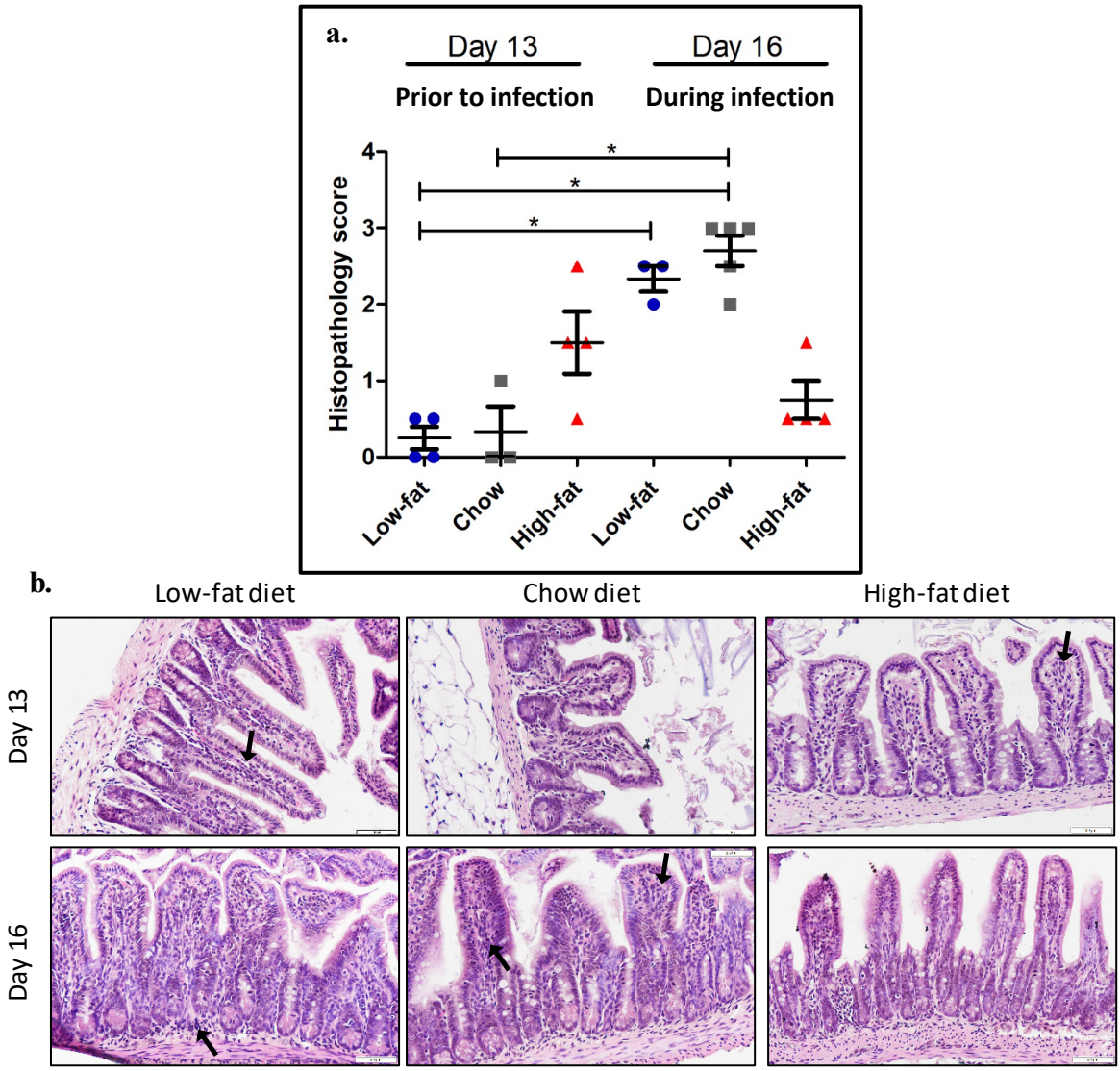



**Figure 11**. Histopathology score of ileum sections**. a.** Histopathology score of ileal sections 13 days post dietary change (Day 13) and 3-days post-infection (Day 16). **b.** The ileal samples were scored on a scale of 0-3 for 2 parameters: infiltration of inflammatory cells (mostly mononuclear cells) to the villi and infiltration of mono- and polymorphonuclear cells to the crypts, yielding a maximum score of 6. In our model, polymorphonuclear cells were mainly located at the bottom of the crypts. The gradient of the inflammatory cell infiltration was based on: 3= highly increased; 2= moderately increased, 1= mildly increased and 0= normal (scale bar, 50µm). One-way ANOVA and Dunnett's Multiple Comparison Test in relation to D0 (error bars represent mean ± SEM). * $P < 0.05$.

We propose that the resultant dampening of immune stimulation in our model is a consequence of both the presence of the pathogen and increased dietary fat intake. In support of this, very recent work has shown that mice fed a HF diet and infected with *Borrelia burdorferi* showed suppressed innate immunity suggesting an immune-regulatory role of dietary fat intake which may favour infection (Zlotnikov et al., 2017).

Analysis of the microbiota during *L. monocytogenes* infection (**Fig. 12a**) indicates a clear separation of communities resulting from the presence of the pathogen in the context of diet. In particular, LF-fed animals undergo a profound rearrangement of the microbial community structure from D13 to D16 as a consequence of infection, in both principal components. An increased Firmicutes/Bacteroidetes ratio in the HF group is maintained during *L. monocytogenes* infection (**Fig. 12b**).

The microbial families affected by infection (D16) (**Fig. 13**, **Fig. S4**) resemble those that are influenced by diet alone (D13) (**Fig. 7**) however we see a stronger representation of the numbers of operational taxonomic units (OTUs) for both Bacteroidaceae and Rikenellaceae in the LF fed group and Ruminococcaceae and Lachnospiraceae in the HF fed mice following Listeria infection (**Fig. 13**). This suggests that Listeria infection in the context of HF diet potentially amplifies OTUs associated with diet-induced inflammation. Interestingly the representation of the Clostridiales family as indicated by the number of significant OTUs has increased abundance in the LF fed group (**Fig. 13**). This family has recently been associated with *L. monocytogenes* clearance upon infection (Becattini et al., 2017).

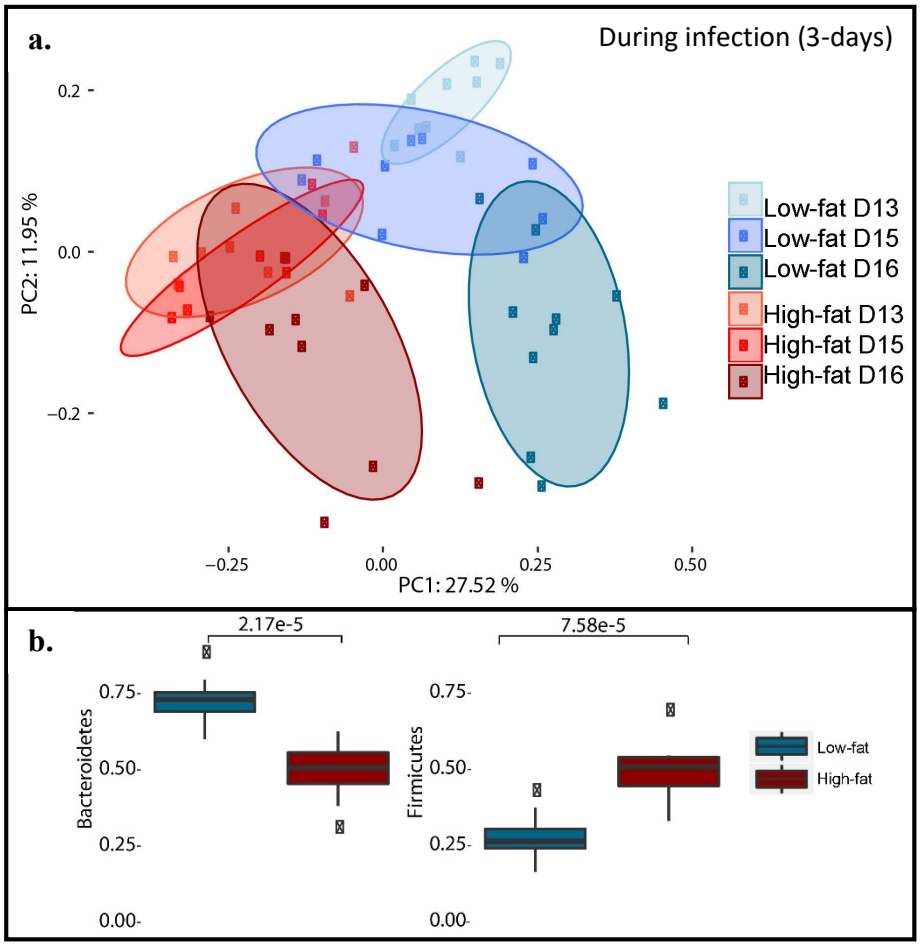



**Figure 12.** Impact of dietary modulation upon infection with *L. monocytogenes*. **a.** Comparison of the changes in bacterial communities during controlled feeding. Unweighted Unifrac PCoA faecal microbiota distances between groups of mice fed different diets (blue representing LF; red representing HF) over indicated time points (D13, D15, D16). P-values were measured using an Adonis test (P-value= 0.00099, R squared is 0.148). **b.** Changes in bacterial percentage of abundance during dietary shifts (D13 to D16) of the most abundant phyla, Bacteroidetes and Firmicutes. Statistical analysis was conducted using Mann-Whitney nonparametric test. Results represent mean ± SEM.

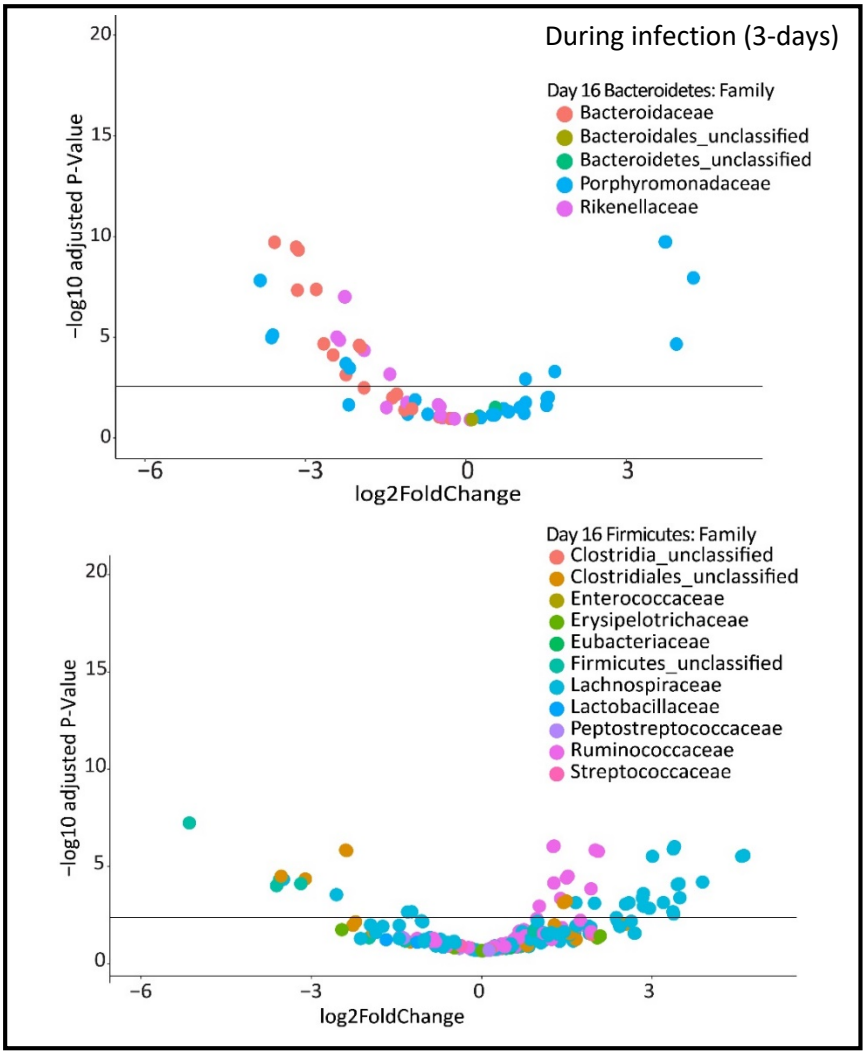



**Figure 13.** Changes in bacterial abundance between low-fat (LF) and high-fat (HF) diets at the family level for the most abundant phyla. A volcano plot showing the fold change between HF and LF diets at D16. Each point represents an Operational Taxonomic Unit (OTU). The x-axis represents in the log2 of the fold change while the y-axis is the negative log10 of DESeq2 P-values adjusted for multiple testing using the False Discovery Rate method. Points to the right of the plot with positive log2 fold-change values represent bacterial taxa with increased abundance in the mice on the HF diet relative to the mice on the LF diet and those with negative log2 fold-change values represent bacterial taxa with increased abundance in the LF diet relative to the HF diet. The horizontal black line represents the cut off for statistical significance, an adjusted p-value of 0.05.

Correlating microbial genera with host gene expression (**Fig. 14**) highlights the general down regulation of host gene expression following Listeria infection in the HF-fed group. Directional changes to specific microbial genera influenced by HF diet (**Fig. 14**) were similar to those seen to be induced by diet alone (**Fig. 8**). However, the correlations with associated host gene expression profiles were generally reversed, indicating that *L. monocytogenes* infection in the context of HF diet was a significant negative modulator of selected host genes.

### 4.5. High-fat diet increases susceptibility to systemic *L. monocytogenes* infection

Whilst this study revealed that diet alone is a driver of physiological changes in the ileum, it also highlighted an unexpected increase in expression of genes encoding *L. monocytogenes* binding sites (including E-Cad, N-Cad, gC1qR) in the liver (**Fig. 5**). We also noted alterations to expression levels of genes encoding cytokines in the liver that have the potential to influence resistance to infection, including an increase in transcription of the anti-inflammatory cytokine IL-10 in animals fed a HF diet (**Fig. 5**). As these changes occurred prior to infection we went on to determine the influence of diet upon the systemic phase of infection. An intraperitoneal (IP) infection of *L. monocytogenes* EGDe $InlA^m$ was administered after 2 weeks of dietary modulation (**Fig. 15**). The results reveal a clear influence of HF diet upon the systemic phase of *L. monocytogenes* compared to both chow and LF diets. The phenomenon was seen for both the murinized EGDe $InlA^m$ and wild-type EGDe strains (**Fig. S5**) of *L. monocytogenes* suggestive that this effect is not solely due to an increase in mN-Cad. The data suggest that a Westernized-HF-diet alters the physiology of the host beyond the gut to heighten susceptibility to infectious disease.

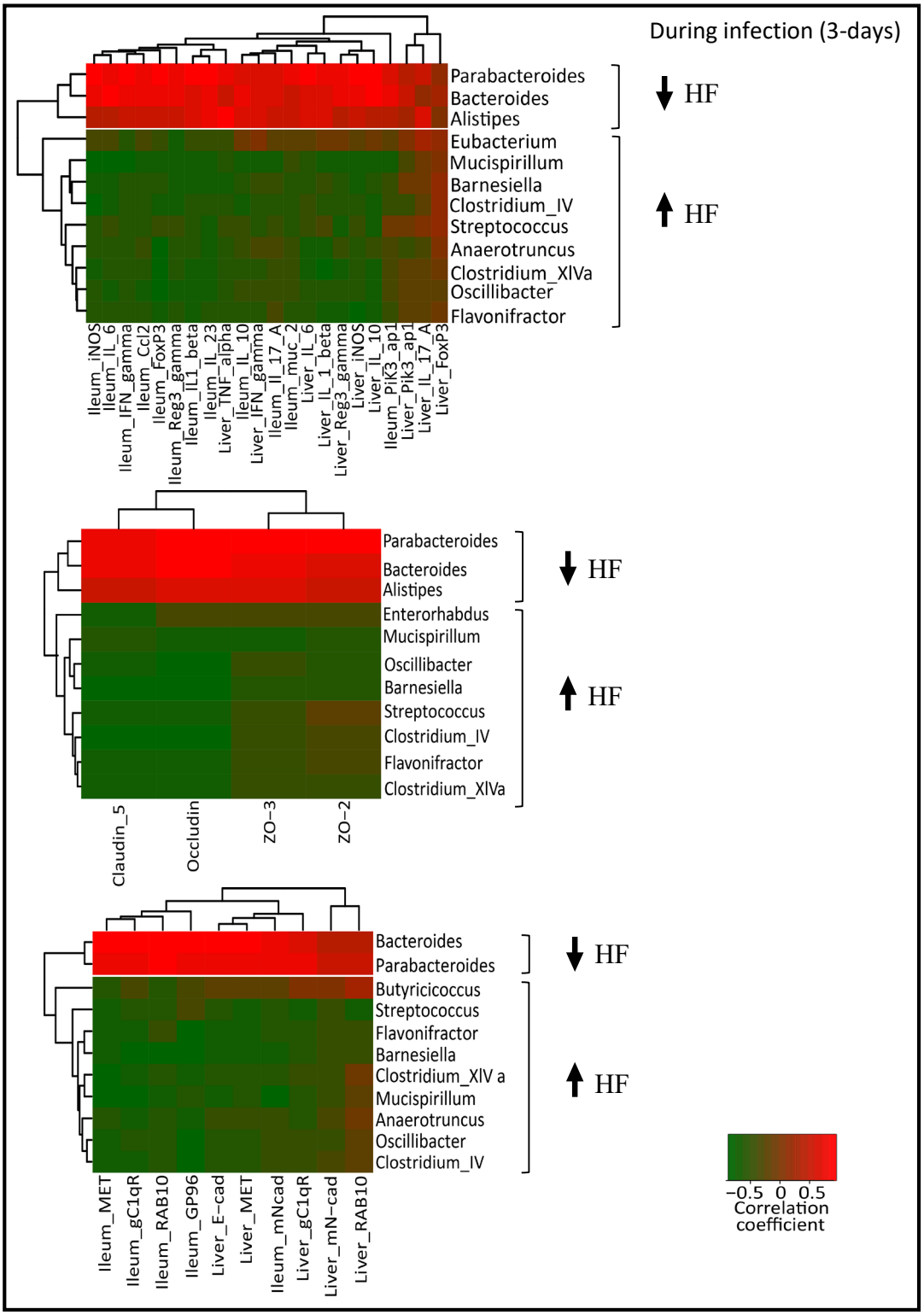

During infection (3-days)
Parabacteroides
Bacteroides
Alistipes
Eubacterium
Mucispirillum
Barnesiella
Clostridium_IV
Streptococcus
Anaerotruncus
Clostridium_XlVa
Oscillibacter
Flavonifractor
HF
HF
Ileum_iNOS
Ileum_IL_6
Ileum_IFN_gamma
Ileum_Ccl2
Ileum_FoxP3
Ileum_Reg3_gamma
Ileum_IL1_beta
Ileum_IL_23
Liver_TNF_alpha
Ileum_IL_10
Liver_IFN_gamma
Ileum_Il_17_A
Ileum_muc_2
Liver_IL_6
Liver_IL_1_beta
Liver_Reg3_gamma
Liver_iNOS
Liver_IL_10
Ileum_PiK3_ap1
Liver_Pik3_ap1
Liver_IL_17_A
Liver_FoxP3
Parabacteroides
Bacteroides
Alistipes
Enterorhabdus
Mucispirillum
Oscillibacter
Barnesiella
Streptococcus
Clostridium_IV
Flavonifractor
Clostridium_XlVa
HF
HF
Claudin_5
Occludin
ZO−3
ZO−2
Bacteroides
Parabacteroides
Butyricicoccus
Streptococcus
Flavonifractor
Barnesiella
Clostridium_XlV a
Mucispirillum
Anaerotruncus
Oscillibacter
Clostridium_IV
HF
HF
Ileum_MET
Ileum_gC1qR
Ileum_RAB10
Ileum_GP96
Liver_E−cad
Liver_MET
Ileum_mNcad
Liver_gC1qR
Liver_mN−cad
Liver_RAB10
−0.5 0 0.5
Correlation
coefficient

**Figure 14.** A correlation between the host regulatory response and the microbiota at the genus level on day 16. Spearman correlation, between the diet dependent relative abundance of bacterial genera (arrows represent abundance in the mice on the high-fat (HF) diet relative to the mice on the low-fat (LF) diet) and the fold change for genes in both ileum and liver. Results shown separately for host-immunity, tight-junctions and host-pathogen interaction genes. Represented are only significant hits, P<0.05.

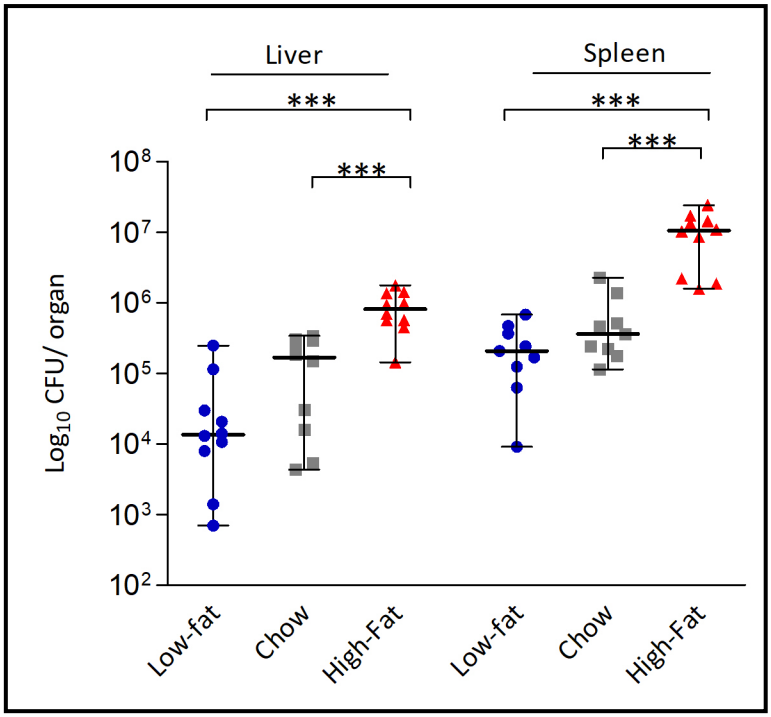


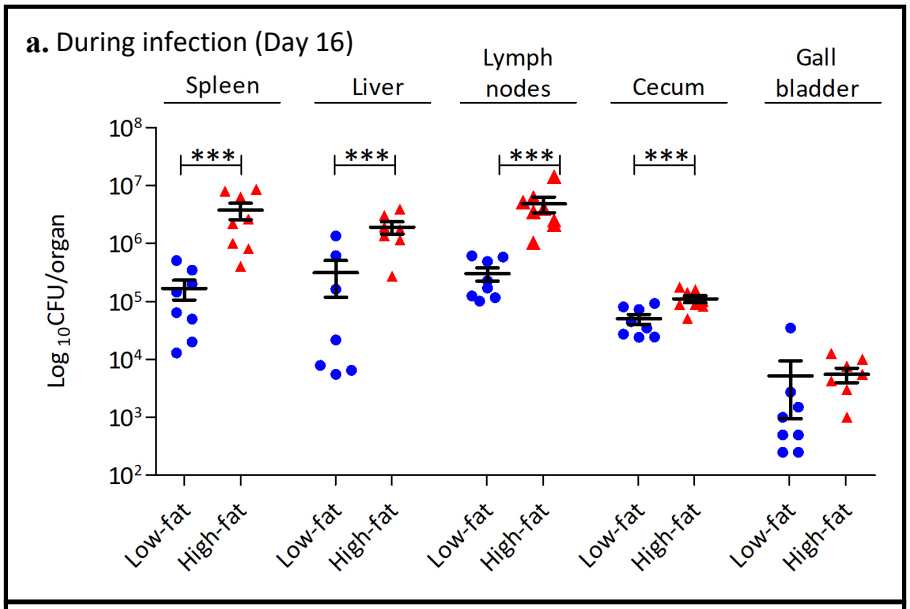

**Figure 15.** Increased dietary fat increases host susceptibility to systemic infection with *L. monocytogenes* EGDe $InlA^m$**.** Bacterial burden of Listeria in spleen and liver of C57BL/6 mice fed for 13 days with different diets and subsequently infected via the IP route (n=10). Standard deviation from the mean, statistical analysis was conducted using One-way ANOVA and Dunnett's Multiple Comparison Test in relation to chow diet. Error bars represent SEM. *** P < 0.001.

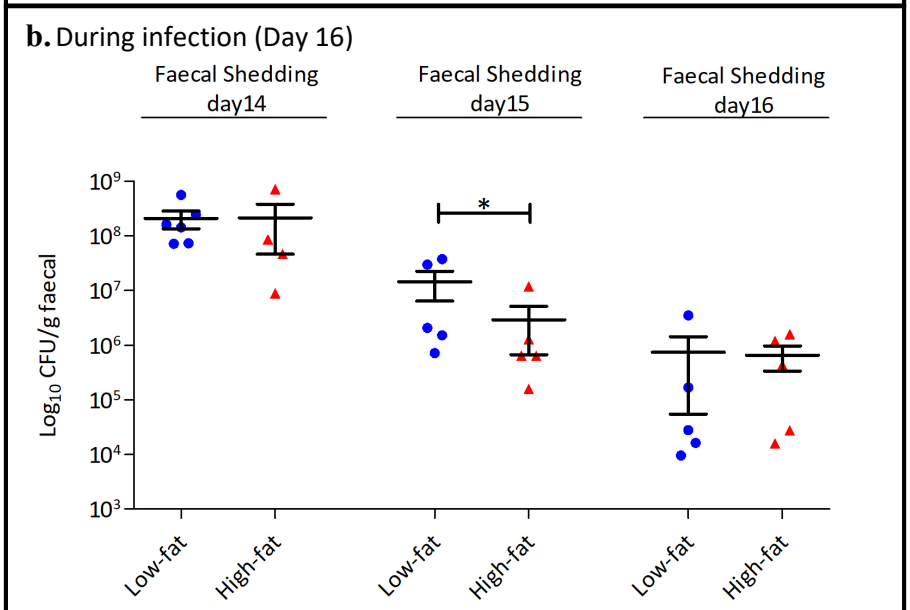
# 5. Conclusions

In the context of a global obesity epidemic and changes in dietary habits towards increased consumption of a Westernized-diet, there is currently surprisingly little information regarding the influence of diet upon the progression of infectious disease. Herein we demonstrate that a HF Westernized-diet significantly and reproducibly increases susceptibility to *L. monocytogenes* in a murine model. HF dietary feeding prior to the onset of obesity influenced parameters in mice that impact both the intestinal and systemic phase of infection suggesting a profound systems-wide alteration in host physiology. We appreciate that we have not examined alterations to listerial expression of virulence factors that may occur as a result of luminal alterations of nutrients. For instance, altered responses in *Listeria* have been reported in response to exposure to various fatty acids *in vitro* (Kallipolitis, 2017) and may therefore have the potential to influence infectivity. It is interesting to note that other studies have demonstrated that a HF food-delivery matrix can increase infectivity of *L. monocytogenes* in murine (Bou Ghanem et al., 2012) or primate (Smith et al., 2008) models. However, the physiological effects on the host of transient HF feeding were not considered in those studies. The data presented herein support emerging evidence that diet can significantly influence infectious disease models (Desai et al., 2016; Hryckowian et al., 2018) and suggests that diet should be a factor in future evaluation of the infectious dose of the pathogen. The work raises the intriguing possibility that a Westernized-diet may be a significant factor influencing host resistance to infection.

# 6. Supplementary files

**Figure S1**




**c.**

| Organs | Low-fat | High-fat |
|---|---|---|
| Spleen | P=0.7618 (NS) | P=0.0205 (*) |
| Lymph nodes | P=0.5049 (NS) | P=0.9654 (NS) |
| Cecum | P=0.1457 (NS) | P=0.2766 (NS) |
| Liver | P=0.3148 (NS) | P=0.0001 (***) |

**Figure S1.** Increased dietary fat from animal source increases host susceptibility to oral infection with *Listeria monocytogenes* EGDe$^{m}$::pIMK2*lux*. Intragastric inoculation of seven-week-old female C57BL/6 mice (ENVIGO, UK) was repeated in a separate duplicate experiment (n=8) using a 200 µl inoculum comprising 2.8 x $10^{9}$ CFU *L. monocytogenes* EGDe$^{m}$::pIMK2*lux*, a bioluminescent murinized strain. After infection (three days), the faecal pellets were collected daily and plated for CFU to determine shedding of *L. monocytogenes*. **a.** Bacterial burden of spleen, liver, lymph nodes and of C57BL/6 mice fed with diets varying in percentage of fat content from the total caloric intake (n=8). **b.** *L. monocytogenes* bacterial shedding per gram of faecal sample in each day after infection. Statistical analysis was conducted using Mann Whitney Nonparametric Test, error bars represent ± SEM **c.** Statistical comparison of CFU levels in spleen, lymph nodes, cecum and liver between the two trials (EGDe InlA$^{m}$ and EGDe$^{m}$::pIMK2*lux*). Statistical analysis was done using Mann Whitney Nonparametric Test, error bars represent ± SEM. * $P < 0.05$; *** $P < 0.001$

**Figure S2**

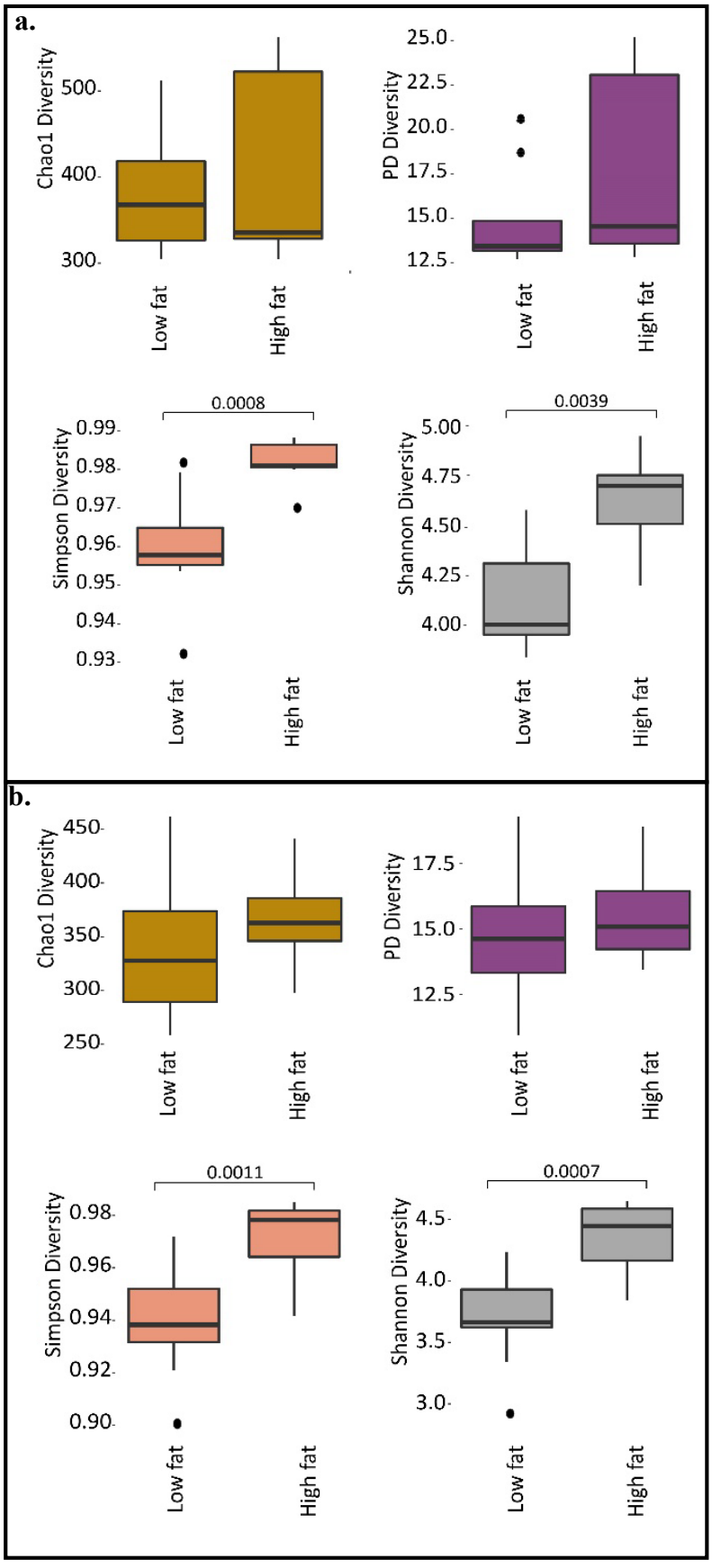

a.
Chao1 Diversity
500
400
300
Low fat
High fat
PD Diversity
25.0
22.5
20.0
17.5
15.0
12.5
Low fat
High fat
Simpson Diversity
0.0008
0.99
0.98
0.97
0.96
0.95
0.94
0.93
Low fat
High fat
Shannon Diversity
0.0039
5.00
4.75
4.50
4.25
4.00
Low fat
High fat
b.
Chao1 Diversity
450
400
350
300
250
Low fat
High fat
PD Diversity
17.5
15.0
12.5
Low fat
High fat
Simpson Diversity
0.0011
0.98
0.96
0.94
0.92
0.90
Low fat
High fat
Shannon Diversity
0.0007
4.5
4.0
3.5
3.0
Low fat
High fat

**Figure S2.** Assessment of diversity within the sample (Alpha-diversity) in the groups fed high-fat or low-fat diets. Chao1 as a measurement of sample coverage. PD (phylogenetic diversity) used to consider affiliation between taxa within diversity. Simpson and Shannon used as an estimation of richness and evenness respectively. **a.** Changes in intestinal microbiota communities caused by short-term increased dietary fat (D0 to D13). **b.** Changes in intestinal microbiota communities upon infection with *L. monocytogenes*, modulated by short-term increased dietary fat (D13 to D16). Statistical analysis was conducted using Mann-Whitney nonparametric test. Results represent mean ± SEM.

**Figure S3**

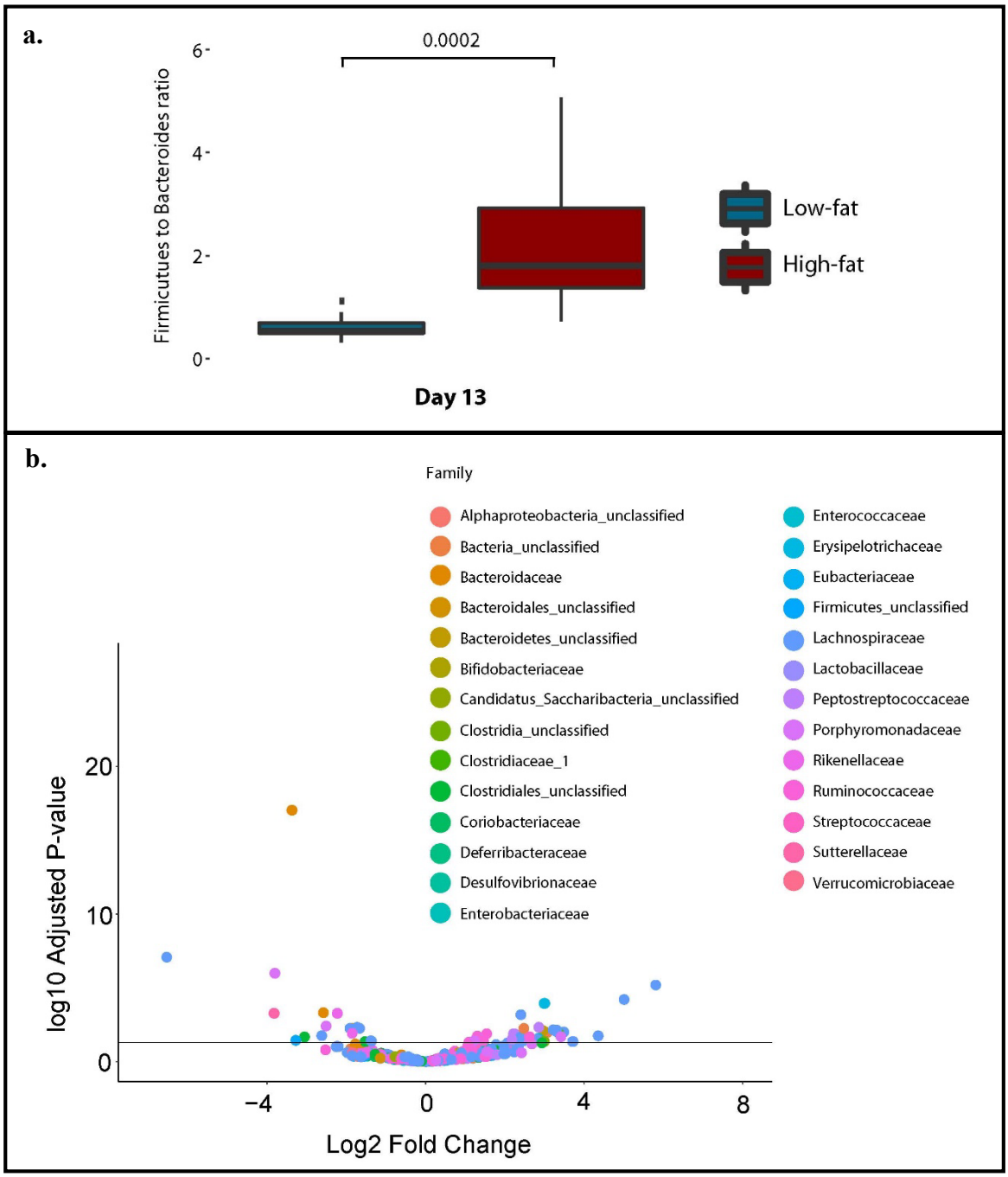

a.
0.0002
Firmicutues to Bacteroides ratio
6
4
2
0
Low-fat
High-fat
Day 13
b.
Family
Alphaproteobacteria_unclassified
Bacteria_unclassified
Bacteroidaceae
Bacteroidales_unclassified
Bacteroidetes_unclassified
Bifidobacteriaceae
Candidatus_Saccharibacteria_unclassified
Clostridia_unclassified
Clostridiaceae_1
Clostridiales_unclassified
Coriobacteriaceae
Deferribacteraceae
Desulfovibrionaceae
Enterobacteriaceae
Enterococcaceae
Erysipelotrichaceae
Eubacteriaceae
Firmicutes_unclassified
Lachnospiraceae
Lactobacillaceae
Peptostreptococcaceae
Porphyromonadaceae
Rikenellaceae
Ruminococcaceae
Streptococcaceae
Sutterellaceae
Verrucomicrobiaceae
log10 Adjusted P-value
20
10
0
−4
0
4
8
Log2 Fold Change

**Figure S3.** β-diversity metrics**. a.** Changes in Firmicutes to Bacteroidetes bacterial ratio of abundancy during dietary shifts on D13. Statistical analysis was conducted using Mann-Whitney nonparametric test. Results represent mean ± SEM. **b.** Changes in total bacterial abundancy between low and high-fat diets at the family level. A volcano plot showing the fold change between high- and low-fat diets at D13. Each point represents an Operational Taxonomic Unit (OTU). The x-axis represents in the log2 of the fold-change while the y-axis is the negative log10 of DESeq2 P values adjusted for multiple testing using the False Discovery Rate method. Points to the right of the plot with positive log2 fold-change values represent bacterial taxa with increased abundancy in the mice on the high fat diet relative to the mice on the low fat and those with negative log2 fold-change values represent bacterial taxa with increased abundancy in the low-fat diet relative to the high-fat diet. The horizontal black line represents the cut off for statistical significance, an adjusted P-value of 0.05.

**Figure S4**

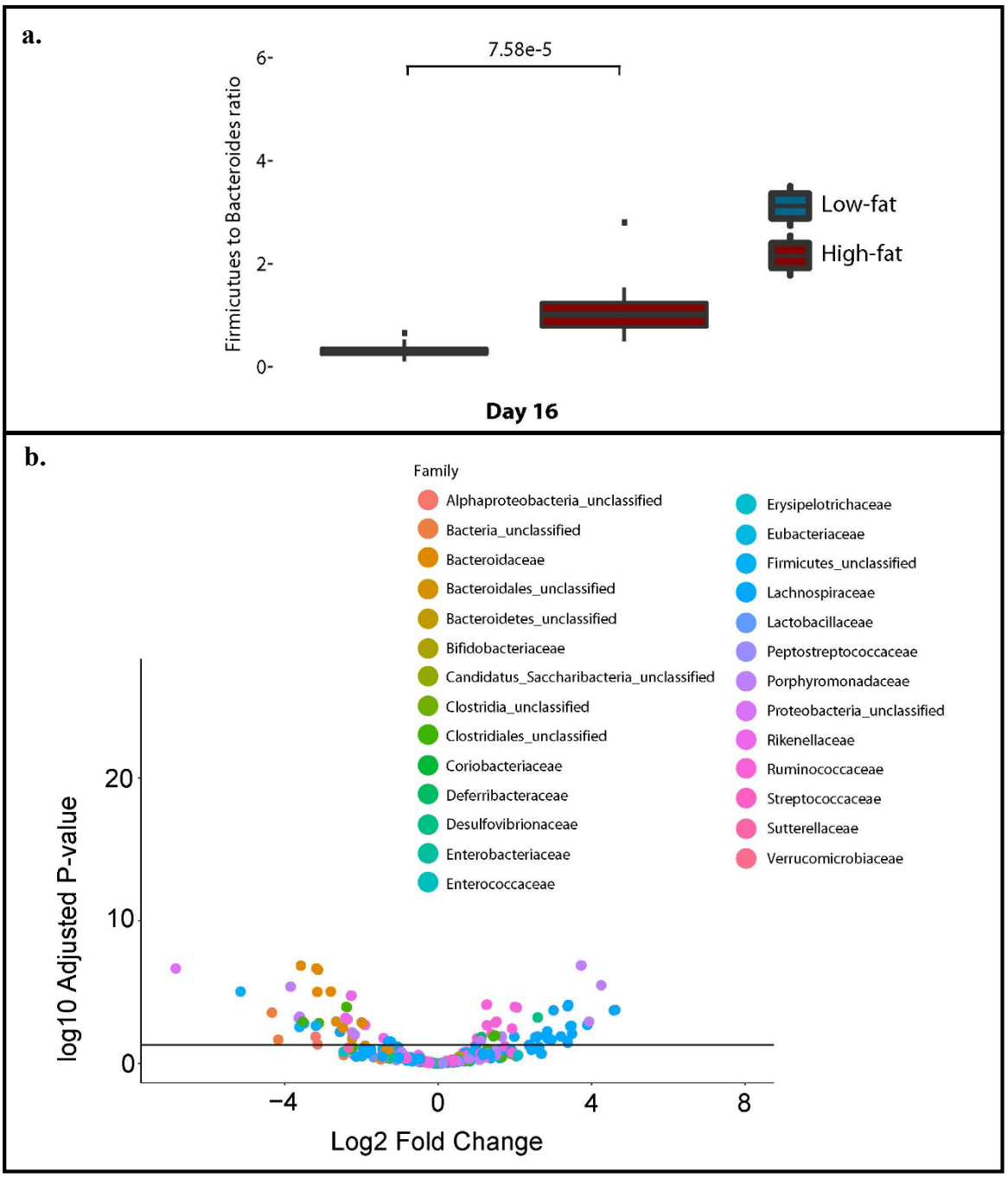

a.
7.58e-5
Firmicutes to Bacteroides ratio
6-
4-
2-
0-
Low-fat
High-fat
Day 16
b.
Family
Alphaproteobacteria_unclassified
Bacteria_unclassified
Bacteroidaceae
Bacteroidales_unclassified
Bacteroidetes_unclassified
Bifidobacteriaceae
Candidatus_Saccharibacteria_unclassified
Clostridia_unclassified
Clostridiales_unclassified
Coriobacteriaceae
Deferribacteraceae
Desulfovibrionaceae
Enterobacteriaceae
Enterococcaceae
Erysipelotrichaceae
Eubacteriaceae
Firmicutes_unclassified
Lachnospiraceae
Lactobacillaceae
Peptostreptococcaceae
Porphyromonadaceae
Proteobacteria_unclassified
Rikenellaceae
Ruminococcaceae
Streptococcaceae
Sutterellaceae
Verrucomicrobiaceae
log10 Adjusted P-value
20
10
0
−4
0
4
8
Log2 Fold Change

**Figure S4.** β-diversity metrics**. a.** Firmicutes/Bacteroidetes bacterial ratio during dietary modulation upon infection, on D16. Statistical analysis was conducted using Mann-Whitney nonparametric test. Results represent mean ± SEM. **b.** Changes in total bacterial abundancy between low-fat (LF) and high-fat (HF) diets at the family level. A volcano plot showing the fold change between high and low fat diets at D16. Each point represents an Operational Taxonomic Unit (OTU). The X axis represents in the log2 of the fold-change while the Y axis is the negative log10 of DESeq2 P values adjusted for multiple testing using the False Discovery Rate method. Points to the right of the plot with positive log2 fold-change values represent bacterial taxa with increased abundancy in the mice on the high fat diet relative to the mice on the low fat and those with negative log2 fold-change values represent bacterial taxa with increased abundancy in the low fat diet relative to the high fat diet. The horizontal black line represents the cut off for statistical significance, an adjusted P-value of 0.05.

**Figure S5**

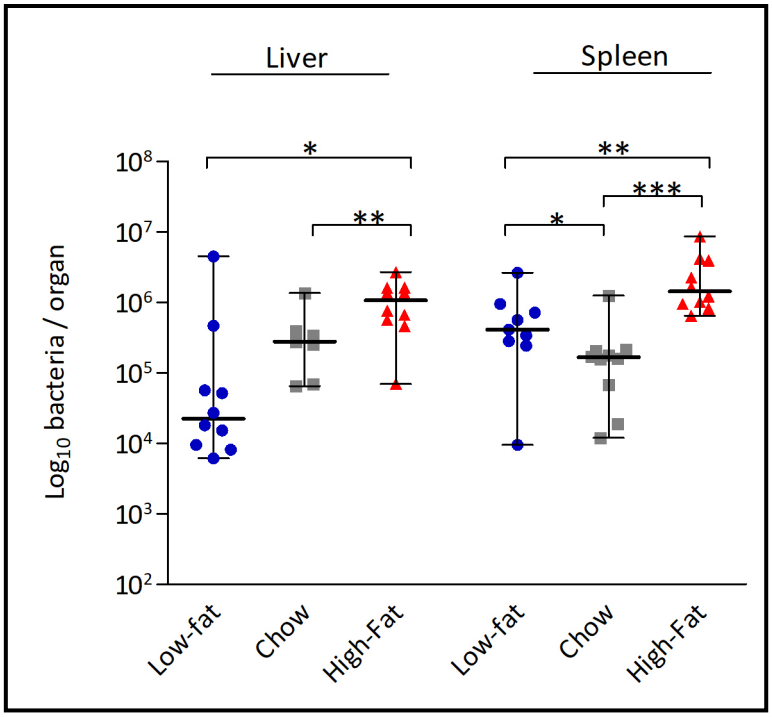



**Figure S5.** Increased dietary fat compromises the host systemic immune response and increases host susceptibility to intraperitoneal (IP) infection with wild-type *L. monocytogenes* EGDe. Bacterial burden in spleen and liver (n=10) C57BL/6 mice fed for 13 days with diets varying in percentage of fat content from the total caloric intake and thereafter infected IP with 5 x $10^4$ CFU (low-fat (LF) and chow groups) or 1 x $10^4$ CFU (high-fat (HF) group) *L. monocytogenes* EGDe. Animals were euthanized at 72h post-infection and the total number of *L. monocytogenes* EGDe CFU per organ were determined by plating homogenized organs. Statistical analysis was conducted using One-way ANOVA and Dunnett's Multiple Comparison Test in relation to chow diet, error bars represent ±SEM). *, P <0.05; **, P <0.01; ***, P <0.001.

**Table S1.** Primers for host transcriptome analysis. HKG (Housekeeping gene); TJP (Tight-junction proteins) Designed in the Assay Design Center from Roche *Universal ProbeLibrary* (version 2.52, 2016). Two housekeeping genes calculated using a normalization algorithm ($V_{(n/n+1)}$) with cut-off value of 0.15. Calibrator calculation using pooling system, using average of the control for each gene in each specific organ. Genes with Ct values superior to 40 were not considered reliable and therefore not calculated.

| **Gene** | | | **Forward primer (5'-3')** | **Reverse primer (5'-3')** | **Probe** |
|---|---|---|---|---|---|
| **HKG Ileum** | B-actin | *actb* | aaggccaaccgtgaaaagat | gtggtacgaccagaggcatac | #56 |
| | Rib-S18 | *rps18* | aagcagacatcgacctcacc | ggaaccagtctgggatcttg | #88 |
| **HKG Liver** | Trypsin inhibitor | *itih4* | cccctggacctcctcttg | agcaggatattgtgctgcaa | #21 |
| | Macroglobulin /bikunin | *ambp* | ccactaataactgggaccctca | ggggccttctgagtaattga | #51 |
| **Host cell invasion** | Ecad | *cdh1* | atcctcgccctgctgatt | accaccgttctcctccgta | #18 |
| | Met | *ryk* | ggcatgcaaagtccaagg | catgcccatagccacaaagt | #33 |
| | GP96 | *grp94* | aatgaggagttaacggtcaagatt | ttcctacacccgtgtctgtg | #62 |
| | *gC1qR* | *c1qbp* | aggaggagccctcacagg | cacaaagttgggagttgatgtc | #92 |
| | RAB10 | *rab10* | gaaaaggcgttcctcacatt | tgggttcttttacaggggtct | #88 |
| | mNcad | *cdh2* | gccatcatcgctatccttct | ccgtttcatccataccacaaa | #18 |
| **TJP** | ZO-1 | *tjp1* | gagcctgctaagccagtcc | cagcatcagtttcgggtttt | #21 |
| | ZO-2 | *tjp2* | tcatcaaagaaatgaccagaaca | tctcagtaacagtgccgttga | #49 |
| | ZO-3 | *tjp3* | aggccgtgcagtttctgt | gcgagactggaccatcttct | #16 |
| | Occludin | *ocln* | ggtctctacgtggatcaatatttgta | aaccccaggacaatggcta | #79 |
| | claudins | *ocld1* | cccttgacccccatcaat | acacctcccagaaggcaga | #20 |
| **Immune response** | TNF-α | *tnfg* | tcttctcattcctgcttgtgg | ggtctgggccatagaactga | #49 |
| | IL1-β | *il1b* | ttgacggaccccaaaagat | agctggatgctctcatcagg | #38 |
| | IL17A | *il17a* | gattttcagcaaggaatgtgg | cattgtggagggcagacaat | #34 |
| | IL23 | *il23* | tccctactaggactcagccaac | tgggcatctgttgggtct | #19 |
| | INF-ʊ | *infg* | atctggaggaactggcaaaa | ttcaagacttcaaagagtctgaggta | #21 |
| | FoxP3 | *foxp3* | tcaggagcccaccagtaca | tctgaaggcagagtcaggaga | #78 |
| | IL10 | *il10* | cagagccacatgctcctaga | gtccagctggtcctttgttt | #41 |
| | IL6 | *il6* | gctaccaaactggatataatcagga | ccaggtagctatggtactccagaa | #6 |
| | Reg3-ʊ | *reg3g* | accatcaccatcatgtcctg | ggcatctttcttggcaactt | #108 |
| | pIK3-ap1 | *pik3r1* | gacggcactttccttgtcc | tgacttcgccgtctaccac | #80 |
| | Ccl2 | *ccl2* | catccacgtgttggctca | gatcatcttgctggtgaatgag | #62 |
| | iNOS | *impdh2* | tcttacgtgccggacgac | cccaggaagaatgagaaaatcat | #100 |
| | Muc2 | *muc2* | gcagccacagatcccaaa | gttggggtcccctgaagt | #33 |

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# Chapter III

# Overcoming osmotic pressure:
# Impact of L-carnitine on *Listeria monocytogenes* gene expression

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**Authors contributions**

Vanessa Las Heras designed experimental protocols, carried out the experimental work and analysed the data. Vanessa Las Heras did all the work related with the cell culture experiments, with significant input from Jorge Pinheiro. The deletion mutants were done by Vanessa Las Heras, with advice and guidance from Justine Rudkin and Jorge Pinheiro. Bjorn Rotter carried out RNA sequencing and analysis, with significant input from Vanessa Las Heras. Justine Rudkin, Colin Hill and Cormac G. M. Gahan devised and co-ordinated the study. Vanessa Las Heras wrote the paper with significant input from Cormac G. M. Gahan and further input from all authors.

# Contents

# 1. Abstract

*Listeria monocytogenes* is a foodborne pathogen able to adapt to a variety of niche-specific stimuli by coordinating a complex gene regulatory network. *L. monocytogenes* is able to respond to increased extracellular salt concentrations through the uptake of compatible solutes (osmolytes), such as L-carnitine which is transported via the OpuC osmolyte uptake system. This response is essential for survival in saprophytic and gastrointestinal environments. In the present study we evaluated the role of L-carnitine in the *L. monocytogenes* osmotic stress response during adaptation to sodium chloride through RNA-seq. In addition, we determined the role of *opuC* in *L. monocytogenes* growth and virulence in the presence of L-carnitine *in vitro*.

The *L. monocytogenes* response to exposure to 3% NaCl (DS) was compared to 3% NaCl and 1mM L-carnitine (DSC) and to the control defined minimal media (without NaCl) (DMM). Sequencing of RNA transcripts (RNA-seq) demonstrated that a total of 766 genes were differentially expressed in DSC in relation to DS, 749 genes were found to be differentially expressed in DMM in relation to DS, while only 51 genes were differentially expressed in DSC in relation to DMM. The gene expression data identifies key systems that are likely to be involved in osmotic homeostasis and adaptation to elevated salt environments (including the gut). Our results demonstrate that L-carnitine uptake reduces the impact of the osmotic stress response upon listerial fitness and cellular homeostasis. Relative to DS conditions, L-carnitine induces the expression of *L. monocytogenes* virulence genes (including *hly, plcA, mpl*) implying that L-carnitine influences virulence signalling events in the presence of salt. Indeed, we show that L-carnitine is fundamental for *L. monocytogenes* growth and invasion of

epithelial cells during osmotic stress using an *in vitro* model which mimics the gut environment. Importantly, we determined the role of the *opuC* system in *L. monocytogenes* fitness and virulence, by demonstrating that it facilitates growth and infection in the presence of L-carnitine. The findings are important for increasing our understanding of how the pathogen adapts to conditions encountered in the lumen of the GI tract which influence the development of invasive disease.

# 2. Introduction

Adaptation to environmental changes is often a crucial factor for the prevalence and proliferation of pathogenic bacteria. In order to overcome such challenges these bacteria are often equipped with an arsenal of specific genes which grant them such adaptive abilities. Among foodborne pathogens *Listeria monocytogenes* is of particular concern due to its ability to survive, and often grow, under a wide range of environments (Gandhi and Chikindas, 2007). *L. monocytogenes* is not believed to belong to a strict environmental niche, since it is widely distributed in nature, and it has been isolated from soil, water, silage and vegetation, from both urban areas and natural environments (Nightingale et al., 2004). The success of *L. monocytogenes* as a foodborne pathogen is mainly attributed to a variety of adaptive mechanisms that allow it to survive a vast range of stress conditions, such as low pH, low temperature and high osmolarity (Bucur et al., 2018).

*L. monocytogenes* osmotic stress response is of particular relevance in regard to bacterial survival in two specific niches namely; the host and food/food-production environments. Elevated osmolarity in the environment triggers genetic and physiological responses in *L. monocytogenes* that support survival and adaptation to threatening conditions such as high-osmotic stress in the intestine (direct adaptation), the low pH in the stomach (through cross-adaptation) or food-preserving agents and surface disinfectants (Bolocan et al., 2016; Sleator et al., 2009). Previous studies have demonstrated that exposure to sublethal osmotic stresses can also provide cross-protection against bile stress in *L. monocytogenes* LO28 (Begley et al., 2002), adaptive heat and acid tolerance in *L. monocytogenes* EGDe (Skandamis et al., 2008;

Wemekamp-Kamphuis et al., 2004), and increased resistance to hydrogen peroxide (Bergholz et al., 2012).

In the presence of elevated salt concentrations, *L. monocytogenes* accumulates compatible solutes in the cytoplasm, such as glycine betaine and L-carnitine, which are known to decrease intracellular osmotic pressure (Duché et al., 2002; Melo et al., 2013). The mechanism of osmoregulation (**Fig. 1**) is mediated through the increased expression of genes encoding proteins involved in the transport of their respective compatible solutes (Bae et al., 2012; Bucur et al., 2018). Glycine betaine, which is found in relatively high concentrations in plants, is primarily transported via the ATP-dependent GbuABC system and the sodium-motive-force-dependent uptake system BetL (Chan et al., 2007). L-carnitine, an amine found in animal tissues in high concentration, is transported across the bacterial membrane through the ATP-dependent OpuCABCD system (Sleator et al., 2001). The uptake of these compatible solutes by their corresponding osmolyte uptake systems represents high-specificity transport (Fraser and O'Byrne, 2002; Verheul et al., 1997).

Upon a decrease of external osmolarity, glycine betaine and L-carnitine are released via a channel-like activity (Verheul et al., 1997), with *lmo1013* and *lmo2064* being proposed to mediate a putative mechanosensitive ion channel (Sleator et al., 2003).

The osmotic stress-response mechanisms of *L. monocytogenes* are mostly controlled by σB-dependent (SigB) promoters, which have been shown to regulate the transcription of all three known osmolyte uptake systems (Fraser et al., 2003). SigB is also known to control the transcription of genes associated with cell wall maintenance and carbon utilization (Abram et al., 2008), and regulates virulence gene expression

in *L. monocytogenes*, playing an important role in both stress adaptation and host infection (Kazmierczak et al., 2003; Sue et al., 2004).

L-carnitine uptake in response to an osmotic stress is of special interest in the context of host infection, given that despite the fact that L-carnitine is mainly identified as an osmoprotective compound, some clues have arisen regarding its involvement in virulence (Sleator et al., 2001; Wemekamp-Kamphuis et al., 2002). Disruption of the *opuC* operon severely affected the ability of *L. monocytogenes* LO28 to colonize the small intestine and cause systemic infection in an intraperitoneal (IP) and intragastric (IG) mice infection model, proving that L-carnitine uptake through OpuC is important for *L. monocytogenes* upon infection.

L-carnitine utilization is a phenotype common to both clinical and food strains of *L. monocytogenes*, indicating the importance of this characteristic in the bacterium survival in natural environments (Dykes and Moorhead, 2000).

The present study aims to address the impact of adaptation to short exposure to increased osmotic pressure in the presence of L-carnitine on the *L. monocytogenes* transcriptomic profile, focusing especially in osmotic stress response and virulence. We suggest that *L. monocytogenes* encounters a moderately elevated salt concentration in the small-intestinal environment (Chowdhury et al., 1996) and must adapt to this environment through L-carnitine uptake. How this process of adaptation influences virulence processes in this environment is currently unclear. Understanding the mechanisms underlying bacterial adaptation in the gastrointestinal environment is fundamental to the development of intervention strategies to limit survival of the pathogen. To our knowledge, this is the first analysis of physiological adaptation mechanisms in *L. monocytogenes* in an environment simulating gastrointestinal entry

by the pathogen, giving us an insight into the links between osmotic stress response and pathogenicity.

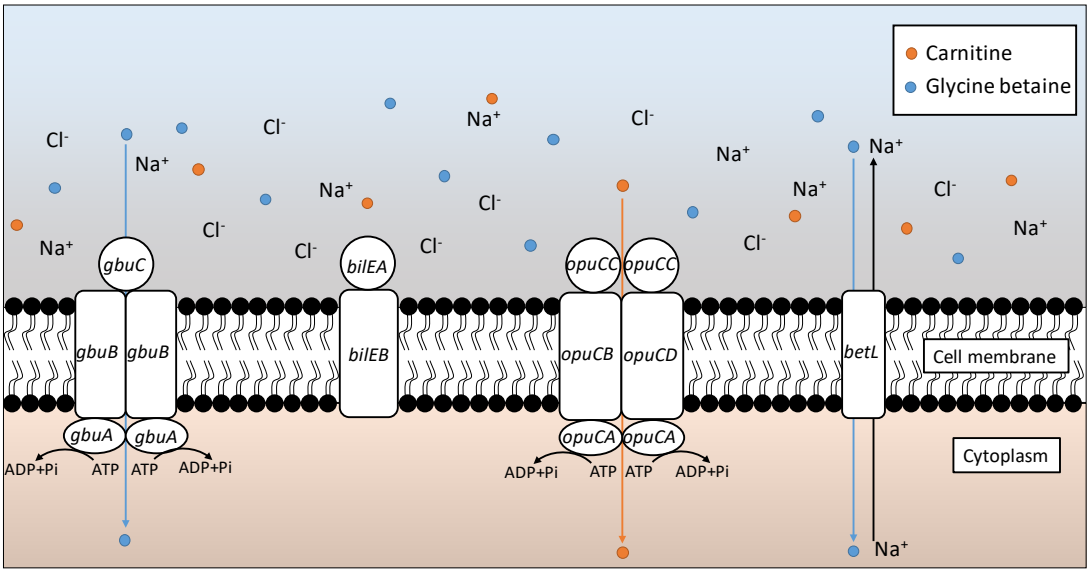



**Figure 1.** *L. monocytogenes* osmotic stress response mechanisms in the context of resistance to increased extracellular osmolarity. In response to increased salt concentrations, *L. monocytogenes* accumulates compatible solutes in the cytoplasm via osmolyte-specific transport systems. Glycine betaine can be accumulated via both BetL and the ATP-dependent GbuABC complex. L-carnitine is transported via the ATP-dependent OpuCABCD system. Despite BilE affinity to the compatible solute uptake systems, this protein is not physically involved in the transport of either of these osmolytes, however it is highly associated with *L. monocytogenes* osmotic stress response.

# 3. Methods

## 3.1. Bacterial strains and growth conditions

Strains used in this study are detailed in **Table S1**. *L. monocytogenes* serotype 1/2a strain EGDe (ATCC BAA-679) and *Escherichia coli* were routinely grown in brain heart infusion broth (BHI, Oxoid) or defined minimal media (DMM, adapted from (Premaratne et al., 1991), **Table S2**) and lysogeny broth (LB, Fisher), at 37°C aerobically with shaking. BHI-agar and LB-agar plates were used for growth on solid media. Growth curves of *L. monocytogenes* strains were determined using overnight cultures diluted 1:100 in fresh media and grown to mid-exponential phase, in which a new dilution was made to reach an initial $OD_{600nm}$ of 0.1 in fresh BHI. Absorbance was measured at 600 nm every 30min, using the Eon Microplate Spectrophotometer (BioTek Instruments). When necessary, antibiotics were added to the media: ampicillin 100 µg/ml and erythromycin 5 µg/ml.

## 3.2. RNA isolation and qRT-PCR

The impact of 2h exposure to osmotic pressure in the presence of L-carnitine was analysed. *L. monocytogenes* EGDe was grown to mid-exponential growth phase, washed and resuspended either of the following media: defined minimal media (DMM), DMM supplemented with 3% NaCl (DS) or DMM supplemented with 3% NaCl and 1 mM L-carnitine (DSC). Samples where then incubated for 2h at 37°C. To harvest the cells, samples were centrifuged at 6000 g for 3min at room temperature, resuspended in RNAlater™ (Sigma), blast frozen in liquid nitrogen and stored at -80°C until RNA extraction. The pellets were resuspended in 900 µl QIAzol Lysis Reagent (Qiagen) and cells disrupted with a FastPrep sample disruptor (MagnaLyser 5000 rpm, 15s, 3 times). Total RNA was extracted using the RNeasy Plus Universal

Kit (Qiagen). RNA quality was measured using Labchip GX II bioanalyzer (Perkin Elmer). For DNA removal, total RNA was incubated with Baseline-Zero DNase (Epicentre) in the presence of RiboLock RNase inhibitor (40 U/µl) (Thermo Fisher Scientific) at 37°C for 30min, followed by purification using Zymo-Spin column (ZymoResesarch). Samples were then mixed with 2 volumes of RNA Binding Buffer and an equal volume of ≥99.8% ethanol (Roth). The mixture was vortexed and transferred to Zymo-Spin™ IC Column and centrifuged at 12000 x g. The RNA bound to the column membrane was washed twice with RNA Wash Buffer and eluted in DNase/RNase free water. RNA concentration was measured using fluorescence-based Qubit™ RNA HS Assay (Thermo Fisher Scientific). RNA-seq validation was done through RT-qPCR of a selection of genes. Primers used are listed in **Table S3**. Briefly, 700ng of total RNA was reversed transcribed into cDNA using the NZY First-Strand cDNA Synthesis Kit (Nzytech). qPCR was performed on 700 ng cDNA in a 10µl reaction volume, using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-RAD). The PCR protocol consisted of 1 cycle of 3min at 95°C, and 55 cycles of 95°C for 10sec, 56°C for 20sec and 72°C for 20sec. Total RNA was treated with Ribo-Zero rRNA removal kit (Illumina), for total RNA-depletion and mRNA enrichment. Beads were hybridized with probes for 10 min at 68°C. 500 ng of total RNA was added to the mixture and incubated sequentially at room-temperature and 50°C, for 5 min each, followed by separation of mRNA from rRNA (bound to the beads). Enriched mRNA was purified by Zymo-Spin column (ZymoResesarch) and run on Labchip GX II bioanalyzer (Perkin Elmer) to confirm rRNA reduction.

### 3.3. RNA-seq and data analysis

cDNA fragment libraries were prepared using NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® (Illumina) with slight modifications. Enriched mRNA

was fragmented for 15min at 94°C and reverse transcribed for cDNA synthesis. Double-stranded cDNA (ds cDNA) was purified using NucleoMag (Macherey nagel) SPRI selection, followed by end-repair and adaptor ligation. After purification using NucleoMag SPRI beads, test RT-qPCR (Applied Biosystems) was performed using KAPA Hifi polymerase (Roche™) with EvaGreen® (Biotium) to determine appropriate cycle numbers for PCR. Using NEBNext Multiplex Oligos for Illumina (Dual Index Primers), high fidelity PCR was performed using KAPA Hifi polymerase to selectively enrich library fragments. The PCR products were purified twice using NucleoMag (Macherey nagel) SPRI beads and the quality of the final library was assessed on Labchip GX II bioanalyzer (Perkin Elmer). Indexed and purified libraries were then loaded onto a flow cell and sequencing was carried out on the Illumina NextSeq 500 platform (paired-end, 2 X 75 bp per read). Transcriptome sequencing quality was assessed through FastQC, followed by removal of Illumina adapter sequences and low-quality base pairs using Cutadapt version 1.9 (Martin, 2011). Reads were mapped to all complete sequenced genomes of reference EGDe strains (ENSEMBL ASM19603v1) using Bowtie 2 v 2.2.4 version, with standard parameters and sensitive-local (Langmead and Salzberg, 2012). The BAM alignment files were then used as input for read counting (htseq-count version 0.6.0). All downstream analysis was done using DESeq2 (R, version 3.2.2). P-value adjustment using the default Benjamini-Hochberg adjustment method (Love et al., 2014). Fold-changes were calculated by comparing expression levels of DMM in relation to DS, DSC in relation to DS and DSC in relation to DMM. Functional annotation of the *L. monocytogenes* EGDe genome was obtained from the Listeriomics COG database (Bécavin et al., 2017). RNA integrity and quantification, ribosomal RNA depletion,

library preparation and sequencing were performed by GenXPro GmbH (Frankfurt, Germany).

### 3.4. Mutant construction

The deletion of *opuCA* and *opuCC* (*lmo1428* and *lmo1426* respectively) from *L. monocytogenes* EGDe was achieved by double homologous recombination (Carvalho et al., 2015), with some modifications. Two flanking regions upstream and downstream of the coding sequences of the genes of interest were synthetized in the plasmid pEX-A128 by Eurofins (**Fig. S1**). These sequences were excised from the pEX-A128 vector by restriction enzymes digestion (BamHI and SacI). The digestion products were separated by agarose gel electrophoresis and purified from the gel using the PureLink™ Quick Extraction Kit (Invitrogen). The sequences were then cloned into the suicide vector pMAD (Arnaud et al., 2004) (**Fig. S2**) by ligation with T4 ligase (Roche™) and transformed into *E. coli*. Transformed *E. coli* were plated in LB-agar plates supplemented with ampicillin. These pMAD constructs were then used to transform electrocompetent *L. monocytogenes* EGDe by electroporation. Transformed *L. monocytogenes* were plated in BHI-agar plates supplemented with erythromycin at 30°C and resistant colonies were selected as pMAD-harbouring isolates. These colonies were then streaked onto BHI-agar plates supplemented with erythromycin and incubated at 43°C. Growth at 43°C in selective media indicates chromosomal integration of the plasmid through homologous recombination. Integrant colonies were sub-cultured into 10 ml BHI and incubated at 30°C with no agitation. Cultures were serial diluted in PBS and plated onto BHI-agar plates, in order to obtain isolated colonies. Individual colonies were simultaneously streaked onto BHI-agar plates and BHI-agar plates supplemented with erythromycin and incubated at 37°C. Isolates that grew in BHI-agar plates but were sensitive to erythromycin indicate chromosomal

excision of the pMAD plasmid by a second homologous recombination. These colonies were selected as possible mutant candidates and PCR screened, with the primers described in **Table S3**. Isolates for which PCR confirmed chromosomal deletion of the genes of interest were subjected to DNA sequencing for confirmation of correct deletion and no additional unwanted mutations.

### 3.5. Cell invasion assays

Human cell lines invasion assays were performed as described (Reis et al., 2010). Briefly, the epithelial cell line C2BBe1 (passage 12) were grown to confluency in Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5 g/l) and L-glutamine (Sigma), supplemented with 10% FBS and apo-transferrin (4 mg/l). Before infection, C2BBe1 cells were washed and fresh media was added accordingly to the condition to be tested: DMEM (Control), DMEM+3% NaCl (DMEMS) or DMEM+3%NaCl+1 mM L-carnitine (DMEMSC). Toxicity assays were performed to validate the concentration of L-carnitine and NaCl used in this experiment. Viability assays upon 2h exposure to the conditions tested were done using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) (data not shown). *L. monocytogenes* EGDe wild type (WT), Δ*opuCA* or Δ*opuCC* were grown to exponential phase, washed and inoculated at a multiplicity of infection of 75 for 1 h. Cells were then incubated with medium supplemented with 20 µg/ml gentamycin (Sigma) for 1 h 30min to eliminate extracellular bacteria, washed and lyzed with 0.2% Triton X100. Bacterial suspensions were serially diluted and plated on BHI-agar plates for CFU determination.

### 3.6. Intracellular multiplication

Assays of intracellular multiplication in mouse J774 macrophage cell line were performed as described (Reis et al., 2010). Cells (≃2 x $10^5$/well) were propagated in DMEM (Sigma) supplemented with 10% FBS. Before infections, cells were washed and fresh media was added accordingly to the condition to be tested: DMEM, DMEMS or DMEMSC. Cells were then infected with either *L. monocytogenes* EGDe WT, Δ*opuCA* or Δ*opuCC* in mid-exponential growth phase at a multiplicity of infection of 10 for 30min, until treated with 20 µg/ml gentamicin. Intracellular growth was measured at, 2, 6, 8 and 20h post-infection, when cells were washed with PBS, lysed in cold 0.2% Triton X-100 and bacterial suspensions were serially diluted and plated on BHI-agar plates for CFU determination.

### 3.7. Statistical analysis

Statistical analyses were conducted with Prism 5 (GraphPad Software). Two-Way ANOVA with Bonferroni post-hoc was used to compare mean differences between groups with two or more factors. If the p-value falls above 0.05, the mean differences were considered statistically non-significant (NS). For statistically significant differences: *, P <0.05; **, P <0.01; ***, P <0.001. As cut-off values for analysis of RNA-seq, genes were considered significantly differentially expressed when the p-value ≤0.05, the fold-change was below -2 and above 2 and the adjusted FDR was below 1.

# 4. Results and Discussion

## 4.1. Exposure to osmotic stress modifies gene expression profiles in *L. monocytogenes in vitro*

Previous studies have focused on the impact of the *L. monocytogenes* cellular response during growth and survival under desiccation and osmotic stress conditions (Dreux et al., 2008; Hingston et al., 2015; Liu et al., 2005). In our study we conducted an RNA-seq analysis of the transcriptomic profile of *L. monocytogenes* in response to bacterial adaptation to a 2h exposure to 3% sodium chloride (NaCl), in an *in vitro* model mimicking the environment that the pathogen would encounter upon entry in the gastrointestinal tract, where the osmolarity is 0.3 M (Chowdhury et al., 1996).

In this study we analysed alterations in the gene expression of the pathogen in response to DMM, DS or DSC through total RNA sequencing of cultures exposed to these conditions for 2h**.** The results demonstrated that the conditions tested induced a clear modulation of the transcriptomic profile of *L. monocytogenes* (**Fig. 2**). Confirmation of RNA-seq results by qRT-PCR of a selection of genes of interest demonstrated a strong Pearson correlation for both DSC vs. DS ($R^2$=0.9925) and DSC vs. DMM ($R^2$=0.9975) (**Fig. 3**).

A total of 766 genes were found to be differentially expressed between the DSC in relation to DS conditions (490 up-regulated genes and 276 down-regulated genes), while 749 genes were differentially expressed between DMM relative to DS (511 genes were up-regulated and 238 were down-regulated) (**Fig. 2, Table S4**). The transcriptional profile of *L. monocytogenes* exposed to DSC was similar to that of bacteria exposed to DMM, with only 51 genes being differently expressed, as opposed to the more dramatic transcriptional shift observed between any other two conditions

(**Fig. 2**). The majority of genes found to be differentially expressed in DMM as compared to DS were also differently regulated in DSC compared to DS, showing a large overlap in the transcriptional changes of both the control and L-carnitine containing media compared to the high-salt condition (**Fig. 2**). Together, these results suggest that the presence of L-carnitine enables the pathogen to recover without the metabolic burden of the mechanisms of the osmotic stress response.

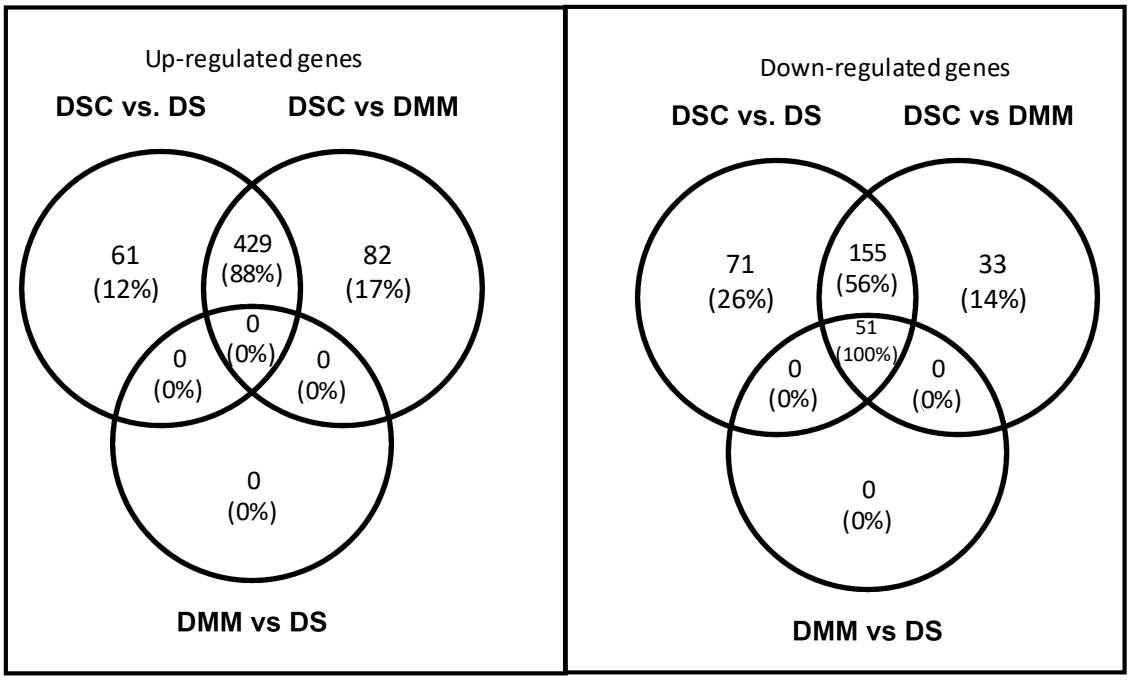



**Figure 2.** Venn diagrams demonstrating the differentially expressed genes in *L. monocytogenes* between the three conditions: DMM (control defined minimal media), DS (DMM supplemented with 3%NaCl) and DSC (DS supplemented with 1mM L-carnitine). **a.** Genes up-regulated. **b.** Genes down-regulated.

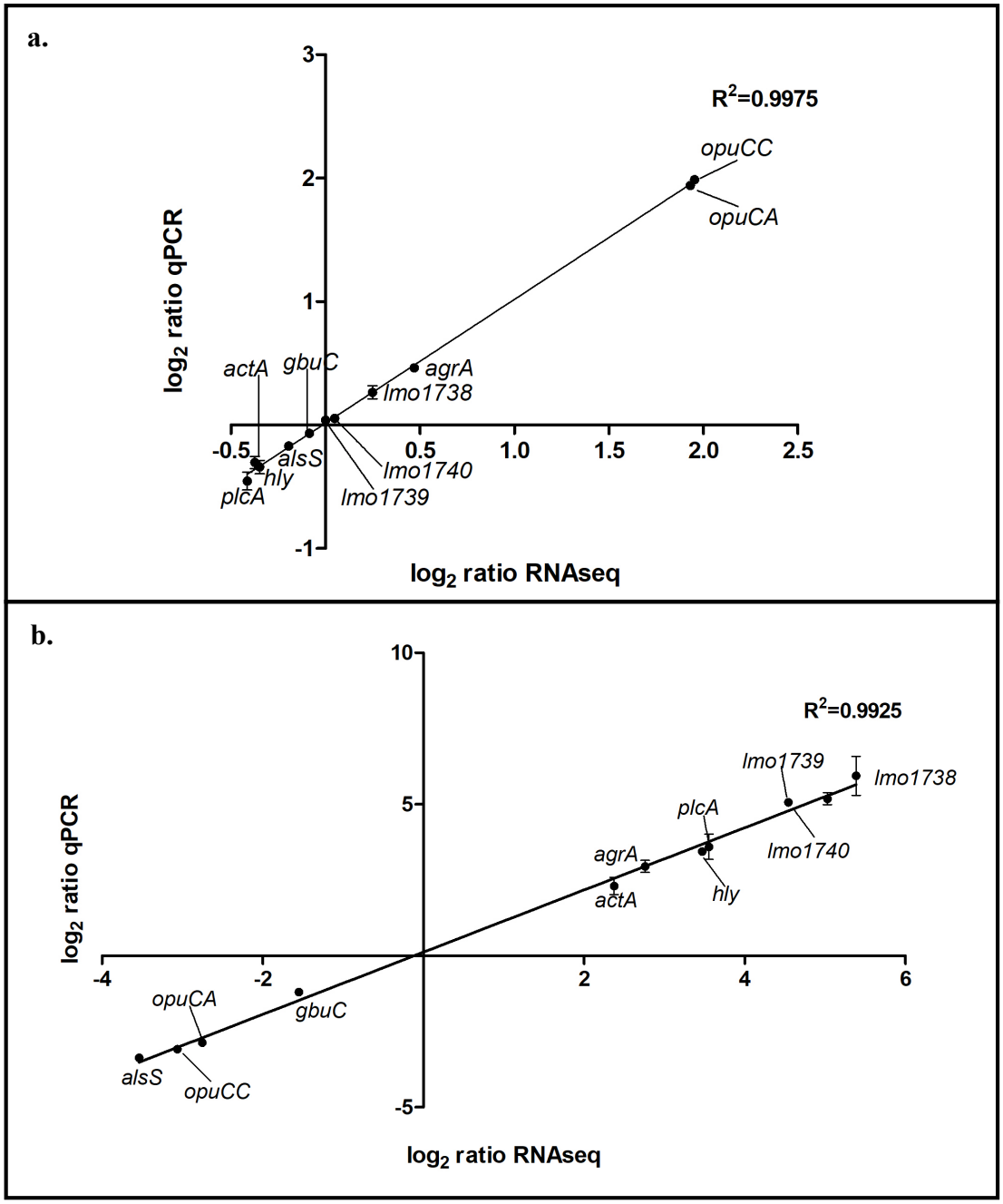



**Figure 3.** RNA-seq validation through qRT-PCR. Data represented as Log2 fold-change between **a.** DSC and DMM; **b.** DSC and DS. Results represent mean ± SD of three technical replicates.

To better elucidate the transcriptomic changes that occurred across the *L. monocytogenes* genome, we categorized the genes differently expressed according to their COG functional categories (**Fig. 4**). The analyses between DSC and DMM resulted in only genes from a few categories being assigned. However, when analysing the conditions DSC vs. DS and DMM vs DS, there were several genes differently expressed that were associated with most functional categories. Among those, are functional categories associated with essential cellular mechanisms such as signal transduction, DNA replication, recombination and repair, transcription and translation which seem to reflect a strong general response from the bacteria when faced with a high osmotic stress in the absence of compatible solutes (**Table S4**).

Our results also demonstrate that genes associated with amino acid and carbohydrate transport mechanism categories are well represented (**Table S4**). This could be related to the bacterial attempt to restore osmotic homeostasis through expression of these transport systems, a well understood mechanism in bacteria faced with an extracellular increase of NaCl (Zhang et al., 2013).

Previous studies have shown that osmotic desiccation stress affects the expression of genes associated with cellular motility, membrane lipid biosynthesis, potassium uptake, energy production and virulence, supporting an interconnection between stress response and regulation of *L. monocytogenes* metabolic and biosynthetic pathways seen in our study (**Fig. 4**) (Cossat et al., 2011; Hingston et al., 2015; van der Veen et al., 2007). Moreover, others have demonstrated that *L. monocytogenes* growth in BHI supplemented with sodium lactate severely affects the pathogens transcriptomic profile, with RNA-seq results identifying differentially expressed genes involved in transporter and phosphotransferase systems (PTS), signal transduction, and more importantly virulence (Suo et al., 2018). In fact, *L. monocytogenes* exposure to mild-

stresses have been linked to adaptive tolerance responses, in a growth-phase dependent manner (O'Driscoll et al., 1996; Poimenidou et al., 2016).

A recent study has demonstrated that pre-exposure to NaCl improved the survival of *L. monocytogenes* in an *in vitro* digestion model (Pettersen et al., 2019), suggesting that salt adaptation plays a role in survival of *L. monocytogenes* in a model of the gut lumen. Our results revealed that high osmolarity and availability of L-carnitine can influence regulatory changes in the expression of virulence factors and genes associated with cell motility. Notably, these gene categories were mostly up-regulated in DSC vs. DS and DMM vs. DS, while only two genes (cell motility) were differentially expressed in DSC vs. DMM (**Fig. 4**). Considering that the intestinal environment presents both high osmolarity and high availability of L-carnitine we believe our data support the hypothesis that adaptation to high salt stress and uptake of L-carnitine *in vivo* can promote *L. monocytogenes* virulence gene expression in the host, with potential to influence pathogenesis.

Altogether, these results demonstrate the connection between different metabolic mechanisms in *L. monocytogenes*, supporting the hypothesis that exposure to osmotic stress may not only affect cellular robustness but also modulate a cascade of intracellular events and virulence gene expression.

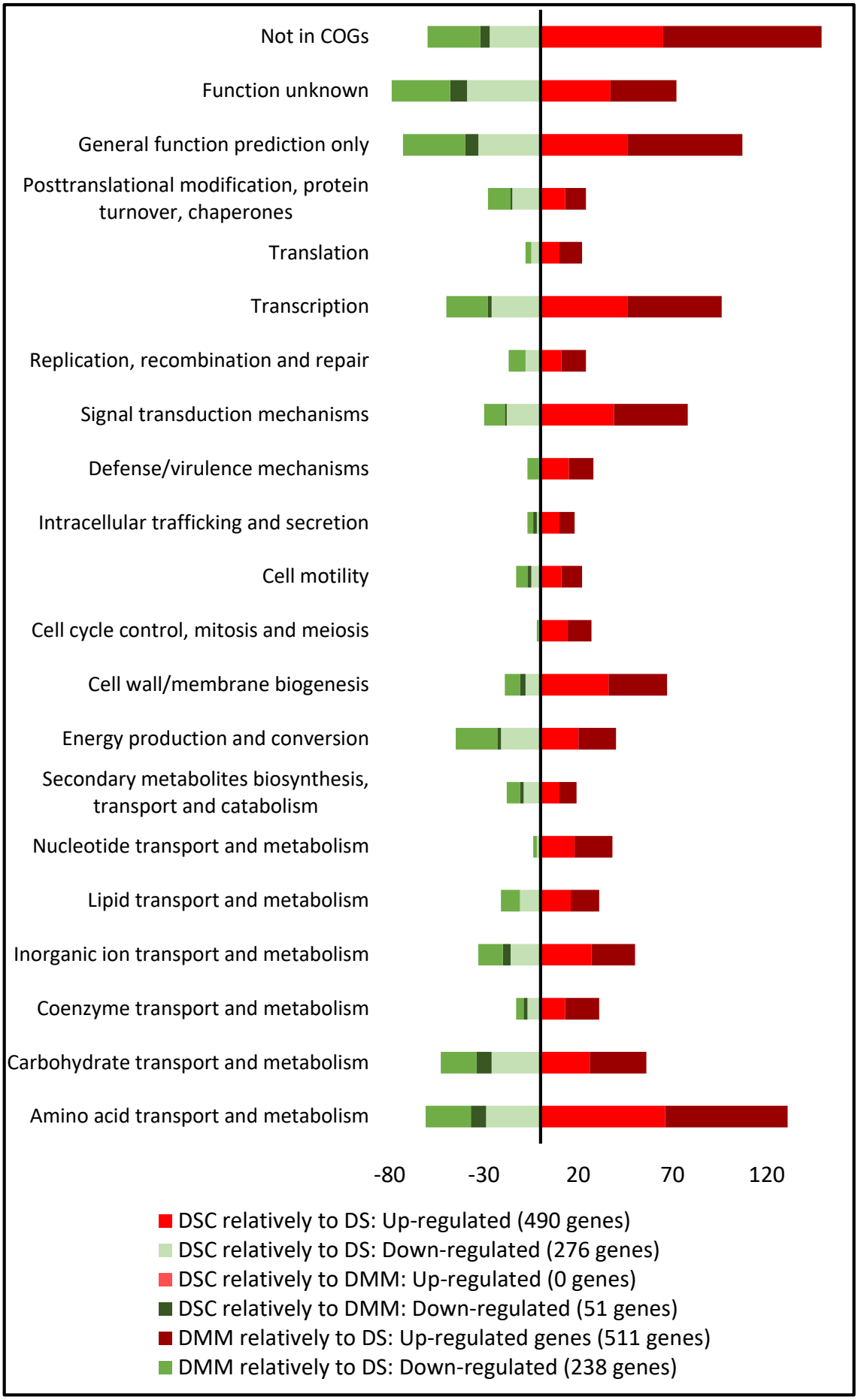

Not in COGs
Function unknown
General function prediction only
Posttranslational modification, protein turnover, chaperones
Translation
Transcription
Replication, recombination and repair
Signal transduction mechanisms
Defense/virulence mechanisms
Intracellular trafficking and secretion
Cell motility
Cell cycle control, mitosis and meiosis
Cell wall/membrane biogenesis
Energy production and conversion
Secondary metabolites biosynthesis, transport and catabolism
Nucleotide transport and metabolism
Lipid transport and metabolism
Inorganic ion transport and metabolism
Coenzyme transport and metabolism
Carbohydrate transport and metabolism
Amino acid transport and metabolism
-80
-30
20
70
120
DSC relatively to DS: Up-regulated (490 genes)
DSC relatively to DS: Down-regulated (276 genes)
DSC relatively to DMM: Up-regulated (0 genes)
DSC relatively to DMM: Down-regulated (51 genes)
DMM relatively to DS: Up-regulated genes (511 genes)
DMM relatively to DS: Down-regulated (238 genes)

**Figure 4.** Genes differentially expressed in response to a 2h exposure to 3% NaCl (DS) in relation to 3% NaCl and 1mM L-carnitine (DSC) or a control (DMM). Numbers of differentially regulated genes organized in different COG functional categories, respective numbers of up-regulated (in red) and down-regulated (in green) genes in relation to the conditions tested.

### 4.2. Impact of short-term NaCl exposure on the transcriptional response to osmotic stress

Among other genes associated with general cell metabolism essential for homeostasis, cell motility and genetic regulation mechanisms, *L. monocytogenes* exhibits the expected osmotic stress response in the presence of salt, with changes occurring in the expression of the *gbu* and *opuC* gene clusters (**Table 1**).

Our data shows higher level of expression of *gbu* and *opuC* genes in DS, in relation to both DMM and DSC, an osmotic stress response triggered by the increased extracellular NaCl. Interestingly, the decreased expression of the *opuC* operon observed in response to short-term exposure to DSC could be explained by an accumulation of the compatible solute in the cytosol. Indeed, previous studies have shown that osmolyte uptake is inhibited by the cellular build-up of these compounds (Verheul et al., 1997). The accumulation of either glycine betaine or L-carnitine can influence the regulation of both Gbu and OpuC in *L. monocytogenes*, demonstrating a cross-talk between the two osmolyte uptake systems. Therefore, these data support our findings, in which we observed that a short-exposure to L-carnitine in the presence of extracellular NaCl ultimately led to a down-regulation of *gbu* and *opuC* genes expression, suggesting solute accumulation-driven inhibition.

Our findings reinforce the hypothesis that when challenged with an osmotic pressure *L. monocytogenes* uptakes L-carnitine which successfully re-establishes the osmotic status of the cell, preventing a stronger generalized stress response and normalizing gene expression profiles, as seen by the proximity of the transcriptomic phenotypes between the DMM and DSC conditions.

| **Osmotic stress response** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **DSC relative to DS** | | | **DSC relative to DMM** | | | **DMM relative to DS** | | |
| **Locus Tag** | **Gene name** | **Fold-change** | **Locus Tag** | **Gene name** | **Fold-change** | **Locus Tag** | **Gene name** | **Fold-change** |
| *lmo1014* | *gbuA* | -2.6 | *lmo1425* | *opuCD* | -2.4 | *lmo1014* | *gbuA* | -2.8 |
| *lmo1015* | *gbuB* | -2.6 | *lmo1426* | *opuCC* | -2.1 | *lmo1015* | *gbuB* | -2.8 |
| *lmo1016* | *gbuC* | -2.9 | *lmo1427* | *opuCB* | -2.3 | *lmo1016* | *gbuC* | -3.1 |
| *lmo1425* | *opuCD* | -8.6 | *lmo1428* | *opuCA* | -3.0 | *lmo1425* | *opuCD* | -2.4 |
| *lmo1426* | *opuCC* | -8.3 | | | | *lmo1426* | *opuCC* | -2.2 |
| *lmo1427* | *opuCB* | -7.4 | | | | | | |
| *lmo1428* | *opuCA* | -6.7 | | | | | | |

**Table 1.** Genes encoding known osmolyte uptake systems in response to adaptation to short-exposure to increased extracellular NaCl concentration. *Betl* and *bilE* are not significantly differentially expressed in neither of the conditions tested. DMM- defined minimal media control; DS- DMM supplemented with 3% NaCl; DSC- DS supplemented with of 1 mM L-carnitine. Ratio of gene expression between the two conditions tested is measured by the fold-change.

Among the top-10 most differentially expressed genes across the conditions, one can find the operon containing the *lmo1738*, *lmo1739* and *lmo1740* genes (**Fig. 5**). These genes seem to be affected under osmotic stress in the absence of L-carnitine, as they are down-regulated in DS in relation to both DMM and DSC (**Table 2**). To our knowledge, this is the first time that this operon has been reported to be associated with osmotic stress response in *L. monocytogenes*.

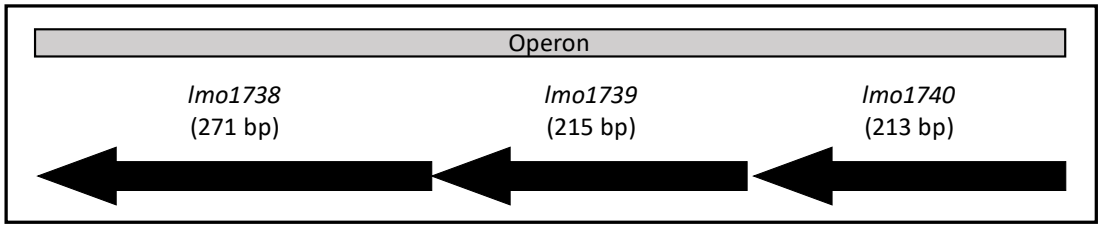



**Figure 5.** Genomic organization of the *lmo1738-1740* operon in *L. monocytogenes* EGDe.

| **DSC relative to DS** | | **DSC relative to DMM** | | **DMM relative to DS** | |
|---|---|---|---|---|---|
| **Locus Tag** | **Fold-change** | **Locus Tag** | **Fold-change** | **Locus Tag** | **Fold-change** |
| *lmo1738* | 41.9 | *lmo1738* | 1.2 | *lmo1738* | 49.7 |
| *lmo1739* | 32.7 | *lmo1739* | 1.0 | *lmo1739* | 33.8 |
| *lmo1740* | 23.3 | *lmo1740* | 1 | *lmo1740* | 22.7 |

**Table 2.** Relative expression of the genes in the *lmo1738-1740* operon. Gene expression from *L. monocytogenes* exposed to different media is presented as fold-change (DMM- defined minimal media control; DS- DMM supplemented with 3% NaCl; DSC- DS supplemented with of 1 mM L-carnitine).

The gene *lmo1738* encodes a putative protein with a phosphate and amino acid ATP-binding cassette transporter-like system domain, belonging to the type-2 periplasmic binding fold-protein superfamily (PBP AatB like domain) (**Fig. S3**). Interestingly, this domain shows similarities to the conserved domain of OpuCC, a PBP OpuCC like domain, which suggests that both proteins have a similar function. Additionally, its subcellular location is predicted to be a membrane anchored/cell surface protein, having been previously detected in both the membrane proteome and secretome of *L. monocytogenes* (**Table S5**).

Moreover, the gene seems to be highly conserved in sequenced pathogenic and non-pathogenic strains of *Listeria* (**Fig. S4**), suggesting that the operon is relevant for the saprophyte lifestyle of *Listeria*. Despite its similarities to proteins in the *opuC* operon, and the fact that *in silico* analysis suggests glutamine/histidine as possible solutes with higher affinity to Lmo1739 and Lmo1740, the solute transported by this system is currently unknown.

The gene *lmo1738* has been previously described to be up-regulated in response to iron limitation (Ledala et al., 2010) and also appears to be particularly important in general stress responses, as it is under positive SigL-dependent expression under cold-stress (Mattila et al., 2012) and induced under acid stress (Feehily and Karatzas, 2013).

Studying the role of the *lmo1738-1740* operon in response to osmotic stress could be important in understanding its possible connection with *opuC* and cellular L-carnitine uptake in *L. monocytogenes*. This is especially relevant when considering that a previous study has demonstrated that an o*puCA* deletion mutant in *L. monocytogenes* EGD background was still able to accumulate high levels of intracellular L-carnitine, suggesting the existence of a second L-carnitine uptake system (Fraser and O'Byrne,

2002). Moreover, upon deletion of *sigB* (*lmo0895*) in *L. monocytogenes* EGDe *lmo1738* was found to be down-regulated, an indication that it could be under SigB-dependent regulation (Hain et al., 2008). The discovery of *lmo1738* as an important gene in the current study is an example of how transcriptomic analysis can guide an understanding of bacterial adaptation. Further work in our laboratory will examine the functional role of this system in stress resistance and osmolyte uptake.

### 4.3. Expression of virulence genes in response to adaptation to osmotic stress

The ability of *L. monocytogenes* to tolerate high-osmotic pressures is of particular interest in the context of host infection since the pathogen must survive the relatively high-salt concentrations in the gastrointestinal tract (Chowdhury et al., 1996). Indeed, the deletion of the three known osmotic stress-response systems significantly affects gastrointestinal infection in a murine model (Wemekamp-Kamphuis et al., 2002). It is therefore important to consider how adaptation to osmotic stress impacts virulence gene expression in *L. monocytogenes* in the presence and absence of L-carnitine as this will provide insights into adaptive processes that occur in the gut lumen (**Table 3, Table S6**).

Our results indicate that upon an increase of extracellular NaCl, the presence of L-carnitine increased the expression of *inlC*, *mpl*, *actA*, *plcA*, *plcB* and *hly*, genes involved in *L. monocytogenes* virulence and cell defence (**Table 3**). *L. monocytogenes* is able to invade and multiply inside non-phagocytic cells, a feature that allows the pathogen invasion into epithelial cells in the gastrointestinal tract. *L. monocytogenes* cell infection cycle includes adhesion/invasion, mediated through internalins like InlA and InlB (Phelps et al., 2018). Following invasion, the pathogen must escape from the vacuole, a process enabled by the expression of a series of virulence factors including

genes such as *hly*, *plcA*, *plcB* and *mpl* (Joseph et al., 2006). In the host cytosol, *Listeria* is able to multiply and becomes mobile through polymerization of host actin comets, enabled mainly by *actA* (Alvarez and Agaisse, 2016). Our work demonstrates that L-carnitine increases expression of these important virulence genes relative to conditions in which salt concentrations are elevated.

Our results show that *sigB* is down-regulated in both DSC and DMM in relation to DS (**Table 3**). The *sigB* gene encodes a major stress regulator of *L. monocytogenes* with an extensive regulon which responds to multiple environmental stimuli (Dorey et al., 2019). For these reasons, the impact of osmotic stress on *sigB* expression observed in our data is not surprising. *SigB* plays a critical role in *L. monocytogenes* adaptation to the gastrointestinal tract (Toledo-Arana et al., 2009). Taking into consideration our data and the high level of salt concentration and L-carnitine availability in the gastrointestinal environment, it is plausible to hypothesize that L-carnitine uptake by *L. monocytogenes* in response to osmotic stress in the host might trigger an important SigB-mediated response, which might promote infection *in vivo*.

In addition to genes encoding the classical virulence factors the *agrBDCA* operon which encodes the Agr system of *L. monocytogenes*, is increased in expression in both DSC and DMM, in relation to DS (**Table 3**, **Table S6**). The Agr system is a quorum sensing system that has been previously associated with stress adaptation, biofilm formation and virulence, suggesting that this system comprises an important link between *L. monocytogenes* saprophytic and infectious phenotypes (Pinheiro et al., 2018; Riedel et al., 2009). The findings shown here indicate that the roles of this system are important for the successful adaptation to niche-specific stresses, potentially favouring survival and virulence in the presence of L-carnitine.

| Defence/virulence mechanisms DSC relative to DS | | | |
|---|---|---|---|
| | **Locus Tag** | **Gene name** | **Fold-change** |
| **Up-regulated** | *lmo0925* | | 2.0 |
| | *lmo0959* | *dacA* | 2.1 |
| | *lmo2371* | | 2.1 |
| | *lmo0987* | | 2.3 |
| | *lmo1786* | *inlC* | 2.3 |
| | *lmo0540* | | 2.3 |
| | *lmo1916* | | 2.8 |
| | *lmo1638* | | 2.9 |
| | *lmo1746* | | 3.0 |
| | *lmo1636* | | 3.3 |
| | *lmo1652* | | 3.3 |
| | *lmo0203* | *mpl* | 3.5 |
| | *lmo0155* | | 3.6 |
| | *lmo1651* | | 3.8 |
| | *lmo2769* | | 4.2 |
| | *lmo0153* | | 4.9 |
| | *lmo0204* | *actA* | 5.2 |
| | *lmo0154* | | 5.2 |
| | *lmo0205* | *plcB* | 5.7 |
| | *lmo0051* | *agrA* | 6.8 |
| | *lmo0050* | *agrC* | 9.3 |
| | *lmo0048* | *agrB* | 9.4 |
| | *lmo0202* | *hly* | 11.1 |
| | *lmo0201* | *plcA* | 11.7 |
| | *lmo0049* | *agrD* | 15.0 |
| | *lmo1831* | *pyrE* | 49.7 |
| **Down-regulated** | *lmo0263* | *inlH* | -12.3 |
| | *lmo2157* | *sepA* | -12.0 |
| | *lmo2067* | | -8.6 |
| | *lmo0895* | *sigB* | -4.2 |
| | *lmo2190* | *mecA* | -3.2 |
| | *lmo0264* | *inlE* | -2.7 |

**Table 3.** Genes associated with defence or virulence differentially expressed *in L. monocytogenes* in the presence of L-carnitine during adaptation to osmotic stress identified through RNA-seq analysis. DS (DMM supplemented with NaCl). DSC (DS supplemented with 1 mM L-carnitine). Fold-change represents the ratio of expression levels of DSC in relation to DS.

Our data also demonstrate an up-regulation of *dacA* in DSC in relation to DS (**Table 3, Table S6**). The c-di-AMP diadenylate cyclase is part of a signal transduction mechanism of *L. monocytogenes* and is encoded by *dacA*. Accumulation of c-di-AMP in the cytoplasm, caused by down-regulation of *dacA* was reported to severely decrease cellular L-carnitine uptake and virulence in a murine infection model (Huynh et al., 2016). Increased expression of *dacA* highlights the importance of L-carnitine uptake upon the osmotic stress response in the context of host infection, especially due to its ability to induce virulence gene expression.

Increased expression of genes associated with the bacterial mechanisms of host cell invasion may indicate that in the presence of L-carnitine under increased osmotic pressure the pathogen's virulence is promoted. Despite the fact that the role of L-carnitine has been extensively described for its important role in *L. monocytogenes* adaptation to growth and survival under osmotic stress, our results emphasize the importance of this compatible solute uptake response in the pathogenic lifecycle of *Listeria*.

### 4.4. Role of o*puC* and L-carnitine in *L. monocytogenes* growth and virulence under osmotic stress

The *opuC* operon of *L. monocytogenes* consists of four genes: *opuCA* that encodes a ATP binding site, *opuCB* and *opuCD* that encode two transmembrane permeases and *opuCC* which encodes the extracellular subunit with an L-carnitine binding site (Fraser et al., 2000; Meadows and Wargo, 2015). Here, two single deletion mutants were constructed in the *L. monocytogenes* EGDe background, Δ*opuCA* and Δ*opuCC*, to further explore the role of the different genes in the *opuC* operon in *L. monocytogenes* survival and virulence (**Fig. 6**).

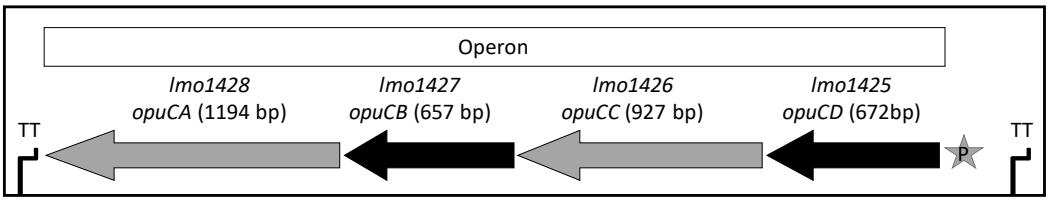



**Figure 6.** Schematic representation of the organization of the *opuC* operon (operon 234) in *L. monocytogenes*. Values representing the gene size (in base pairs). TT indicates transcription terminator sites. Star represents the location of the operon promoter upstream of *opuCA*. The grey arrows represent the genes deleted in this study.

When compared with the wild-type control (WT) neither of the mutations significantly impact *L. monocytogenes* growth under normal conditions in BHI or DMM (**Fig. 7**).

When growing in DMM supplemented with 3% NaCl (DS), all strains presented impaired growth, demonstrating the impact of osmotic stress in *L. monocytogenes* phenotype in the absence of compatible solutes (**Fig. 8a**). Upon addition of 1 mM L-carnitine (DSC) the WT strain demonstrated a relative recovery of growth potential in response to osmotic stress, compared to that observed in the DS condition (**Fig. 8b**). Both mutants, however, were not able to recover and the growth curves presented are similar to the ones seen in the absence of L-carnitine in the presence of 3% NaCl (DS).

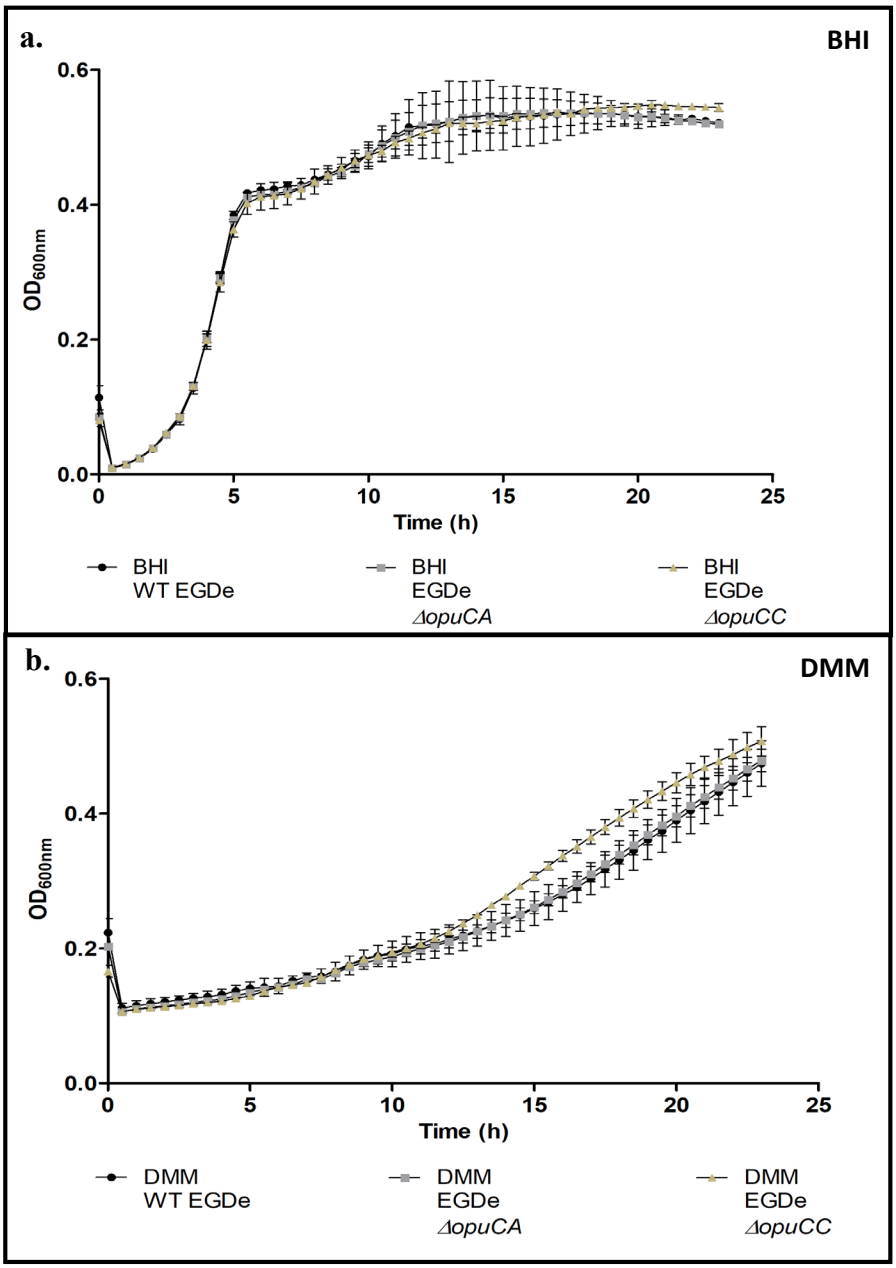



**Figure 7.** Growth curves of *L. monocytogenes* EGDe WT, Δ*opuCA* and Δ*opuCC* strains in **a.** rich media (BHI) and **b.** minimal media (DMM). Growth curves in the tested conditions were obtained by incubating the cultures at 37°C with agitation for 23h. Measurement of optical density ($OD_{600}$ nm) was performed every 30min. Two-way ANOVA with Bonferroni post-test indicated no significant differences between the strains in neither of the conditions. Results represent mean ± SD of three independent experiments.

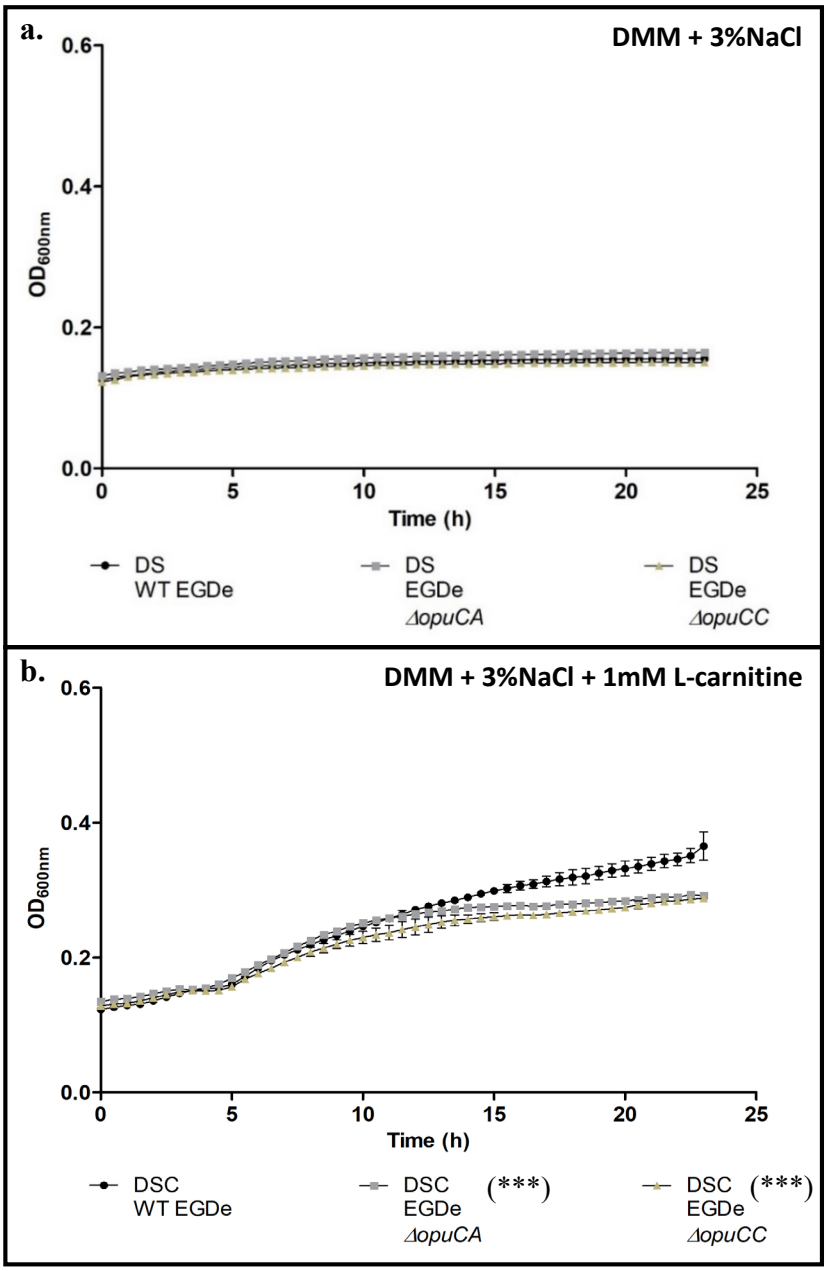



**Figure 8.** Growth curves of *L. monocytogenes* EGDe WT, Δ*opuCA* and Δ*opuCC* strains **a.** under osmotic stress (3% NaCl, DS) and **b.** upon addition of 1 mM L-carnitine (DSC). Cultures were incubated at 37°C with agitation for 23h. Measurement of optical density ($OD_{600}$ nm) was performed every 30min. Two-way ANOVA with Bonferroni post-test indicated no significant differences between the strains in DS. In DSC, the statistical test indicated a significant difference (*** $P < 0.001$) of the WT strain in relation to the two mutants. Results represent mean ± SD of three independent experiments.

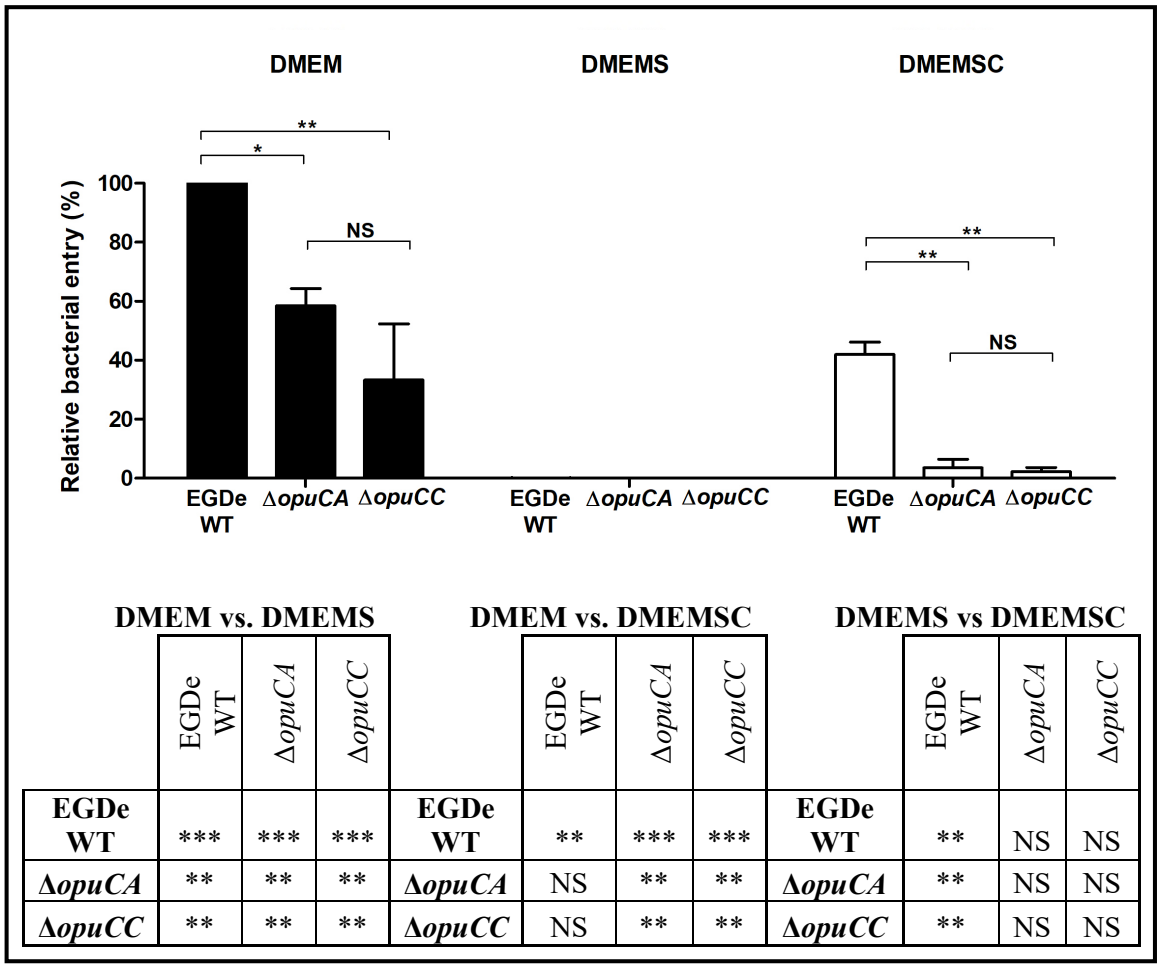
The data highlight the role of L-carnitine in osmoregulation and the importance of the transporter system OpuC. These results show that both *opuCA* and *opuCC* genes are involved in *L. monocytogenes* EGDe growth upon increased extracellular concentration of NaCl when L-carnitine is available. The presence of L-carnitine is shown to be able to promote bacterial growth in response to osmotic stress, since the addition of this compatible solute rescues the growth phenotype of the WT strain (**Fig. 8b**).

Our data demonstrates that, even if another functional L-carnitine uptake system exists in *L. monocytogenes*, L-carnitine uptake through OpuC is still fundamental for bacterial growth, highlighting the importance of this gene cluster in cellular fitness.

When taking into consideration the impact of osmotic stress in the regulation of genes essential for cellular homeostasis (**Fig. 2**), these results support the hypothesis that responses to environmental stimuli and consequent transcriptional shifts shape the phenotype of *L. monocytogenes* in the direction of improving cellular fitness, potentially modulating both stress adaptation and virulence. To evaluate this hypothesis, and in order to mimic the gastrointestinal environment, we determined the impact of *opuCA* and *opuCC* deletions upon *L. monocytogenes* ability to invade epithelial cells in the presence or absence of salt stress (**Fig. 9**).

When compared with the WT, both mutant strains exhibit a significant defect on the ability to invade epithelial cells in DMEM only. Furthermore, all strains displayed a severe impairment in their ability to invade the host cell once the infection occurred in the presence of 3% NaCl (DMEMS). Interestingly, in the presence of 3% NaCl and L-carnitine the WT strain was able to at least partially recover the virulence phenotype.

However the *opuC* mutant strains were severely impaired in invasive potential under these conditions (**Fig. S5**).




**DMEM vs. DMEMS**

| | EGDe WT | Δ*opuCA* | Δ*opuCC* |
|---|---|---|---|
| **EGDe WT** | *** | *** | *** |
| **Δ*opuCA*** | ** | ** | ** |
| **Δ*opuCC*** | ** | ** | ** |

**DMEM vs. DMEMSC**

| | EGDe WT | Δ*opuCA* | Δ*opuCC* |
|---|---|---|---|
| **EGDe WT** | ** | *** | *** |
| **Δ*opuCA*** | NS | ** | ** |
| **Δ*opuCC*** | NS | ** | ** |

**DMEMS vs DMEMSC**

| | EGDe WT | Δ*opuCA* | Δ*opuCC* |
|---|---|---|---|
| **EGDe WT** | ** | NS | NS |
| **Δ*opuCA*** | ** | NS | NS |
| **Δ*opuCC*** | ** | NS | NS |

**Figure 9.** Both *opuCC* and *opuCA* are essential for *in vitro* invasion of host cells. Invasion of C2BBe1 epithelial cell line by *L. monocytogenes* EGDe WT, Δ*opuCA* and Δ*opuCC* strains, shown as intracellular CFU counts relative to WT in DMEM (fixed at 100%). Statistical analysis was conducted using Two-way ANOVA with a Bonferroni post-test. Statistical analysis represented in the graph refers to the comparison between strains within the same condition. Inter-condition statistical analysis is represented in the table. Results represent mean ± SD of three independent experiments (*, P <0.05; **, P <0.01; ***, P <0.001).

In order to understand if these results were due to decreased survival in the media or a defect in the *in vitro* virulence of the Δ*opuCA* and Δ*opuCC* strains we studied the adhesion step of the host cell invasion (**Fig. 10a**), complemented by the quantification of viable bacteria outside of the host cell 1h (**Fig. 10b**) and 2h post-infection (**Fig. 10c**) under the same conditions as the invasion assays were performed. Our experiment revealed that, when compared to the WT, there was no significant impairment in adhesion to host cell or extracellular survival of either mutant strains, under any of the conditions tested.

In the face of these results, it is possible that the increased epithelial cell invasion seen in the WT strain in DMEMSC in relation to DMEMS could be reflective of the increased expression of virulence genes seen in **Table 3**.

Increasing the L-carnitine concentration in the DMEMSC medium from 1 mM to 2 mM did not increase the relative bacterial entry of the WT strain (**Fig. S6**). This indicates that an increase in L-carnitine availability does not induce *L. monocytogenes* epithelial cell invasion in a concentration-dependent manner, i.e., when a specific concentration of the compatible solute is reached the induction of epithelial invasion reaches a plateau phase.

Both Δ*opuCA* and Δ*opuCC* mutant strains showed no impairment in their ability to multiply intracellularly in J774 murine macrophage-like cells in neither of the conditions tested, when compared to the WT (**Fig. 11**). Our findings suggest that while important for invasion of host cells under osmotic stress, *L. monocytogenes* OpuCA and OpuCC are not fundamental for intracellular replication.

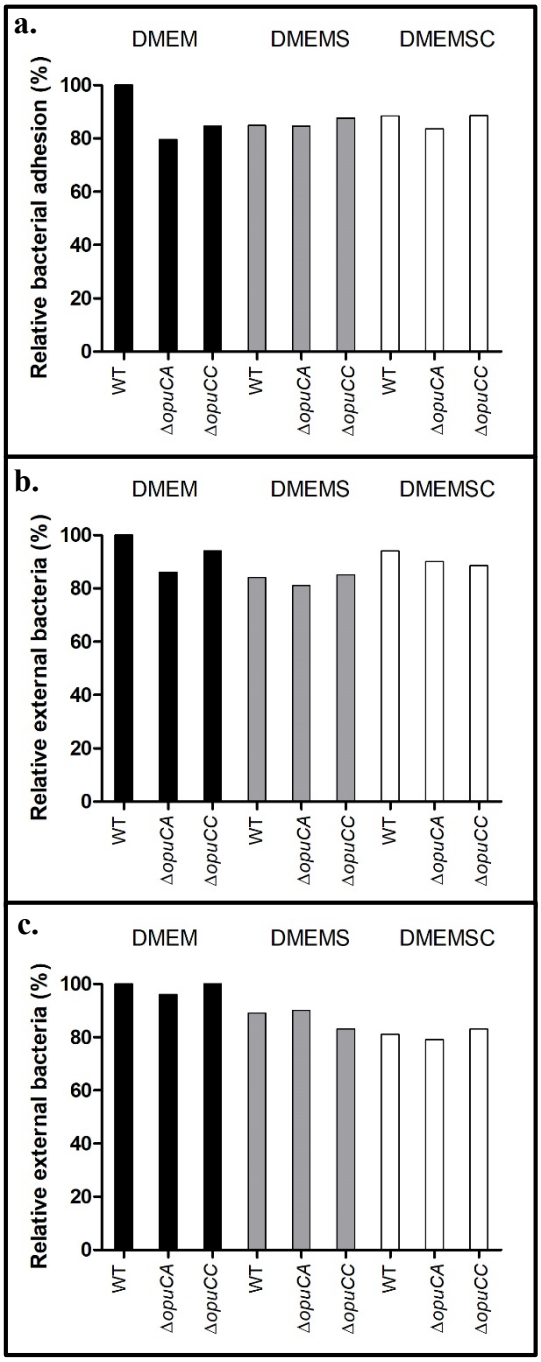



**Figure 10**. Host cell adhesion and extracellular survival is not affected by neither of the conditions tested. **a.** Adhesion assays representing cells adhering to the host cells 30min post-infection. **b.** Characterization of pathogen survival 1h and **c.** 2h post-infection in the tested media conditions (DMEM, DMEMS- DMEM supplemented with 3%NaCl or DMEMSC- DMEM supplemented with 3%NaCl and 1mM L-carnitine). Results shown as CFU counts in the supernatant in relation to the control (WT in DMEM). Result of a single experiment.

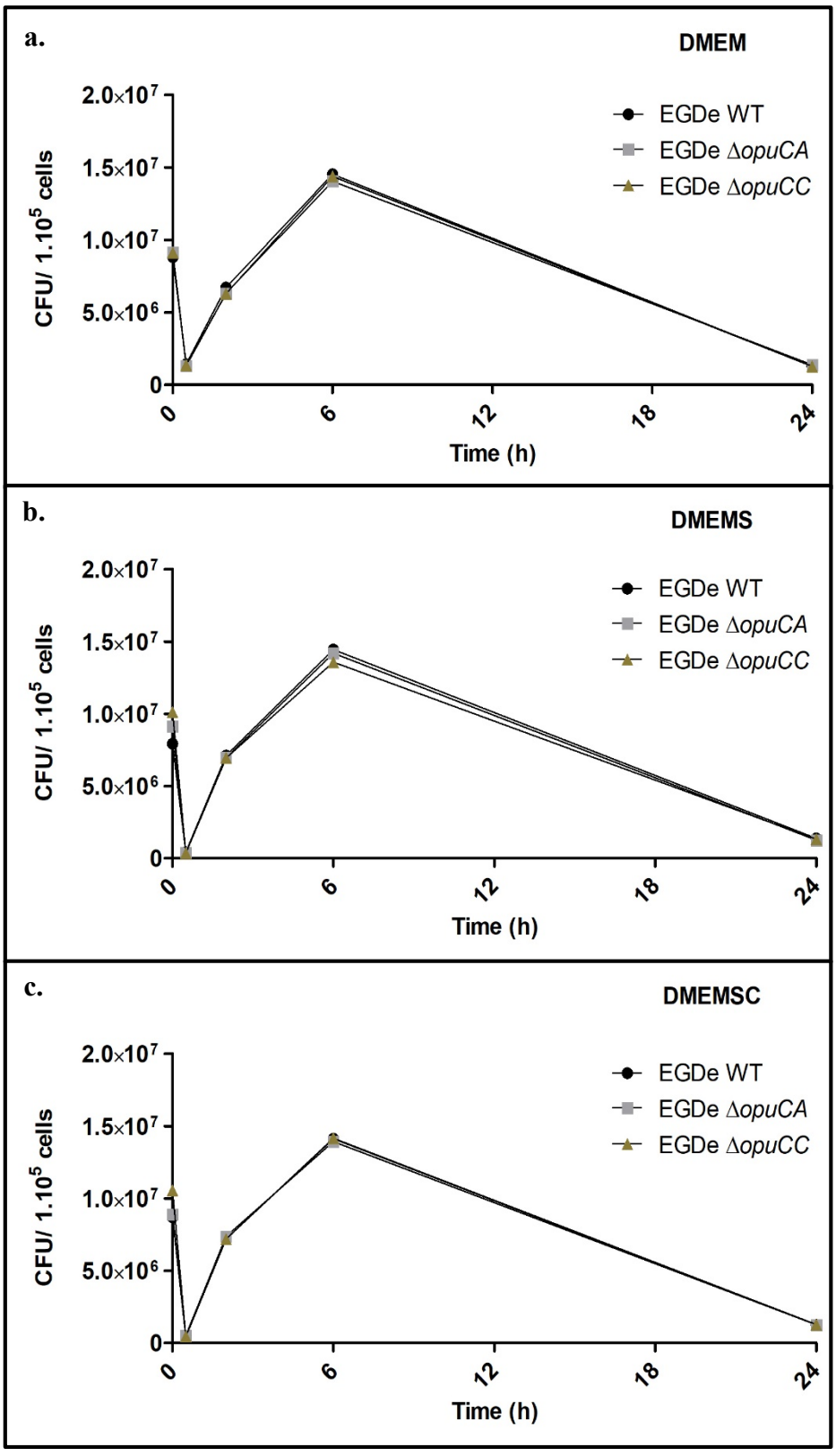



**Figure 11.** Intracellular replication of *L. monocytogenes* EGDe WT, Δ*opuCA* and Δ*opuCC* in J774 macrophages in **a.** DMEM, **b.** DMEMS and **c.** DMEMSC. Data presented is a result of a single experiment.

Previous studies have focused on determining the intracellular transcription profile of *L. monocytogenes* compared to growth in BHI (Chatterjee et al., 2006; Joseph et al., 2006). Transcriptomic data from cytosolic bacteria in mouse P388D1 macrophage cells 4h and 8h post infection showed no differential expression of *opuC* genes (Chatterjee 2006), while intracellular bacteria recovered from Caco-2 human epithelial cells 6 h post infection, showed overexpression of 2.6 and 2.3 fold-change of *opuCA* and *opuCC* respectively, but not the other genes of the operon (Joseph 2006). It is possible that these genes might be expressed and needed at a certain moment during the intracellular phase of *L. monocytogenes* although not necessary for intracellular multiplication.

# 5. Conclusion

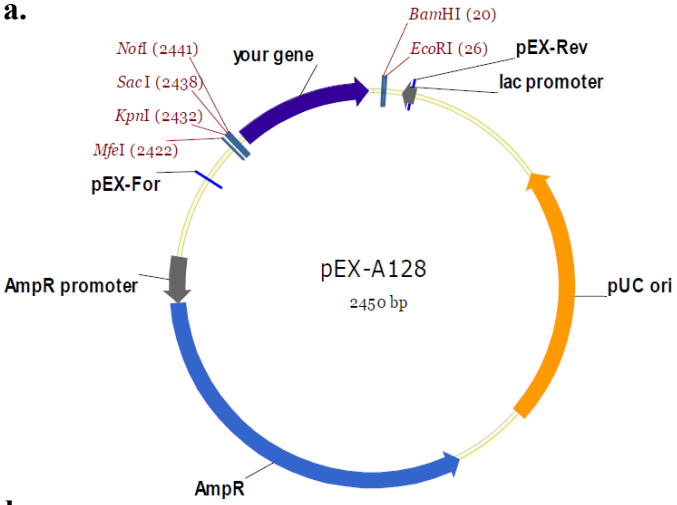
Adaptation to osmotic stress shapes the *L. monocytogenes* gene transcription profile, with L-carnitine playing a major role in *L. monocytogenes* fitness and cellular homeostasis once the pathogen is under osmotic pressure. Here we demonstrate that L-carnitine clearly enables recovery of *L. monocytogenes* from the impact of increased osmotic pressure. Importantly, we show that L-carnitine promotes virulence gene expression during *L. monocytogenes* adaptation to osmotic stress and that the uptake of this compatible solute through OpuC is essential in order to establish infection in epithelial cells. Given the high osmotic pressure and L-carnitine availability in the gastrointestinal environment, these results allow us to hypothesize that L-carnitine uptake in response to increased osmotic pressure might be a trigger to promote infection *in vivo*.

Further studies should focus on the *L. monocytogenes* virulence gene expression profile in the gastrointestinal environment to gain further insights into the role of osmotic stress and L-carnitine availability in *L. monocytogenes* adaptation to the gastrointestinal environment upon infection. It would also be interesting to further investigate the role dietary L-carnitine in *L. monocytogenes* virulence *in vivo*, by focusing on the role of OpuC in the pathogens' ability to establish intestinal infection.

# 6. Supplementary files

**Figure S1**

**a.**




**b.**

***opuCA* 5'-3'**

GGATCCCCAAAATAACCAAAAGGCCCAGAAGCATTTGCTTTTGGGCCTTTTTAAGTG
TGTTATAATTAAATAAATACTATATTGCAGCTTATTACAATGCTCATTTTCACGATTAC
AAGTTTAGCATCGATGTCACACGTGCCACAAGTACGAAATCATCTTGTTTAGTAACTT
CCGTTTGAATGTAACACTGTCTTTAAGCTGTTTTTTTGTATTTAAAAAGTTTAAATCTA
TACTAGTTAGGGAAATTAGTTATCGATTGCCAATTTTTTGGTAGTGGTAAAGAAAAAA
TGGAGGTGTGTAGGCGTGTTAGGATAAGCTATGGACGCAATTGTTACATTTTTTCAA
GAAAACGGCCATAACTTGCTTGTTCAAACATGGCAACATTTATTTATCTCCCTATCTG
CTGTTATTTTAGGTATTGCAGTTGCGGTACCAACTGGGATTTTACTCACGCGTTCACC
AAAAGTAGCGAACTTTGTTATTGGTGTTGTTAGTGTTTTGCAAACCGTTCCGTCTCTTG
CTATTTTGGCGTTTATTATTCCGTTTCTTGGCGTTGGAACGTTACCAGCGATTATTGCG
CTATTTATTTACGCGCTTTTACCAATTTGTCGAC

***opuCC* 5'-3'**

GGATCCTGACGAATTGGCAACTAATTGTTAATGTCGAAATTCCTAACTCCATCTCCGT
TATTATGGCTGGTATTCGTTTATCTGCTGTATACGTTATCGCTTGGGCGACGCTTGCTT
CTTACATTGGAGCTGGTGGACTAGGAGACTTTATTTTTAATGGTTTAAACTTATACCG
TCCAGACTTAATTCTTGGTGGGGCAATTCCGGTTACCATTTTAGCCCTTGTAGTAGAA
TTCGCGCTTGGAAAATTAGAATATCGCTTAACACCAAAAGCCATCCGTGAAGCTCGG
GAAGGGGGAGAATAACATGAAGAACTAAGGAGGTGCCAAAAGATGGACACATTAA
AACAATTAATTGATTATTACCAAACAAATGGAAGTTATGTCATGGAAGAGTTCTGGC
GGCATTTCTTAATGAGTGCTTACGGAGTTATCTTCGCAGCAATCATAGCGATACCGCT
TGGAGTATATATTGCAAGAAAAAAACGCTTAGCTGGTTGGGTTATCCAAATCGCTAA
TATCATCCAAACAATTCCGGCACTAGCAATGTTAGCCGTACTTATGCTTATCATGGGC
TTAGGGACGAATACAGTCGTCTTGTCCTTGTTCCTATATTCTTTATTACCAATTCTGTC
GAC

**Figure S1. a.** pEX-A128 map. **b.** DNA sequences of *opuCA* and *opuCC* loci synthesized in the pEX-A128 plasmid. Sequences in blue are restriction sites (BamHI), in green the gene start codon, in red the gene stop codon and in purple restriction sites (SalI).

**Figure S2**

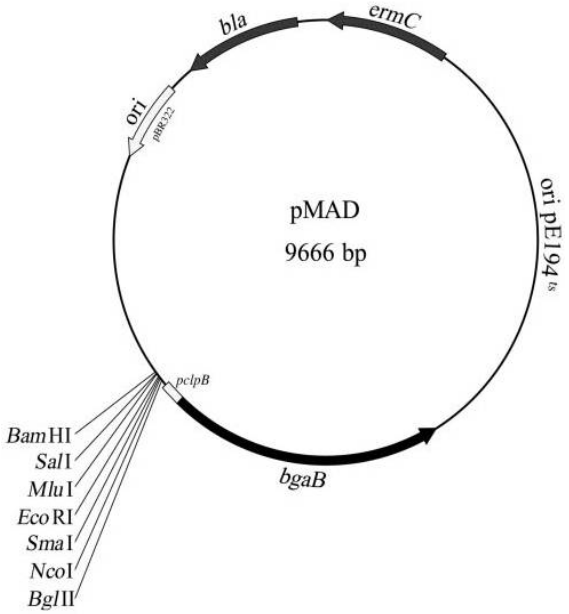



**Figure S2.** pMAD map (Arnaud et al., 2004).

**Figure S3**

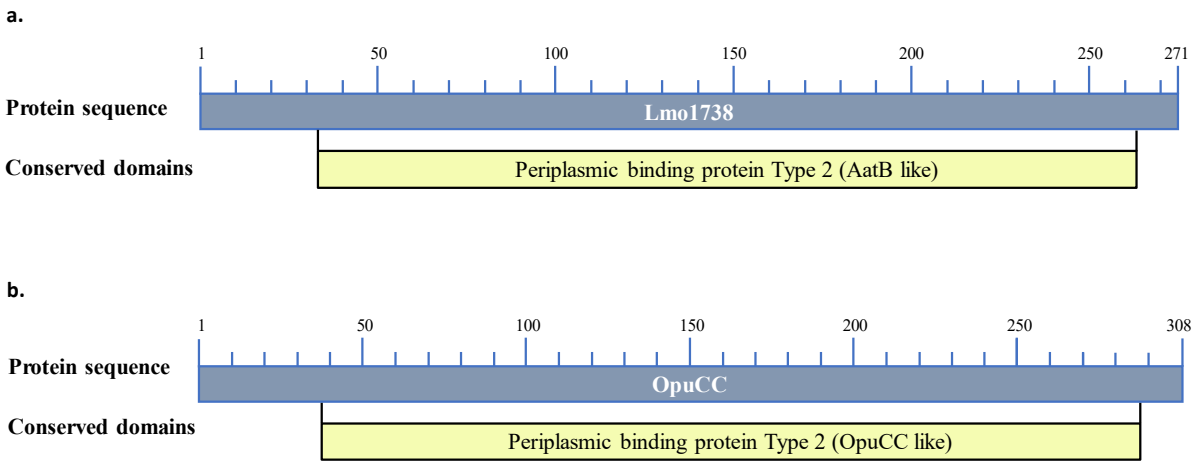



**Figure S3.** The *lmo1738* gene encodes a putative unit of an ATP-binding cassette transporter-like system. Representation of the protein conserved domains of **a.** Lmo1738 and **b.** OpuCC of *L. monocytogenes* EGDe. *In silico* analysis predicts that the gene *lmo1738* encodes a protein belonging to the polar amino acid-binding ATP-binding cassette transporter-like system protein family. The Lmo1738 amino acid sequence contains a specific periplasmic binding protein type 2 (PBP2) AatB conserved domain, as determined by the NCBI Conserved Domains database. OpuCC, the carnitine binding subunit of OpuC also contains a PBP2 conserved domain. Numeric scale indicates amino acid position of the protein sequence.

**Figure S4**

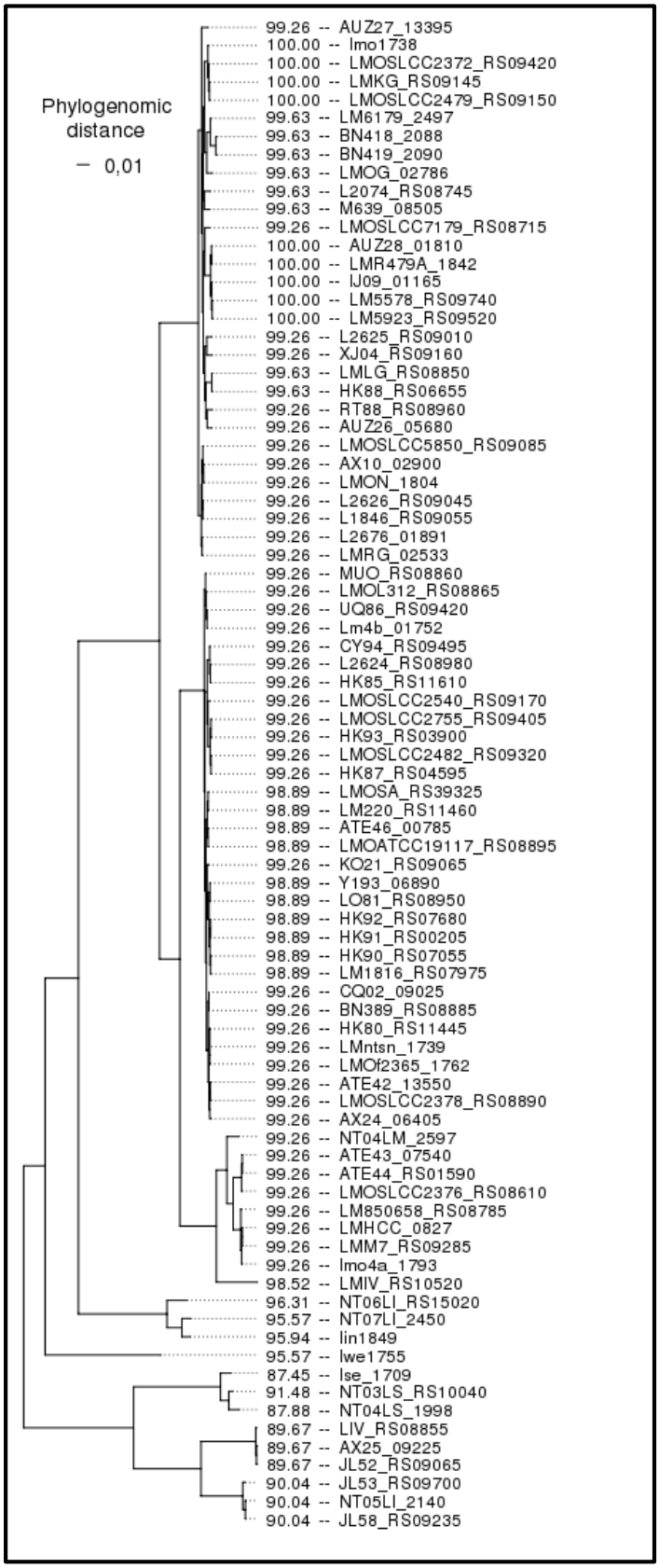



**Figure S4.** Phylogenetic tree of *Listeria lmo1738* orthologues. The *lmo1738* gene highly conserved among *Listeria* species.

**Figure S5**

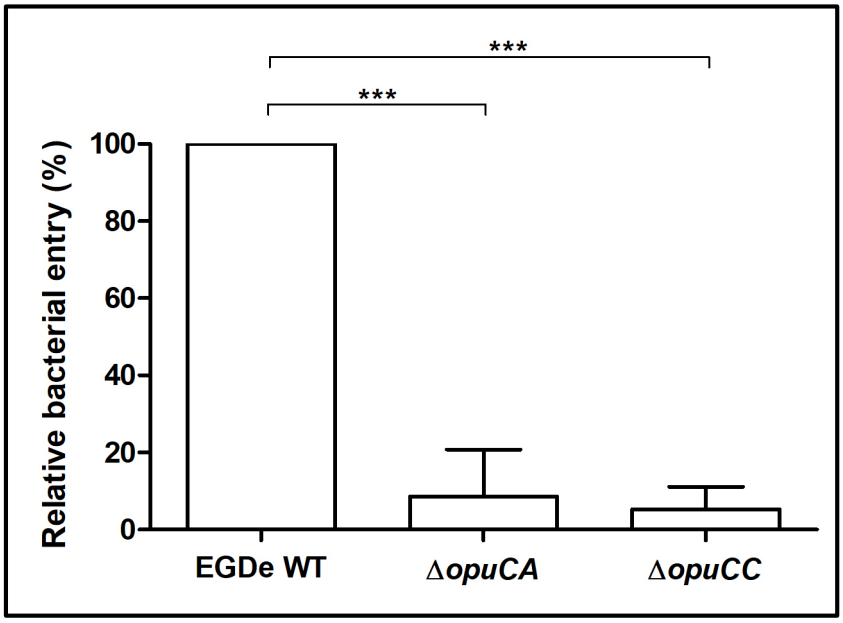



**Figure S5.** Percentage of invasion of Δ*opuCA* and Δ*opuCC* strains in DMEMSC. Invasion of C2BBe1 epithelial cell line by *L. monocytogenes* EGDe WT, Δ*opuCA* and Δ*opuCC* strains, shown as intracellular CFU counts relative to WT in DMEMSC (fixed at 100%). Statistical analysis was conducted using Two-way ANOVA with a Bonferroni post-test. Results represent mean ± SD of three independent experiments (***P<0.001).

**Figure S6**

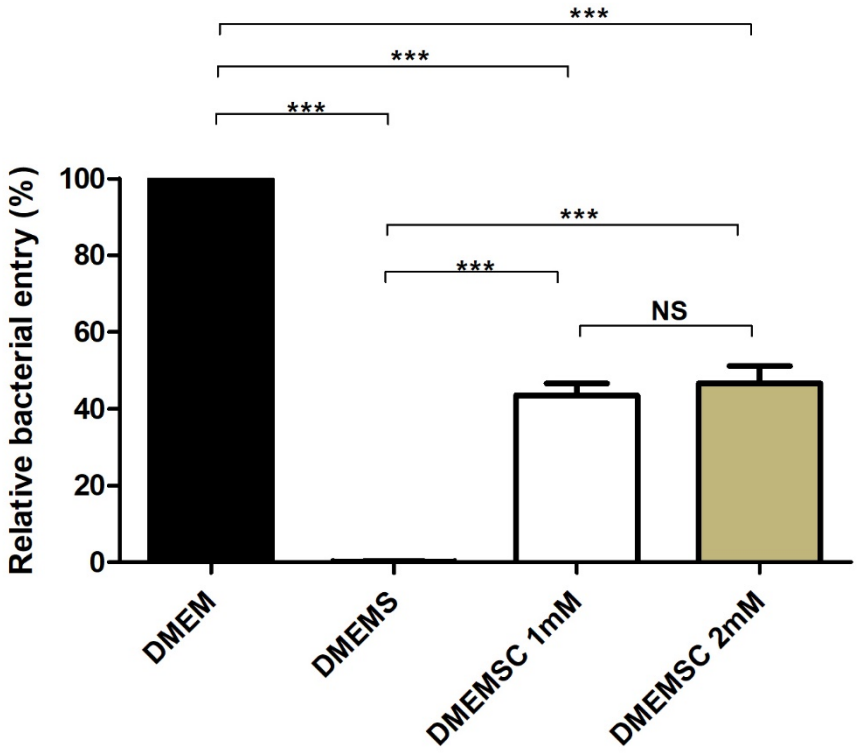



**Figure S6.** Increasing L-carnitine supplementation to DMEMS from 1mM to 2mM does not increase invasion of C2BBe1 epithelial cell line by *L. monocytogenes* EGDe. Statistical analysis was conducted using Two-way ANOVA with a Bonferroni post-test. Results represent mean ± SD of three independent experiments (***P<0.001).

**Table S1.** Plasmids and bacterial strains.

| Plasmid/Strain | Description | Source |
|---|---|---|
| *Escherichia coli* | | |
| DH5α | Cloning host strain; $F^-$ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17*($r_k^-$, $m_k^+$) *phoA supE44 thi-1 gyrA96 relA1* $\lambda^-$ | Life Technologies |
| *Listeria monocytogenes* | | |
| EGDe | Wild type; serotype 1/2a | (Glaser et al., 2001) |
| EGDe Δ*opuCA* | EGDe *opuCA* deletion mutant | This study |
| EGDe Δ*opuCC* | EGDe *opuCC* deletion mutant | This study |
| Plasmids | | |
| pEX-A128 | Standard cubcloning vector; $Amp^r$ | Eurofins Genomics |
| pEX-A128(*opuCA*) | pEX-A128 with 5'- and 3'-flanking regions of *opuCA* locus; $Amp^r$ | This study |
| pEX-A128(*opuCC*) | pEX-A128 with 5'- and 3'-flanking regions of *opuCC* locus; $Amp^r$ | This study |
| pMAD | Gram-negative/Gram-positive shuttle vector; thermosensitive replication; $Amp^r$ $Ery^r$ | (Arnaud et al., 2004) |
| pMAD(*opuCA*) | pMAD with 5'- and 3'-flanking regions of *opuCA* locus; $Amp^r$ $Ery^r$ | This study |
| pMAD(*opuCC*) | pMAD with 5'- and 3'-flanking regions of *opuCC* locus; $Amp^r$ $Ery^r$ | This study |

**Table S2.** Defined minimal media (DMM) composition (adapted from Premaratne et al., 1991).

| Compound | Amount per litre |
|---|---|
| $KH_2PO_4$ | 6,56 g |
| $Na_2HPO_4$* 7 $H_2O$ | 30,96 g |
| $MgSO_4$ * 7 $H_2O$ | 0,41 g |
| Ferric citrate | 88 mg |
| L-glutamine | 0,6 g |
| L-leucine | 0,1 g |
| DL-isoleucine | 0,1 g |
| DL-valinie | 0,1 g |
| DL-methionine | 0,1 g |
| L-arginine HCl | 0,1 g |
| L-cysteine HCl | 0,1 g |
| L-histidine HCl | 0,1 g |
| L-tryptophan | 0,1 g |
| L-phenylalanine | 0,1 g |
| Adenine | 2,5 mg |
| Biotin | 0,5 mg |
| Riboflavine | 5 mg |
| Thiamine HCl | 1 mg |
| Pyridoxal HCl | 1 mg |
| Para-aminobenzoic acid | 1 mg |
| Calcium panthothenate | 1 mg |
| Nicotinamide | 1 mg |
| Thioctic acid | 5 mg |
| $H_2O$ (deionised & autoclaved) | |

**Table S3.** List of primers.

| **Name** | **Sequence (5' - 3')** |
|---|---|
| *Mutant construction* | |
| *opuCA*-SeqF | TTTCTATCGTGATGAATGGG |
| *opuCA*-SeqR | ATGTAAGAAGCAAGCGTCG |
| *opuCC*-SeqF | ACCGTTCCGTCTCTTGCT |
| *opuCC*-SeqR | ATAACAGATAATGCGAGTGGC |
| pMAD-SeqF | TGATGGTCGTCATCTACCTGCC |
| pMAD-SeqR | CCTACGTAGGATCGATCCGACC |
| | |
| *RT-qPCR* | |
| qPCR-*16S*-F | TGGAAACTGGAAGACTGG |
| qPCR-*16S*-R | GTTCCTCCACATATCTACG |
| qPCR-*actA*-F | CTATACAAGTGGAGCGTC |
| qPCR-*actA*-R | CTATCCGATGATGCTATGG |
| qPCR-*agrA*-F | AGTTAGTATCACGAATGCCTAC |
| qPCR-*agrA*-R | ACTTCCGAATTTCCTGAGC |
| qPCR-*alsS*-F | CCAGAATTAATCGTTAGTCGTC |
| qPCR-*alsS*-R | GTTACAAGCACAACACCAG |
| qPCR-*gbuC*-F | GTTGGACACCTCACTGGAT |
| qPCR- *gbuC* -R | GCACTTTGTAGGCAGAAGG |
| qPCR-*hly*-F | TTCGAGCCTAACCTATCC |
| qPCR-*hly*-R | GTCTTGATTAGTCATACCTGG |
| qPCR-*lmo1738*-F | GCAGAATATGGCATCAAAGC |
| qPCR-*lmo1738*-R | CGTCCGTCACAGTATAACC |
| qPCR-*lmo1739*-F | AAGAAGCAGAACGATTACTTGG |
| qPCR-*lmo1739*-R | TGTCCGCCTGATAATTGGTA |
| qPCR-*lmo1740*-F | CTCGTCCTTAGTCTACATTCTCG |
| qPCR-*lmo1740*-R | CAGTGTAACATCTCTACTCATTGC |
| qPCR-*opuCA*-F | TGGTGGACAACAACAACG |
| qPCR- *opuCA* -R | GCTCCGAATGGTTCATCC |
| qPCR- *opuCC*-F | ACCTCGGATCAACTATCGT |
| qPCR- *opuCC* -R | CGGTATATCTTGTCGCTGTAAT |
| qPCR-*plcA*-F | CCAGGTACACATGATACG |
| qPCR-*plcA*-R | CTGCTTCTAGTTGTTGGTA |
| qPCR-*sepA*-F | TACGATTATGGAAGGCGATA |
| qPCR-*sepA*-R | TTGGACGATGCTCATAGTAT |

**Table S4.** Total numbers of differentially regulated genes in different COG functional categories, respective numbers of up-regulated and down-regulated genes in relation to the conditions tested. Total numbers of genes presented do not correspond to the sum of genes in categories, as several genes were classified in more than one COG functional categories.

| Category of genes encoding for | DSC relatively to DS | | | DSC relatively to DMM | | | DMM relatively to DS | | |
|---|---|---|---|---|---|---|---|---|---|
| | Up 490 | Down 276 | Total 766 | Up 0 | Down 51 | Total 51 | Up 511 | Down 238 | Total 749 |
| **Amino acid transport and metabolism** | 66 | 29 | 95 | | 8 | 8 | 65 | 24 | 89 |
| **Carbohydrate transport and metabolism** | 26 | 26 | 52 | | 8 | 8 | 30 | 19 | 49 |
| **Coenzyme transport and metabolism** | 13 | 7 | 20 | | 2 | 2 | 18 | 4 | 22 |
| **Inorganic ion transport and metabolism** | 27 | 16 | 43 | | 4 | 4 | 23 | 13 | 36 |
| **Lipid transport and metabolism** | 16 | 11 | 27 | | | | 15 | 10 | 25 |
| **Nucleotide transport and metabolism** | 18 | 2 | 20 | | | | 20 | 2 | 22 |
| **Secondary metabolites biosynthesis, transport and catabolism** | 10 | 9 | 19 | | 2 | 2 | 9 | 7 | 16 |
| **Energy production and conversion** | 20 | 21 | 41 | | 2 | 2 | 20 | 22 | 42 |
| **Cell wall/membrane biogenesis** | 36 | 8 | 44 | | 3 | 3 | 31 | 8 | 39 |
| **Cell cycle control, mitosis and meiosis** | 14 | 1 | 15 | | | | 13 | 1 | 14 |
| **Cell motility** | 11 | 5 | 16 | | 2 | 2 | 11 | 6 | 17 |
| **Intracellular trafficking and secretion** | 10 | 2 | 12 | | 2 | 2 | 8 | 3 | 11 |
| **Defense/virulence mechanisms** | 25 | 6 | 31 | | | | 23 | 11 | 34 |
| **Signal transduction mechanisms** | 39 | 18 | 57 | | 1 | 1 | 39 | 11 | 50 |
| **Replication, recombination and repair** | 11 | 8 | 19 | | | | 13 | 9 | 22 |
| **Transcription** | 46 | 26 | 72 | | 2 | 2 | 50 | 22 | 72 |
| **Translation** | 10 | 5 | 15 | | | | 12 | 3 | 15 |
| **Posttranslational modification, protein turnover, chaperones** | 13 | 15 | 28 | | 1 | 1 | 11 | 12 | 23 |
| **General function prediction only** | 46 | 33 | 79 | | 7 | 7 | 61 | 33 | 94 |
| **Function unknown** | 37 | 39 | 76 | | 9 | 9 | 35 | 31 | 66 |
| **Not in COGs** | 65 | 27 | 92 | | 5 | 5 | 84 | 28 | 112 |

**Table S5.** List of proteome studies with *L. monocytogenes* where the protein Lmo1738 has been detected.

| **Localization** | **Growth phase, temperature** | **Media** | **Strain *L. monocytogenes*** | **Reference** |
|---|---|---|---|---|
| Membrane | Exponential 37°C | BHI | EGDe | (Wehmhöner et al., 2005) |
| Membrane | Exponential 37°C | BHI | EGDe | (Renier et al., 2012) |
| Membrane | Exponential 37°C | BHI | LI0521 | (Zhang et al., 2013) |
| Secretome | Exponential 37°C | BHI | EGDe | (Trost et al., 2005) |
| Secretome, cytoplasm | Exponential 37°C | BHI | LI0521 | (Zhang et al., 2013) |

**Table S6.** Differentially expressed genes in DMM relatively to DMM supplemented with 3% NaCl (DS) associated with *L. monocytogenes* defence and virulence mechanisms.

| **Defence/virulence mechanisms DMM relative to DS** | | | |
|---|---|---|---|
| | **Locus tag** | **Gene name** | **Fold-change** |
| **Up-regulated** | *lmo0049* | *agrD* | 18.9 |
| | *lmo1831* | *pyrE* | 16.6 |
| | *lmo0050* | *agrC* | 12.2 |
| | *lmo0048* | *agrB* | 12.0 |
| | *lmo0051* | *agrA* | 9.4 |
| | *lmo0201* | *plcA* | 8.8 |
| | *lmo0202* | *hly* | 8.7 |
| | *lmo0153* | | 5.5 |
| | *lmo0154* | | 4.8 |
| | *lmo0205* | *plcB* | 4.7 |
| | *lmo2769* | | 4.2 |
| | *lmo0204* | *actA* | 4.0 |
| | *lmo1636* | | 3.8 |
| | *lmo0155* | | 3.4 |
| | *lmo1638* | | 3.1 |
| | *lmo1916* | | 3.0 |
| | *lmo1746* | | 2.8 |
| | *lmo2371* | | 2.7 |
| | *lmo0203* | *mpl* | 2.5 |
| | *lmo0605* | | 2.4 |
| | *lmo1952* | *lysA* | 2.4 |
| | *lmo1786* | *inlC* | 2.3 |
| | *lmo0925* | | 2.2 |
| | *lmo0923* | | 2.0 |
| **Down-regulated** | *lmo1062* | | -2.0 |
| | *lmo2114* | | -2.1 |
| | *lmo2752* | | -2.1 |
| | *lmo2751* | | -2.3 |
| | *lmo2580* | | -2.3 |
| | *lmo2067* | | -2.4 |
| | *lmo0895* | *sigB* | -2.8 |
| | *lmo2190* | *mecA* | -3.1 |
| | *lmo0264* | *inlE* | -3.1 |
| | *lmo2157* | *sepA* | -3.2 |
| | *lmo0263* | *inlH* | -4.0 |

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# Chapter IV

# Impact of dietary supplementation with L-carnitine on host susceptibility to *Listeria monocytogenes* infection

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**Authors Contributions**

Vanessa Las Heras carried out the experimental work, designed experiments and analysed the data. Vanessa Las Heras wrote the paper with significant input from Cormac Gahan and input from all authors. Adam Clooney carried out bioinformatics analyses with significant input from Vanessa Las Heras. Cara Hueston designed and performed the infection studies with significant input from Vanessa Las Heras, Jorge Pinheiro and Maria Sanchez. Vanessa Las Heras and Jorge Pinheiro carried out the histological analyses. Vanessa Las Heras carried out the RNA and DNA extractions, with significant input from Maria Sanchez. Conall Strain carried out the SCFA extraction and analysis. Paul Cotter, Catherine Stanton, Colin Hill and Cormac Gahan devised and co-ordinated the study and analysed the data. The final manuscript was read and approved by all authors.

# Contents

# 1. Abstract

The primary source of L-carnitine in omnivorous diets is red meat, the consumption of which is increasing as part of the Western-diet. Previous studies have demonstrated that uptake of L-carnitine via the bacterial OpuC transport system is important for full virulence of *Listeria monocytogenes* in the gastrointestinal tract. However, the influence of increased dietary L-carnitine upon bacterial virulence is currently unclear. Here we administered mice with a two-week dietary supplementation with L-carnitine and focused upon changes to the intestinal landscape, microbiota and host susceptibility to oral infection with *L. monocytogenes*. We demonstrate that increased dietary L-carnitine in our model caused an expected modulation of the expression of genes in pathways involved in bile acid metabolism and transport. Dietary L-carnitine also induced modulation of the host immune response towards a reduction in the expression of genes encoding cytokines (TNF-α, IL-1β and IL-6) in both ileum and liver. We also observe that dietary supplementation significantly altered the composition of the intestinal microbiota and resulted in a reduction of short-chain fatty acids (SCFA) in the cecum. Despite inducing significant physiological changes to the host gastrointestinal tract, L-carnitine did not increase host susceptibility to oral infection with *L. monocytogenes*. However, we confirmed that L-carnitine uptake through the OpuC osmolyte transporter system is important for full virulence in the gastrointestinal tract. Overall the data indicate significant alteration to host parameters influenced by short-term L-carnitine consumption in mice and suggest that existing carnitine levels in the gut are sufficient to mediate the effects of OpuC upon *L. monocytogenes* infection.

# 2. Introduction

L-carnitine is a quaternary amine present in most animal tissues and is an essential metabolite that plays a critical role in cell physiology (Vaz and Wanders, 2002). In mammalian tissues, L-carnitine is an indispensable intermediary of cellular metabolism, with a significant role in the transport of activated long-chain fatty acids (LCFAs) from the cytosol into the mitochondrial matrix (McGarry and Brown, 1997), as well as transferring the products of peroxisomal β-oxidation into the mitochondria (Verhoeven et al., 1998). L-carnitine can be synthesized by cells through a specific biosynthetic pathway but is mostly obtained from a diet rich in meat (Demarquoy et al., 2004) and is found in relatively high concentrations in animal tissues (Bremer, 1983). L-carnitine is primarily absorbed from the small intestinal lumen by active transport into enterocytes, followed by diffusion into the circulatory system (Famularo et al., 2004).

Animal products such as fish, meat (both beef and poultry) and milk constitute dietary sources containing the highest amounts of L-carnitine (Shils and Shike, 2006). Studies on the current dietary shifts to the so-called Western-diet, indicate an increased consumption of ready-to-eat meat products, which could suggest an increased dietary L-carnitine intake (Cordain et al., 2002).

In bacteria, L-carnitine is most frequently transported into the cytosol by binding to an ATP-binding cassette (ABC) transporter system, to be then used as a compatible solute, a final electron acceptor for respiration or as a sole energy source (Meadows and Wargo, 2015). Recently it has become evident that the host microbiota metabolizes a significant proportion of the dietary L-carnitine (Ghonimy et al., 2018). This microbiota-mediated metabolic pathway promotes the conversion of L-carnitine

to trimethylamine which is then oxidized in the liver to trimethylamine-*N*-oxide (TMAO) (Gut and Conney, 1993). Previous reports have identified the gut microbiota as a variable influencing bioavailability of intestinal L-carnitine (Shang et al., 2014), demonstrating the role of Firmicutes, Bacteroides and Proteobacteria in TMAO synthesis in both humans and mice (Zhu et al., 2014). Moreover, evidence shows that TMAO accumulation decreases upon antibiotic treatment in healthy patients, demonstrating that TMAO production is dependent on the metabolism of intestinal commensal bacteria (Koeth et al., 2013). Furthermore, vegetarians and vegans have a lower ability to convert L-carnitine into TMAO, a results linked to the differences in microbiota composition (Koeth et al., 2013). TMAO can be either stored as an osmolyte in the tissues or cleared by the kidneys (Velasquez et al., 2016). TMAO accumulation in the plasma has been linked to an increased risk of cardiovascular and kidney disease (Tang et al., 2015; Tang et al., 2013).

L-carnitine is also an important compound to relevant human bacterial pathogens such as the foodborne *Listeria monocytogenes*. *L. monocytogenes* is the etiological agent of listeriosis, a potentially fatal invasive infectious disease, with associated symptoms varying from mild gastroenteritis to severe meningitis, encephalitis, or even septicaemia (Acheson and Schlech, 2000). Listeriosis has a high fatality rate of 20-30% (Swaminathan and Gerner-Smidt, 2007) and affects primarily high-risk groups, including infants, elderly, pregnant women and also immunocompromised patients (Allerberger and Wagner, 2010). Interestingly, food contamination with *L. monocytogenes* is of special concern in ready-to-eat meat products, with previous outbreaks traced to consumption of such products in a number of countries including Italy (Duranti et al., 2018), Denmark (Mølbak et al., 2016) and the Czech Republic (Gelbíčová et al., 2018). Indeed, the largest globally recorded listeriosis outbreak was

recently reported in South Africa and is linked to consumption of a contaminated ready-to-eat meat product. The outbreak lasted from early 2017 until mid-2018 and officially involved 1049 laboratory-confirmed cases including 209 fatalities, constituting a 20% mortality rate (Chersich et al., 2018).

The prevalence of *L. monocytogenes* in food products is frequently associated with its ability to survive and grow at high osmolarity and refrigerated temperatures (Gandhi and Chikindas, 2007). Moreover, an *in vitro* study indicated that food matrix composition influences *L. monocytogenes* virulence gene expression, by demonstrating that growth in liver pates (rich in L-carnitine) with increased salt content induced virulence gene expression (Olesen et al., 2010).

Upon increased osmotic pressure, *L. monocytogenes* imports L-carnitine into the cytosol through the OpuCABCD transporter system, were it functions as an osmolyte (Fraser et al., 2000). Previous studies have demonstrated the relevance of OpuC for *L. monocytogenes* survival and virulence in the small intestine and for the establishment of systemic infection in mice (Sleator et al., 2001; Wemekamp-Kamphuis et al., 2002). The OpuC system is significantly upregulated in the cytosol of epithelial Caco-2 cells (Joseph et al., 2006) and mice spleen (Camejo et al., 2009). L-carnitine uptake through the OpuC transport system has been implicated in protecting *L. monocytogenes* against bile acid stress as well as salt stress, and this cross-protection may be important for survival in the small intestine (Watson et al., 2009).

Considering the relationship between L-carnitine and the virulence of *L. monocytogenes* as well as increasing consumption of dietary L-carnitine we investigated whether dietary supplementation with L-carnitine might increase host susceptibility to infection. We used a systems biology approach to determine the role

of L-carnitine dietary supplementation on the ability of *L. monocytogenes* to adapt to the high osmolarity in the gastrointestinal environment and establish infection in an *in vivo* murine model. In particular, we studied the impact of increased L-carnitine intake in the modulation of the host intestinal environment, including host physiological changes and microbiota modulation. We show that the OpuC system is essential for full virulence of *L. monocytogenes*. However, whilst elevated dietary L-carnitine alters specific characteristics of the gut environment there is no increased susceptibility to infection.

# 3. Methods

## 3.1. Bacterial strains and growth conditions

Details of the bacterial strains used in this study are in **Table S1**. *L. monocytogenes* EGDe InlA$^{m}$, the murinized strain of *L. monocytogenes*, and *E. coli* were routinely grown in brain heart infusion broth (BHI, Oxoid) and lysogeny broth (LB, Fisher) respectively, at 37°C aerobically with shaking. Growth on solid media was preformed using BHI-agar and LB-agar plates. When necessary, antibiotics were added to the media: ampicillin 100 µg/ml and erythromycin 5 µg/ml.

## 3.2. Mutant construction

The deletion of *opuCC* (*lmo1426*) from the murinized *Listeria* strain *L. monocytogenes* EGDe InlA$^{m}$ was achieved by double homologous recombination as previously described (**Chapter III**). The vectors and primers used are detailed in **Fig. S1**, **Fig. S2** and **Table S2**.

## 3.3. Animal dietary intervention and infection

Seven-week-old female C57BL/6 mice (ENVIGO, UK), n=90, were housed in a controlled environment with access to chow (Teklad Global 2018S Rodent Diet, ENVIGO, UK) and water *ad libitum*. The mice were then either kept on the normal regimen (n= 10) or given water supplemented with 1.3% L-carnitine (Sigma-Aldrich) for 13 days (n= 10). Thereafter, animals were orally inoculated by intra-gastric gavage (IG) with *L. monocytogenes*. Overnight cultures of the murinized wild type strain *L. monocytogenes* EGDe InlA$^{m}$ (WT) (n=10 normal regimen, n=10 1.3% L-carnitine), and the *opuCC* deletion mutant *L. monocytogenes* EGDe InlA$^{m}$Δ*opuCC* (Δ*opuCC*) (n=10 normal regimen, n=10 1.3% L-carnitine) were pelleted (7000 g for 5min),

washed twice with PBS and resuspended in PBS. Inoculum were administrated as 200 µl with 5 x $10^9$ CFU.

Mice were infected for a period of 72h (Monk et al., 2010) and were maintained on their specific diets for that period. Mice were euthanized and the internal organs aseptically removed and homogenized in PBS using stomacher bags. For CFU per organ enumeration, dilutions were plated on BHI-agar plates. During the infection period, on days 14 (day 1 post-infection), 15 (day 2 post-infection) and 16 (day 3 post-infection) faecal pellets were collected and plated for CFU enumeration to determine shedding of both strains of *L. monocytogenes*.

### 3.4. RNA extraction and RT-qPCR analysis

Ileum and liver samples were collected for the analysis of the host regulatory response to increased dietary L-carnitine intake. Upon collection, the samples were stabilized with RNAlater™ (Sigma) and stored at -80°C until total RNA extraction (RNeasy Plus Universal Mini Kit, Qiagen). The total RNA extracted was depleted of DNA by DNase treatment (TURBO DNA-free™ Kit, Ambion) and the transcriptomic analysis was done through RT-qPCR. RNA was converted to cDNA using the NZY First-Strand cDNA Synthesis Kit (Nzytech) and qPCR protocol was carried out using LightCycler® 480 Probes Master (Roche™) with the Universal Probe Library from Roche. The amplification setup used was 45 runs in 384 well plates with the MonoColor hydrolysis probe detection format. Primers used are outlined in **Table S3**.

### 3.5. Faecal samples and microbiota profiling

For analysis of mouse gut microbiota based on 16S rRNA gene amplicon sequencing, faecal samples were collected to study dietary microbiota modulation prior to infection (on days 0 and 13). Total DNA was extracted form faecal pellets using QIAmp fast

DNA stool mini Kit (Qiagen) and microbiota analysis was done through 16S rDNA sequencing. The quality of raw sequences was visualised with FastQC. This was followed by quality filtering using trimmomatic to ensure only high quality reads were retained for further analysis (Bolger et al., 2014). Briefly, the first 15 bases were removed from both forward and reverse reads while the forward was cropped at 245 bases and the reverse at 220. Finally, a sliding window (4:20) with a minimum length of 200 bases was applied. The remaining high quality reads were processed and merged using the DADA2 pipeline (Callahan et al., 2016). Further chimera filtering of the resulting Ribosomal Sequence Variants was performed with the ChimeraSlayer Gold database (Edgar et al., 2011). Sequences were classified (phylum to genus) using the Mothur implementation of the Ribosomal Database Project (RDP) classifier and the RDP database (v11.4). All assignments obtaining a bootstrap value of less than 80% assigned as the preceding rank and unclassified. Species classification was carried out using SPINGO against the RDP (v11.4) with default parameters (similarity score of 0.05 and a bootstrap cut-off of 0.8) (Allard et al., 2015). All downstream analysis was performed in R version 3.4.3. Alpha and Beta diversity was calculated using the R package phyloseq (McMurdie and Holmes, 2013). Differences in alpha diversity were assessed using the nonparametric Mann-Whitney test. Differential abundant analysis was performed using DESeq2 (Love et al., 2014). The Adonis function in the vegan library was used to assess group level differences in the microbiota.

### 3.6. Tissue staining and Microscopy

Ileal samples were collected for histology analysis and the identity of sample groups was blinded to the investigator. The tissue sample was stored in methacarn (60% absolute methanol, 30% chloroform and 10% glacial acetic acid) for 2h and

dehydrated with 70% ethanol for 2h, both at room temperature. Samples were then paraffin embedded (dehydration and permeation in molten wax in the histokinette in a 13h overnight cycle; wax blocking of the samples using the console system TissueTek for 2h). The wax molds containing the embedded tissue were cut using the Leica RM2135 rotary Microtome. For goblet cell analysis ileal paraffin sections of 5 µm were stained with Alcian Blue and Periodic Acid Schiff (PAS) and counterstained with Schiff Reagent and Nuclear Fast Red Solution. Sections were mounted in DPX mounting reagent (Sigma) and imaged using the Olympus BX51 microscope (Olympus DP71 camera), with a 20× objective. Image analysis was performed using ImageJ. For histological scoring paraffin-embedded ileal sections (5 µm) were stained with haematoxylin and eosin according to standard procedures. The sections were blindly scored using a light microscope (Olympus BX51, Olympus, Germany). The histology score was adapted from Drolia et al. (Drolia et al., 2018) with some modifications. The ileal samples were scored on a scale of 0-3 for 2 parameters: infiltration of inflammatory cells (mostly mononuclear cells) to the villi and infiltration of mono- and polymorphonuclear cells to the crypts, with a possible maximum score of 6. The gradient of the inflammatory cell infiltration was defined as: 3=highly increased; 2=moderately increased, 1=mildly increased and 0=normal.

### 3.7. Short-chain fatty acid (SCFA) quantification

External standards were prepared from stock solutions of acetate (20 mM), propionate (20 mM), isobutyrate (2 mM), butyrate (20 mM), isovalerate (2 mM) and valerate (20 mM) (Sigma). Stocks were diluted in acidic Milli-Q water (1 in 10 dilution of Milli-Q water and 36.5-38% hydrochloric acid). In addition, an internal stock was prepared consisting of 2-ethylbutyric acid and formic acid. 100 mM stock solutions of 2-ethylbutyric acid and formic acid were diluted to 10 mM in acidic Milli-Q water. All

stocks were stored at -20°C. A 7-point standard curve was generated (SCFA 0.1 mM, 0.5 mM, 1 mM, 2 mM, 4 mM, 8 mM & 10 mM; BCFA 0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM & 1 mM) in acidic Milli-Q water with 1 mM internal standard. Cecum samples were homogenized in 800 µl of acidic MilliQ water, using a vortex mixer for 10min. Homogenised samples were centrifuged at 20000 g for 5min to remove the pellet containing bacteria and other solids. The supernatants were syringe filtered with 0.22 µM filters (Corning) and duplicate aliquots of 270 µl of filtrate were mixed with 30 µl of 10 mM internal standard. The samples were then vortexed briefly, followed by a centrifugation step of 20000 g for 3min. The supernatant was transferred to 250 µl inserts (Agilent) placed in amber glass 2 ml GC vials (Agilent) sealed with silicone/PTFE screw caps (Agilent). Samples and standards were analysed by gas chromatography flame ionisation detection (GC-FID) using a Varian 3800 GC system, fitted with a DB-FFAP column (30 ml x 0.32 mm ID x 0.25 µm df; Agilent) and a flame ionisation detector with a CP-8400 auto-sampler. Helium was used as the carrier gas, at an initial flow rate of 1.3 ml/min. The oven temperature initiated at 50°C, was maintained for 30sec, and raised to 140°C at 10°C/min and held for 30sec, before being increased to 240°C at 20°C/min, and held for 5min (total run time 20min). The temperatures of the injection port and the detector were 300°C and 240°C, respectively. A split-less injection of 0.2 µl was carried out for each sample or standard using a 10 µl syringe (Aglient) installed to a CP-8400 auto-sampler (Varian). Varian Star Chromatography Workstation version 6.0 software was used for peak integration. Vials containing 1800 µl of water were run between each sample duplicates as blanks to check for any potential carryover. Standards (2 mM) were included in each run for calibration checks.

### 3.8. Luciferase reporter system for analysis of *in vivo L. monocytogenes* virulence gene expression

The expression of virulence genes during *L. monocytogenes* murine infection was analysed using a luciferase tagging system developed by our group (pPL2*lux* system) (Bron et al., 2006). Constructs of pPL2*lux* coupled to the promoters of the genes of interest *hly*, *plcA* and *hpt* were used to study their expression in *L. monocytogenes* EGDe *in vivo*. A pPL2*lux*$P_{help}$ plasmid with constitutive expression of luminescence was utilized as a positive control. Seven-week-old female C57BL/6 mice (ENVIGO, UK), n=30 were housed in a controlled environment with access to chow (Teklad Global 2018S Rodent Diet, ENVIGO, UK) and water *ad libitum*. The mice were then either kept on the normal regimen (n=5 x 3) or given water supplemented with 1.3% L-carnitine (Sigma-Aldrich) for 13 days (n=5 x 3). Thereafter, all animals were orally inoculated by intra-gastric gavage (IG) with *L. monocytogenes*. Overnight cultures of *L. monocytogenes* EGDe::pPL2*lux*-$P_{hly}$, *L. monocytogenes* EGDe::pPL2*lux*-$P_{plcA}$, *L. monocytogenes* EGDe::pPL2*lux*-$P_{hpt}$, *L. monocytogenes* EGDe::pPL2*lux*$P_{help}$ and the *L. monocytogenes* EGDe WT were pelleted by centrifugation (7000 g for 5 min), washed twice with PBS and resuspended in PBS. Inoculum was administrated as 200 µl containing 5 x $10^9$ CFU. Immediately after infection and 4h post infection the animals were anesthetized with isoflurane and imaged using a Xenogen IVIS 100 system. Eight hours after infection, the mice were sacrificed by cervical dislocation, and animals as well as individual organs were examined for bioluminescence using a Xenogen IVIS 100 system. Cecum, liver and spleen were then homogenized in PBS, serial diluted and CFU were determined by plaiting onto BHI-agar plates.

### 3.9. Statistical analysis

Statistical analyses conducted with Prism 5 (Graph-Pad Software). Mann-Whitney nonparametric test was used to compare the means of 2 groups. One-way ANOVA with a Dunnett's Multiple Comparison Test was used for pair-wise comparison of means from more than 2 groups in relation to the control, or with Tukey's post-hoc test for comparison of means relative to the mean of a control group.

For p-value above 0.05, the mean differences were considered statistically non-significant (NS). For statistically significant differences: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

# 4. Results and Discussion

## 4.1. Experimental design: Impact of dietary supplementation with L-carnitine in the gastrointestinal environment

Previously we demonstrated that upon increased osmotic stress L-carnitine induced *L. monocytogenes* invasion of the human intestinal cell line C2BBe1 *in vitro* (**Chapter III**). This result was associated with an increased relative expression of virulence genes, induced by the presence of L-carnitine, in response to osmotic stress in minimal media. Moreover, we demonstrated that *opuCC* is essential for optimal bacterial growth under osmotic stress and epithelial cell invasion by *L. monocytogenes.*

To further analyse if these results would translate to our murine model of infection, we established a protocol designed to understand if a short-term increase in dietary L-carnitine would affect host susceptibility to infection with *L. monocytogenes*. Moreover, we analysed the role of the *opuCC* gene in *L. monocytogenes* survival in the intestine and the influence of this system upon the capacity to establish infection (**Fig. 1**).

Here we analysed the impact of a two-week dietary supplementation of L-carnitine to C57BL/6J female mice by administration through drinking water (1.3%). At day 13 (D13), animals were orally infected with either the *L. monocytogenes* EGDe $InlA^m$ wild-type strain (WT) or the *opuCC* deletion mutant *L. monocytogenes* EGDe $InlA^m\Delta opuCC$ (Δ*opuCC*), while continuing the respective control or L-carnitine supplemented dietary regimen. The L-carnitine concentration used has been previously reported to affect intestinal microbiota metabolism (albeit with a longer duration of dietary supplementation) (Almeida et al., 2016; Koeth et al., 2013).

Moreover, it is representative of the current L-carnitine uptake present in the Western-diet (Shils and Shike, 2006).

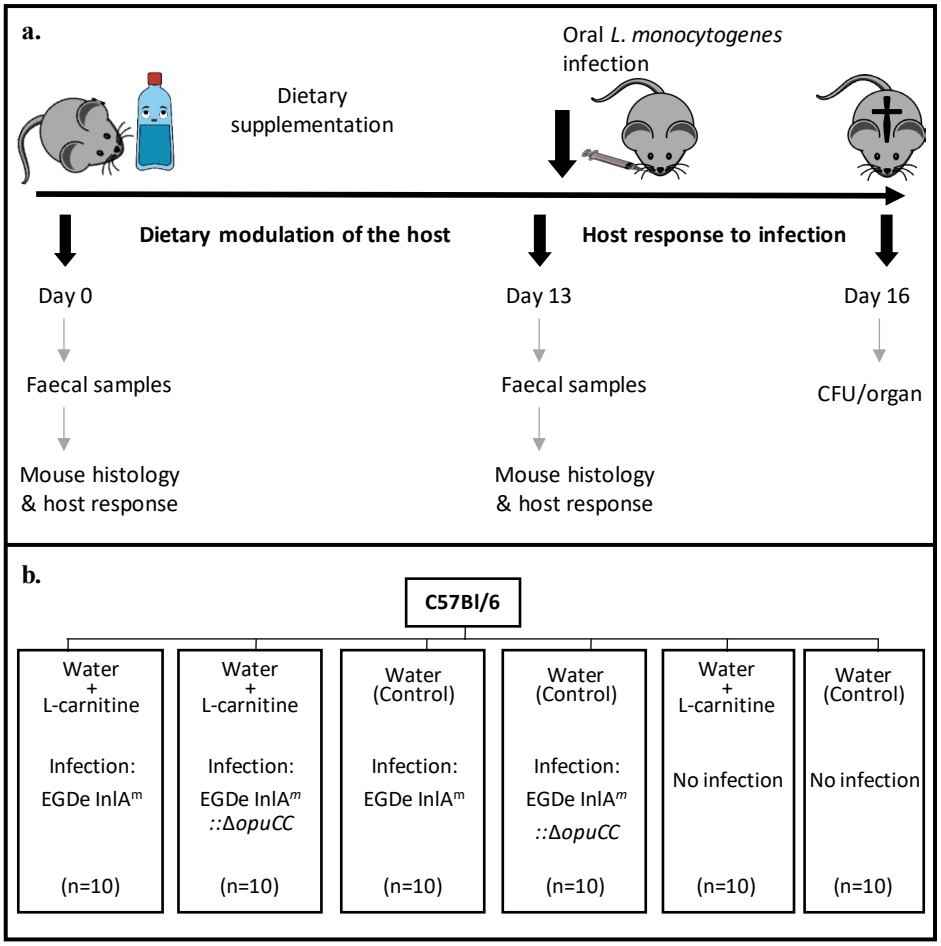



**Figure 1.** Study overview: Impact of L-carnitine dietary supplementation on *L. monocytogenes* infection. **a.** C57BL/6 female mice were administrated a dietary supplement of 1.3% L-carnitine through drinking water for 13 days. Animals were then orally infected with 5 x $10^9$ CFU of *L. monocytogenes* for 72h, after which the animals were euthanized and infection progression was determined by quantification of CFU in visceral organs. Bacterial load in faecal samples was analysed over time, to follow *L. monocytogenes* shedding during infection. **b.** Animals were infected with either the WT strain or the Δ*opuCC* strain, while in the presence or absence of the L-carnitine supplement. Uninfected control groups were kept to identify any possible adverse effects of the dietary supplement in regard of changes in animal weight, food intake or signs of stress (data not shown).

### 4.2. Impact of dietary modulation on host physiology prior to infection with *L. monocytogenes*

Diet composition is a critical factor modulating intestinal physiology (Shanahan et al., 2017; Vancamelbeke and Vermeire, 2017). We investigated the host physiological response to a two-week dietary increase of L-carnitine prior to oral infection with *L. monocytogenes*. This represents the immediate environment that *L. monocytogenes* is exposed to once it reaches the intestine.

Even though L-carnitine is an essential metabolite for cellular energetic homeostasis and its uptake occurs mainly in the intestine (Vaz and Wanders, 2002) (Flanagan et al., 2010), relatively little is known about the impact of L-carnitine in intestinal physiology. L-carnitine absorption in the gastrointestinal lumen is mediated by organic cation transporters (OCTN). Mutations in OCTN, causing the impairment of L-carnitine uptake, have been linked with colonic atrophy and inflammation, suggesting a fundamental role of L-carnitine uptake in maintenance of colonic homeostasis (Shekhawat et al., 2007). In the same study, impairment of L-carnitine uptake was associated with decreased lymphocytic and macrophage infiltration in intestinal villi (Shekhawat et al., 2007).

Our blinded histological analysis demonstrates that two-week increased dietary intake of L-carnitine had no impact on villus height or number of goblet cells (**Fig. 2**). We specifically focused upon goblet cell numbers as these cells represent a preferential site for invasion by *L. monocytogenes* (Nikitas et al., 2011) and a previous study by our group demonstrated that a high-fat diet influences goblet cell number (Las Heras et al., 2019). Moreover, the infiltration level of lymphocytes and macrophages into the

villi and base of the crypts is also not significantly affected by the dietary intervention (**Fig. 3**).

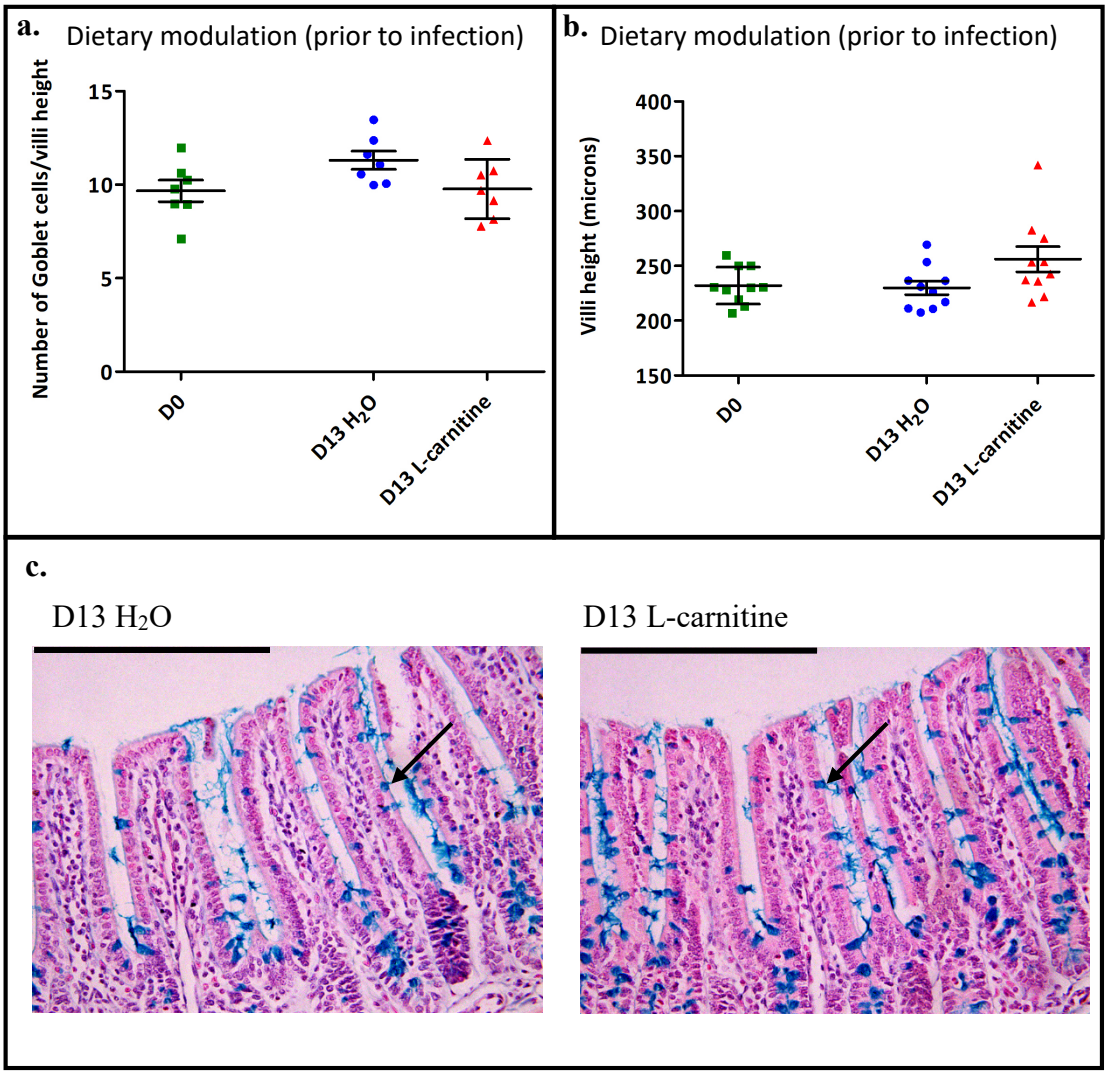



**Figure 2.** Blinded histological analysis of 5 µm ileal sections stained with Alcian Blue and Periodic Acid (PAS) and counterstained with Schiff reagent and Nuclear Fast Red solution. Examples of goblet cells are indicated by arrows (scale bar, 200 µm). **a.** Number of goblet cells per villi length. **b.** Villi height in microns. **c.** Representative histological images. Left image is representative of untreated group (D13 $H_2O$) and right image is representative of L-carnitine supplemented group (D13 L-carnitine). Statistical analysis was conducted using One-way ANOVA and Dunnett's Multiple Comparison Test in relation to D0. Error bar represent ±SD.

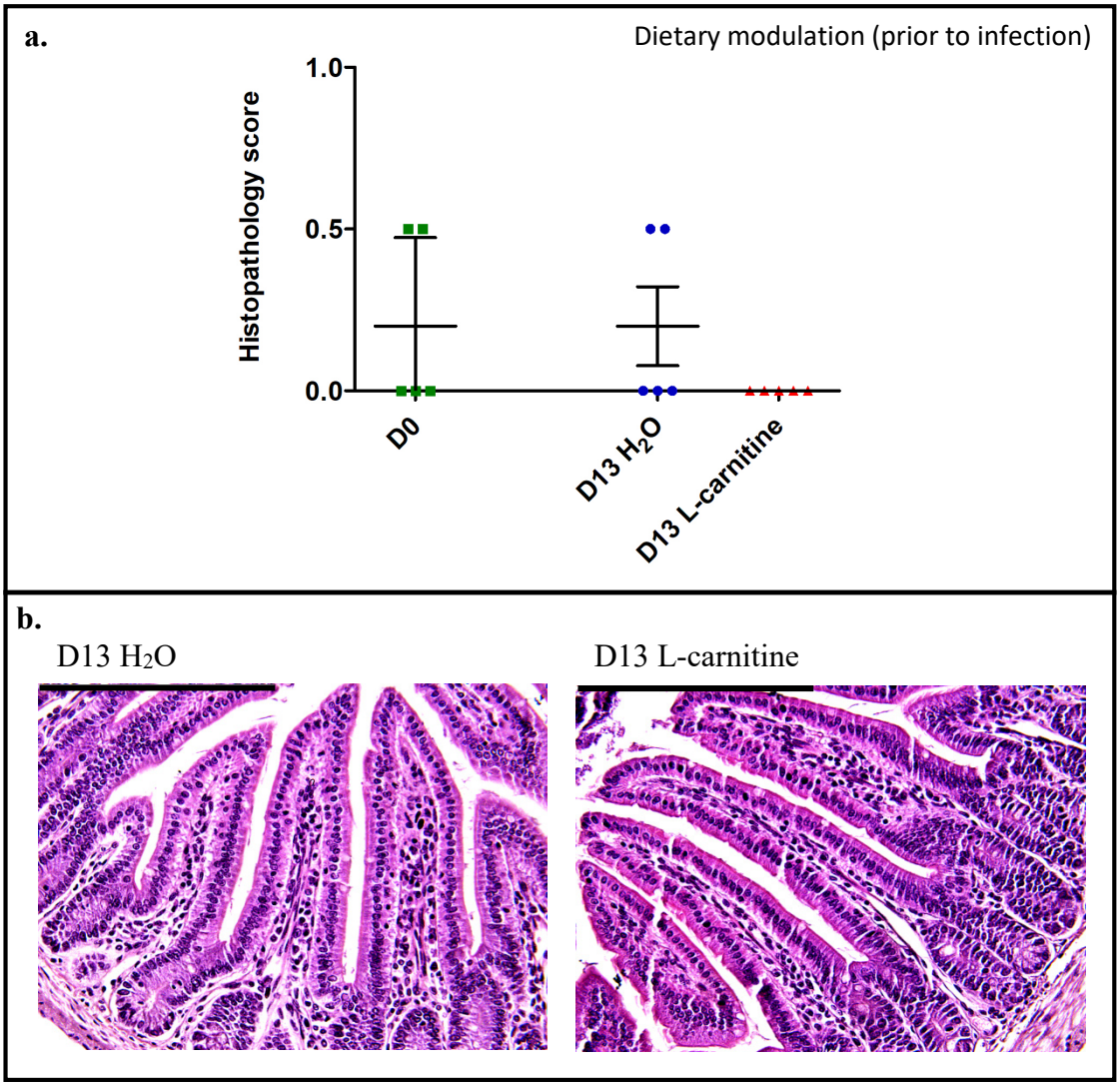



**Figure 3.** Blinded histological analysis of 5 µm ileal sections stained with haematoxylin and eosin. **a.** Histopathology score 13 days post dietary supplementation with L-carnitine. Ileal sections were scored on a scale of 0-3 for 2 parameters: infiltration of inflammatory cells (mono- and polymorphonuclear cells) to the villi and crypts, yielding a maximum score of 6. The gradient of the inflammatory cell infiltration was based on: 3=highly increased; 2=moderately increased, 1=mildly increased and 0=normal (scale bar, 200 µm). **b.** Representative histological images. Left image is representative of untreated group (D13 $H_2O$) and right image is representative of L-carnitine supplemented group (D13 L-carnitine). Statistical analysis was conducted using One-way ANOVA and Dunnett's Multiple Comparison Test in relation to D0. Error bar represent ±SD.

Even though, a previous study has demonstrated that the impairment of L-carnitine uptake causes colonic atrophy (Shekhawat et al., 2007), our results demonstrate that dietary supplementation of this metabolite did not result in changes to the morphology of the murine mucosa.

Regulatory changes in the host occurring in response to dietary intervention were assessed through transcriptional analysis of both ileum and liver. Our analysis determined the host transcriptional profile of a set of genes of interest in response to dietary supplementation with L-carnitine between D0 and D13, prior to infection with *L. monocytogenes* (**Fig. 4**). Our data demonstrated that dietary supplementation with L-carnitine did not affect the expression of genes encoding E-cad or gC1qR, involved in host-pathogen interactions, in either the ileum or liver. Moreover, the expression of genes encoding ZO-1 and Occludin, tight-junction proteins in the ileum, were not significantly changed as a consequence of increased dietary L-carnitine uptake (**Fig. 4**). To our knowledge, the impact of dietary carnitine upon host intestinal physiology has not yet been studied. Overall these results contribute to our understanding of the dietary impacts of L-carnitine on the host, by demonstrating that it did not alter the expression of host genes relevant for intestinal barrier integrity and *L. monocytogenes* pathogenesis *in vivo,* neither does it significantly impact the intestinal cellular landscape.

However, our experiment showed that increased dietary intake of L-carnitine significantly decreased the expression levels of genes encoding the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in both the ileum and the liver (**Fig. 4**). This is in agreement with previous studies which demonstrated that L-carnitine uptake caused a repression of TNF-α, IL-1β and IL-6 production in response to lipopolysaccharide

(LPS)-induced septic shock (Winter et al., 1995). Our data is, thus, consistent with the described concept that L-carnitine can act as an immunosuppressive agent.

Evidence from previous studies indicates that inflammatory processes impair reverse cholesterol transport (Yin et al., 2010). In addition, others have demonstrated that dietary-driven plasma accumulation of TMAO, induced by increased L-carnitine intake, inhibited reverse cholesterol transport in a mouse model (Koeth et al., 2013). To further understand the impact of a short-term dietary increase of L-carnitine in liver metabolism we studied the expression of genes involved in cholesterol metabolism in this organ. The gene encoding the cholesterol 7 alpha-hydroxylase enzyme (*Cyp7a1*), an oxyreductase driving the biosynthesis of bile acid from cholesterol, was down-regulated in the group fed with the dietary supplementation of L-carnitine in relation to the control (**Fig. 4**). The same was seen for *Mrp2* (encoding the ATP-binding cassette, sub-family C, member 2), involved in biliary transport in hepatocytes, and *Ntcp* (encoding for the $Na^+$-taurocholate co-transporting polypeptide) which participates in the hepatic influx. Hence, our data seems to indicate that increased dietary intake of L-carnitine induces a down-regulation of genes that are involved in the bile acid synthetic pathway in the liver, which is a major mechanism of cholesterol elimination form the body. These results are in accordance with previous studies showing that dietary L-carnitine induced accumulation of TMAO resulted in a decrease in total bile acids in small intestine, gallbladder and liver (Koeth et al., 2013).

Bile acids are synthetized from cholesterol and play a critical role in the emulsification and solubilisation of lipids, acting as biological detergents (Hjelm et al., 2000). Bile acids constitute a potent antimicrobial agent, by being part of the physicochemical defence system (Begley et al., 2005; Hofmann and Eckmann, 2006). Bile salt toxicity

has been previously linked with the reduction of bacterial survival and virulence in the gastrointestinal environment (De Boever et al., 2000).

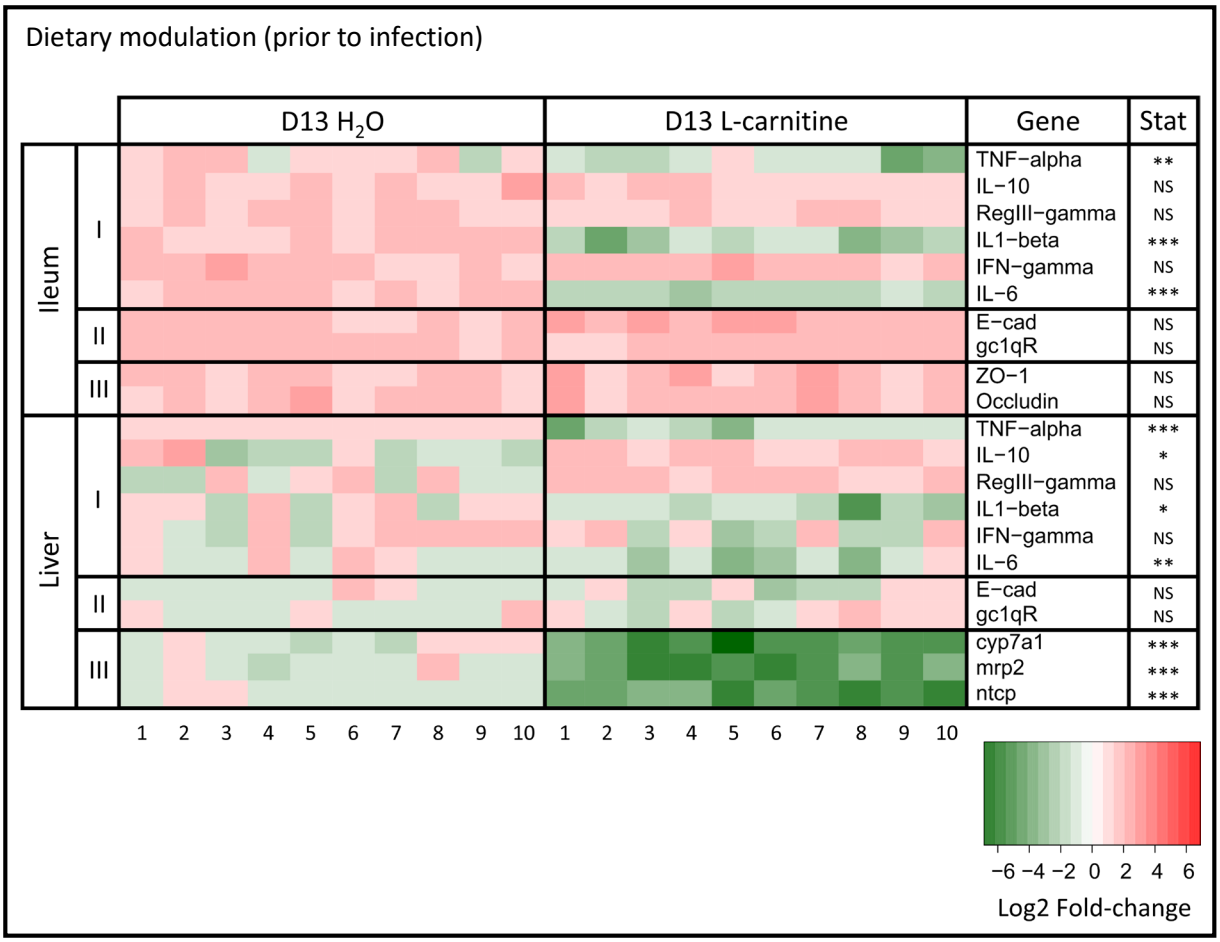



**Figure 4.** Dietary supplementation with L-carnitine has immunosuppressive properties and affects reverse cholesterol transport prior to infection with *L. monocytogenes* (D0 to D13). Impact of dietary challenge in murine gene expression profile by RT-qPCR. Results represented as log2 of the fold-change in response to the dietary intervention (D13 $H_2O$ and D13 L-carnitine in relation to D0). Each individual column represents a single animal. Statistical analysis was conducted using Mann-Whitney nonparametric test. *, P <0.05; **, P <0.01; ***, P <0.001.

Taken together our results indicate a clear impact of increased dietary L-carnitine on the host cytokine production profile in both ileum and liver. Moreover, the dietary challenge affects bile acid synthesis in the liver. The reported changes in the gastrointestinal environment constitute evidence that increased dietary L-carnitine uptake interferes with the host homeostasis at both intestinal and visceral level. Our novel findings also constitute an important contribution to the knowledge of L-carnitine-induced physiological changes in the host.

Given that our results demonstrate that dietary L-carnitine reduces the expression of genes encoding proteins involved in the synthesis and transport of bile acids in the liver, and taking into consideration their antimicrobial properties, it could be interesting to see if this dietary intervention modulates the availability of bile acids in the intestine. Future work should include the quantification of the bile acid profile in the intestine upon increased dietary L-carnitine supplementation, reinforcing a possible correlation between dietary-driven gastrointestinal environment and microbiota composition.

### 4.3. L-carnitine dietary supplementation alters intestinal microbiota composition and metabolism

Microbiota composition and metabolism have an essential role in the regulation of intestinal mucosal homeostasis (Sekirov et al., 2010). Interactions between the host and the intestinal microbiota can modulate a range of physiological events, including host immune homeostasis (McLeod et al., 2019), barrier function (Vancamelbeke and Vermeire, 2017) or colonization resistance against foodborne pathogens (Keith and Pamer, 2019; Libertucci and Young, 2019). Previous studies have demonstrated the link between microbiota metabolism and plasma accumulation of TMAO, providing

evidence that the gut microbiota is essential for the production of TMAO from dietary L-carnitine (Koeth et al., 2013).

Here we examined the role of increased L-carnitine intake upon microbiota composition and metabolism immediately prior to infection with *L. monocytogenes*. Increased dietary intake of L-carnitine did not significantly affect bacterial diversity within samples, also known as Alpha-diversity (**Fig. S3**). However, Beta-diversity metrics, measured by Unweighted UniFrac PCoA analysis of Operational Taxonomical Units (OTUs), demonstrated a significant dietary-driven separation of the microbiota in animals fed the L-carnitine supplement (D13 L-carnitine, in red) from that of the control group (**Fig. 5a,** D13 $H_2O$, in blue).

Additionally, there were no significant time-dependent differences between the microbiota from day 0 and the control group from day 13 (D0, in green, in relation to D13 $H_2O$). In addition, our results show a decreased abundancy of bacteria belonging to the Firmicutes phylum in the L-carnitine fed group, influencing the Firmicutes/Bacteroidetes ratio **(Fig. 5b)** which has previously been shown to influence gastrointestinal barrier function and of a pro-inflammatory response (Scott et al., 2013).

Past studies have demonstrated the fundamental role of the microbiota in the metabolism of quaternary ammines, by demonstrating that microbiota impairment through antibiotic treatment led to a decrease in choline conversion to TMAO (Wang et al., 2011).

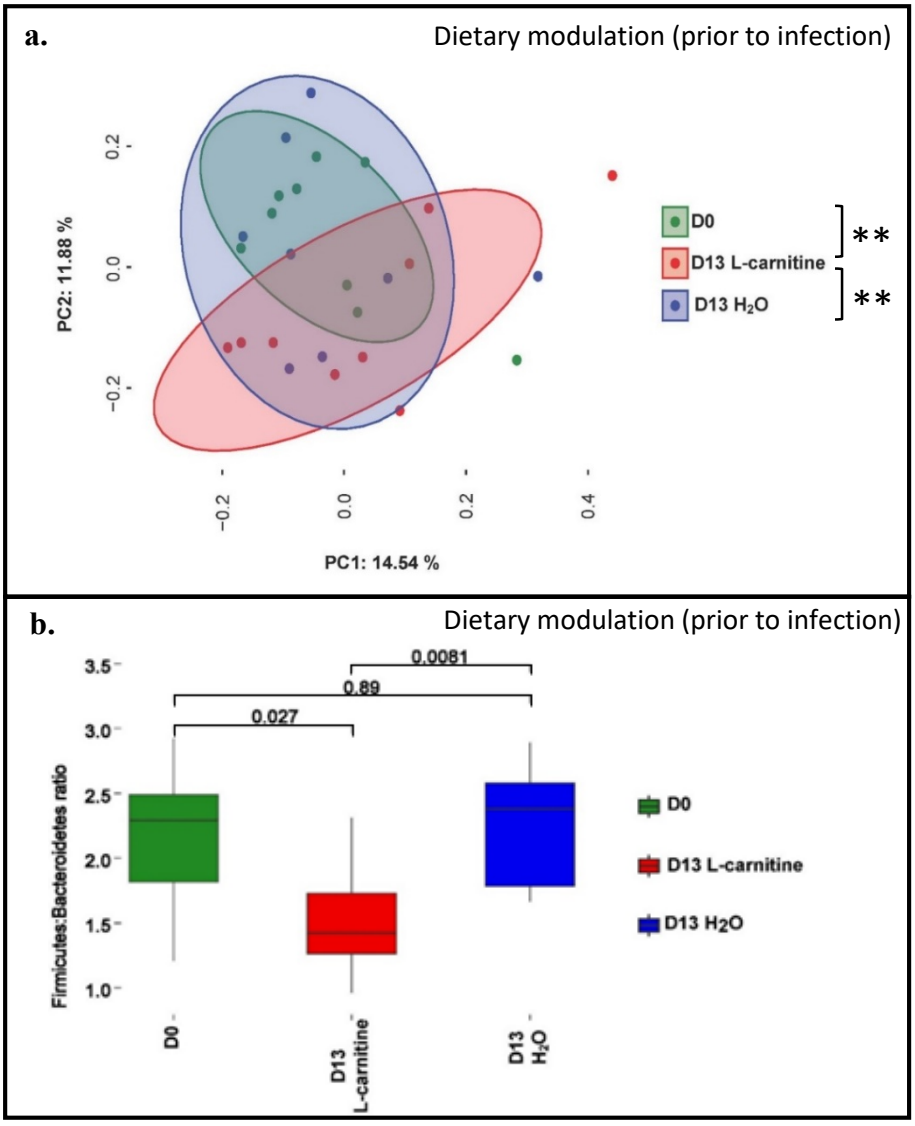



**Figure 5.** Impact of increased L-carnitine dietary intake in microbiota composition. **a.** Beta-diversity analysis of faecal microbiota through Unweighted UniFrac PCoA analysis of OTUS between groups of mice fed different diets. Green represents the control group prior to dietary challenge (D0), red represents the group of mice fed the L-carnitine dietary supplement (D13 L-carnitine) and blue representing the control group, without dietary intervention (D13 $H_2O$), immediately prior to infection. P-values were measured using an Adonis test (P-value=$1e^{-5}$, R squared (proportion of variance explained) is 0.48). **b.** Changes in bacterial abundance upon dietary intervention (D0 in relation to D13) between Bacteroidetes and Firmicutes. **, P <0.01.

Moreover, the same study highlighted that bacteria belonging to the Firmicutes and Proteobacteria phyla have been identified as being involved in choline conversion into TMAO, namely *Anaerococcus hydrogenalis*, *Clostridium hathewayi*, *Clostridium sporogenes*, *Clostridium asparagiforme*, *Escherichia fergusonii*, *Providencia rettgeri*, *Proteus penneri*, and *Edwardsiella tarda* (Wang et al., 2011). However, little is known regarding the underlying metabolic mechanisms behind how dietary L-carnitine may modulate abundancy of specific bacterial taxa in the gastrointestinal tract.

Here we demonstrate that a two-week dietary supplementation with L-carnitine (D13 L-carnitine) affects the relative abundancy of bacteria belonging to the Firmicutes (**Fig. S4**), Bacteroidetes (**Fig. S5**) and Proteobacteria phyla (**Fig. S6**) (**Fig. 6**).

Our results show a dietary driven modulation in abundancy of bacteria belonging to the Firmicutes phylum (**Fig. 6a**). We observed significantly increased abundancy of bacteria in the Lachnospiraceae, unclassified Clostridiales and Ruminococcaceae (*Oscillibacter* genus) families in D13 L-carnitine group in relation to the control group (D13 $H_2O$) (**Table S4**). Moreover, unclassified Clostridiales, Lachnospiraceae family (*Clostridium*_XlVa or unclassified genus) and Erysipelotrichaceae family (*Allobaculum* genus) are more abundant in D13 $H_2O$ in relation to D13 L-carnitine (**Table S5**).

In addition, we demonstrate the increased abundancy of members of the Bacteroidetes phylum (**Fig. 6b**), in particular in the Bacteroidaceae and Porphyromonadaceae families (*Bacteroides* and *Barnesiella* genus) in the L-carnitine fed group (D13 L-carnitine) in relation to the control (D13 $H_2O$) (**Table S6**).

Interestingly, despite the fact that Proteobacteria have been previously associated with the metabolism of quaternary amines in the intestine, more specifically choline (Wang

et al., 2011), Proteobacteria abundancy does not seem to be affected by a two-week increase in L-carnitine dietary intake (**Fig. 6c**). These results seem to indicate that the metabolism of choline and L-carnitine has a different impact on the intestinal microbiota composition, despite a similarity in molecular structure.

Our results demonstrate for the first time that gastrointestinal environmental changes, driven by a short-term increase in L-carnitine dietary intake, significantly modulates the Firmicutes and Bacteroidetes composition of the intestinal microbiota in a murine model. The dietary induced alterations in particular affected Clostridiales abundancy between the two groups (D13 L-carnitine and D13 $H_2O$), suggesting a species-specific response to L-carnitine in the gastrointestinal environment. This has been previously demonstrated in a study in a clinical trial which focused on the impact of one-month dietary supplementation of L-carnitine in patients receiving haemodialysis (Irie et al., 2016). Moreover, we believe that this modulation in abundancy of Clostridiales is driving the decrease in the Firmicutes/Bacteroidetes ratio (**Fig. 5b**). Bacteroidetes respond to increased L-carnitine uptake, suggesting a metabolic preference of these bacteria towards L-carnitine.

These novel data provide an insight into the impact that dietary L-carnitine can potentially have on the microbiota and consequently general health and susceptibility to gut infection.

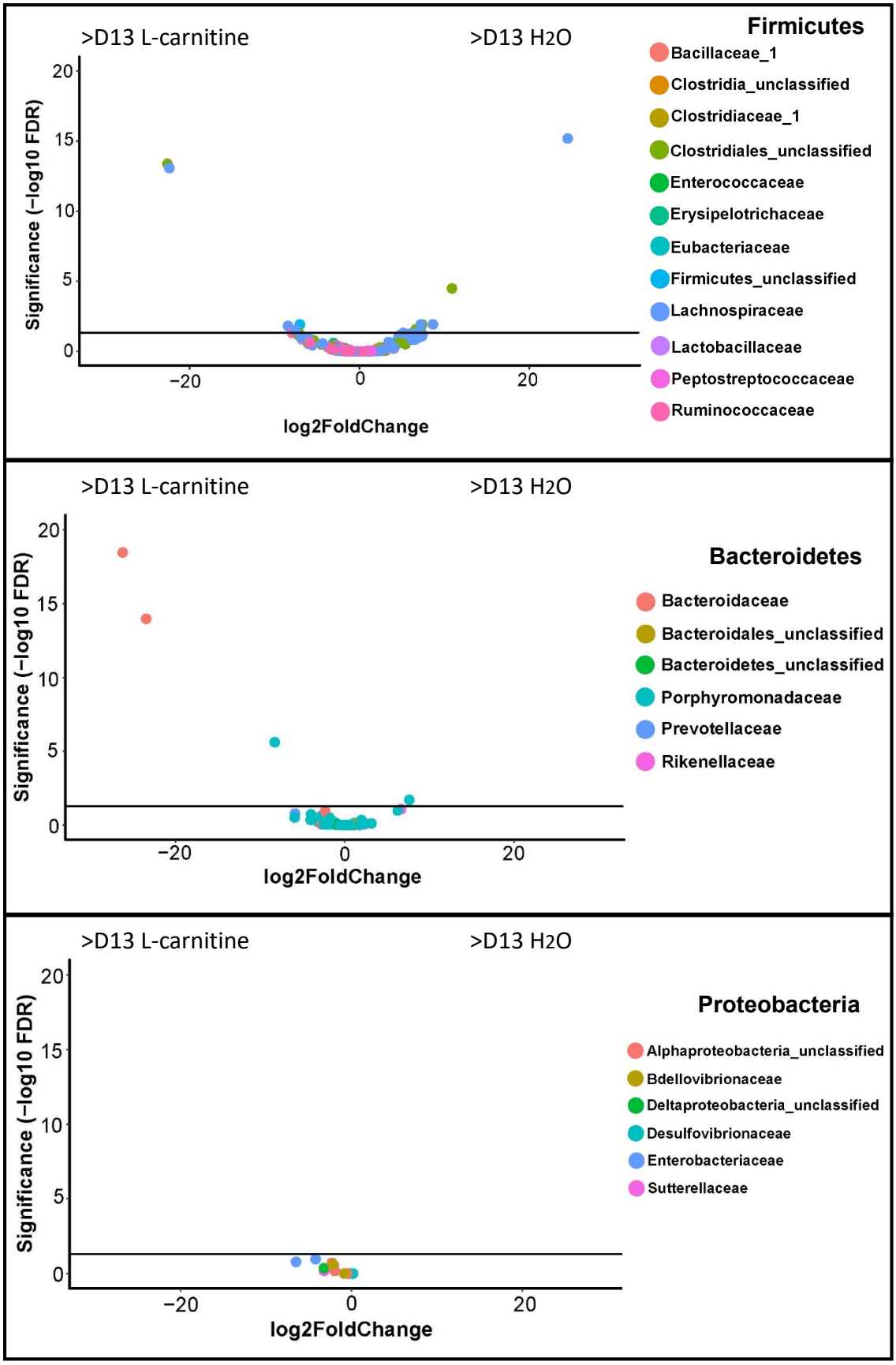

>D13 L-carnitine
>D13 H2O
Significance (−log10 FDR)
log2FoldChange
Firmicutes
Bacillaceae_1
Clostridia_unclassified
Clostridiaceae_1
Clostridiales_unclassified
Enterococcaceae
Erysipelotrichaceae
Eubacteriaceae
Firmicutes_unclassified
Lachnospiraceae
Lactobacillaceae
Peptostreptococcaceae
Ruminococcaceae
Bacteroidetes
Bacteroidaceae
Bacteroidales_unclassified
Bacteroidetes_unclassified
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Proteobacteria
Alphaproteobacteria_unclassified
Bdellovibrionaceae
Deltaproteobacteria_unclassified
Desulfovibrionaceae
Enterobacteriaceae
Sutterellaceae

**Figure 6.** Volcano plots showing fold-change in bacterial abundance at the family level, between the group fed the L-carnitine dietary supplement and the control (D0) in the **a.** Firmicutes, **b.** Bacteroidetes and **c.** Proteobacteria phyla, prior to infection with *L. monocytogenes*. Each point represents an Operational Taxonomic Unit (OTU). The x-axis represents the log2 of the fold-change while the y-axis is the negative log10 of DESeq2 P-values adjusted for multiple testing using the False Discovery Rate method. Points to the right of the plot with positive log2 fold-change values represent bacterial taxa with increased abundance in the mice fed the increased L-carnitine dietary intake (D13 L-carnitine) relative to the control group (D0). The points on the left side of the graph represent families of bacteria with higher abundancy in the control group (D0) in relation to the dietary supplemented group (D13L-carnitine). The horizontal black line represents the cut off for statistical significance, an adjusted P-value of 0.05.

Even though our results demonstrate changes in abundancy of specific bacterial taxa as a consequence of increased dietary intake of L-carnitine, the mechanisms behind metabolic-driven modulation of microbiota composition still remain mostly unknown. However, in this study we considered the changes at the level of short-chain fatty acids (SCFA) production by commensal microbiota, to assess if the dietary intervention studied could modulate bacterial metabolism in the gastrointestinal tract (**Fig. 7**).

SCFA are essential in the link between diet and microbiota metabolism in the intestine. SCFA are synthesized by the intestinal microbiota as by-products of fermentation of food components in the large bowel, existing in straight (here mentioned as SCFA) or branched-chain (mentioned as BSCFA) conformation (Ríos-Covián et al., 2016).

Previous work has demonstrated the role of SCFA in the modulation of intestinal barrier function and immune response (Chambers et al., 2018). The composition of the microbiota determines the availability of SCFA in the intestinal lumen, as they are primarily produced as microbial by-products of fermentation (Davis, 2018).

Here we demonstrate that increased dietary L-carnitine modulates microbiota metabolism and composition towards a decrease in total SCFA, without influencing the production of BCFAs (**Fig. S7**) (**Fig. 7**). Our observations confirm that the concentration of L-carnitine administrated in this study modulates bacterial metabolism in the gastrointestinal tract (**Fig. 7**), without affecting intestinal barrier integrity (**Fig. 4**).

Others have demonstrated the role of L-carnitine dietary supplementation in the metabolism of commensal bacteria in the gastrointestinal tract. These studies focused on the modulation of the microbiota composition by secondary metabolites of L-carnitine microbiota-dependent conversion into TMAO, namely γ-butyro-betaine (γBB) (Claus, 2014; Koeth et al., 2014). In order to understand the impact of dietary L-carnitine on bacterial metabolism, further metabolic analysis of the cecum content is required. future work in our laboratory will focus upon quantification of L-carnitine in the cecum (major site of L-carnitine conversion into both γBB and TMA) (Koeth et al., 2014), followed by the analysis of possible changes in the cecum pH (**Fig. 8**).

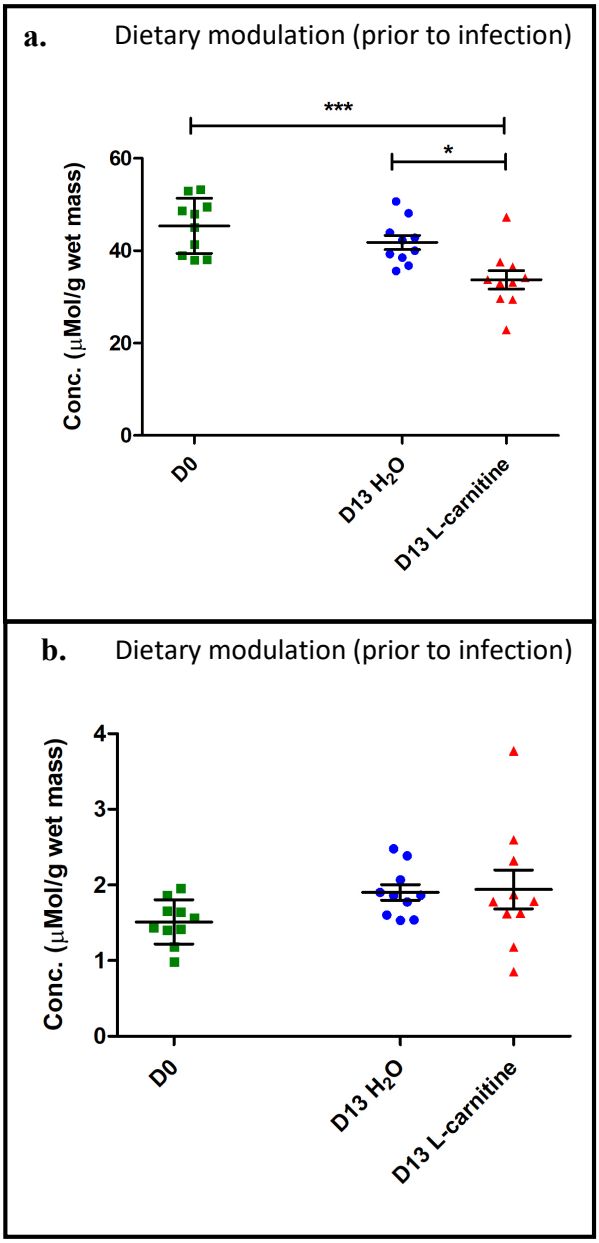



**Figure 7.** Short-chain fatty acid composition of the cecum content of animals in the beginning of the dietary challenge (D0, n=10), control group (D13 $H_2O$, n=10) and fed the L-carnitine dietary supplement for 13 days (D13 L-carnitine, n=10), prior to infection with L. monocytogenes. **a.** Total short-chain fatty acid (SCFA). **b.** Total branched SCFA (BSCFA). Statistical analysis was conducted using One-way ANOVA and Dunnett's Multiple Comparison Test in relation to D0. Error bar represent SD. *, P <0.05; ***, P <0.001.

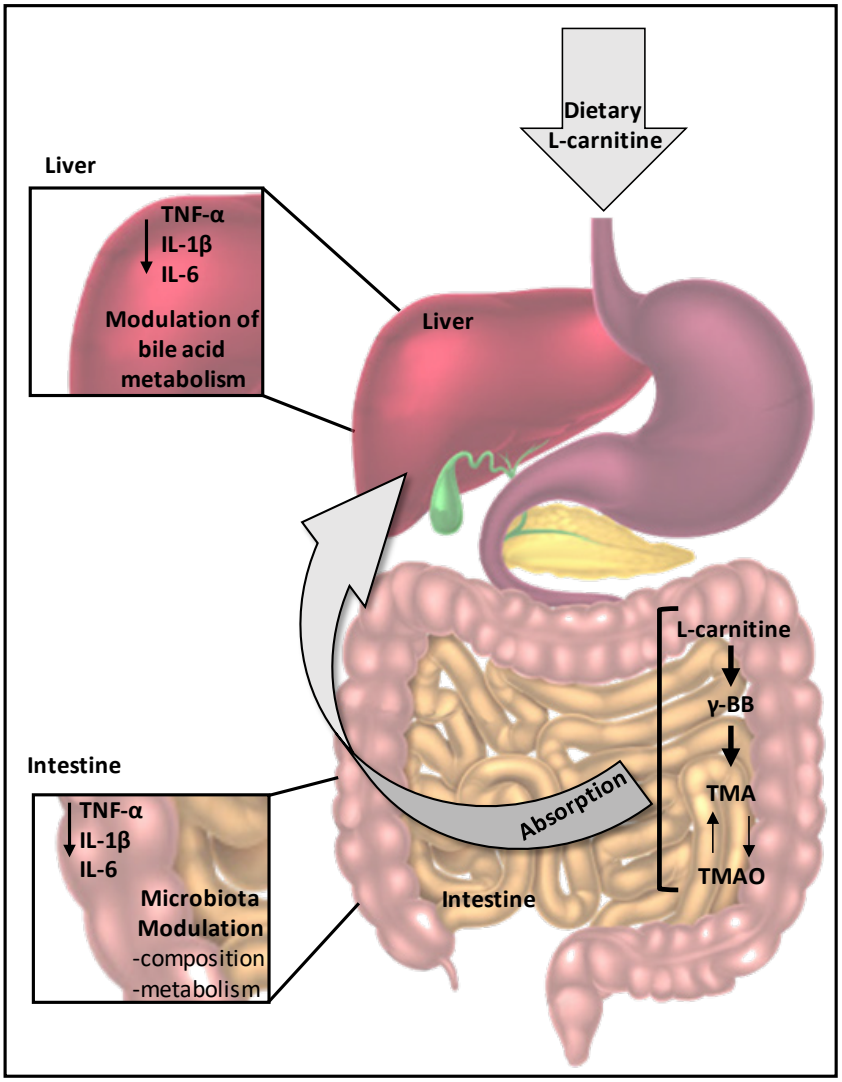



**Figure 8.** The main source of L-carnitine in omnivorous humans is diet (in particular red meat). Dietary L-carnitine is metabolized by the gut microbiota into γ-butyro-betaine (γBB), that is then converted into TMA and TMAO. All these metabolites can be absorbed and further metabolized endogenously in the liver. Our study demonstrates that two-weeks of dietary challenge with L-carnitine induces modulation of the host immune response towards a reduction in the expression of genes encoding cytokines (TNF-α, IL-1β and IL-6) in both ileum and liver, coupled with the modulation of the expression of genes in pathways involved in bile acid metabolism and transport. In addition, we observe a modulation of the intestinal microbiota composition and metabolism, shown by a reduction of SCFA concentration in the cecum. Adapted from (Mitchell, 2018).

### 4.4. L-carnitine uptake by OpuCC has an important role in *L. monocytogenes* gastrointestinal infection

Earlier in this thesis we demonstrated that increased dietary fat intake increases host susceptibility to infection with *L. monocytogenes* (**Chapter II**). The short-term consumption of a high-fat diet induced changes in the host physiological landscape, and resulted in an increase in bacterial translocation through the intestinal epithelial barrier resulting in elevated systemic infection by *L. monocytogenes*.

L-carnitine plays a critical role in intestinal energy homeostasis, by controlling the influx of long-chain fatty acids into the mitochondria. As a result, L-carnitine was previously considered an antioxidant in the gastrointestinal tract (Kumari and Menon, 1988). Indeed, a previous study demonstrated the role of dietary L-carnitine in attenuating injuries associated with necrotizing enterocolitis in the mucosal tissue of mice (Akisu et al., 2002). These results were essentially associated with a reduction of lipid peroxidation, preventing damage caused by oxygen-derived free radicals. Down-regulation of cytokines in response to L-carnitine has also been be associated with modulation of polymorphonuclear leukocyte (PMN) metabolism (Fattorossi et al., 1993). PMNs play a major role as effector cells in acute inflammation, as they are routinely recruited to sites of infection with microbial pathogens. Moreover, an *in vitro* study suggests that L-carnitine can inhibit the proliferation of antigen-presenting cells (APC), including macrophages and dendritic cells, and T-cell activation upon stimulation with lipopolysaccharides (LPS), without inducing cell apoptosis, indicating that L-carnitine uptake exerts an immunosuppressive effect without displaying toxicity (Fortin et al., 2009). Our results are in agreement with these previous studies, indicating a reduction of cytokine expression in both ileum and liver (**Fig. 4**), potentially associated with an immune suppressive effect of L-carnitine.

Here we studied the role of a short-term increase in L-carnitine dietary intake in host susceptibility to infection with *L. monocytogenes*. Moreover, we evaluated the role of *opuCC* in *L. monocytogenes* virulence in the context of an increased uptake of dietary L-carnitine (**Fig. 9**). Our results demonstrate that there is no effect of the dietary supplementation of L-carnitine in the host susceptibility to oral *L. monocytogenes* EGDe $InlA^m$ infection, as no significant differences in bacterial burden were observed between the control and L-carnitine supplemented mice group for either organs (**Fig. S8**) (**Fig. 9**).Taking into consideration that earlier in this study we demonstrated that this dietary intervention does not influence the expression of genes encoding tight-junction proteins (ZO-1 and occludin) nor the expression of RegIII-gamma, involved in the immune response against Gram-positive bacteria, such as *L. monocytogenes,* the present results were not entirely surprising (**Fig. 4**). This is further supported by the fact that the expression of genes involved in host-pathogen interaction mechanisms (E-cad and gC1qR) are not affected by the increased L-carnitine dietary intake (**Fig. 4**). The findings suggest that the changes in cytokine expression associated with dietary L-carnitine are not sufficient to alter susceptibility to this foodborne pathogen.

Overall, our results indicate a reduction of cytokine expression in both ileum and liver, altered expression of genes associated with bile acid metabolism in the liver (**Fig. 4**), and dietary induced microbiota dysbiosis at D13 in the group fed the L-carnitine dietary supplement (**Fig. 5a**). Despite these results, the dietary challenge does not potentiate the translocation of *L. monocytogenes* through the epithelial infection to influence systemic infection to either spleen (**Fig. 8a**) and liver (**Fig. 8b**).

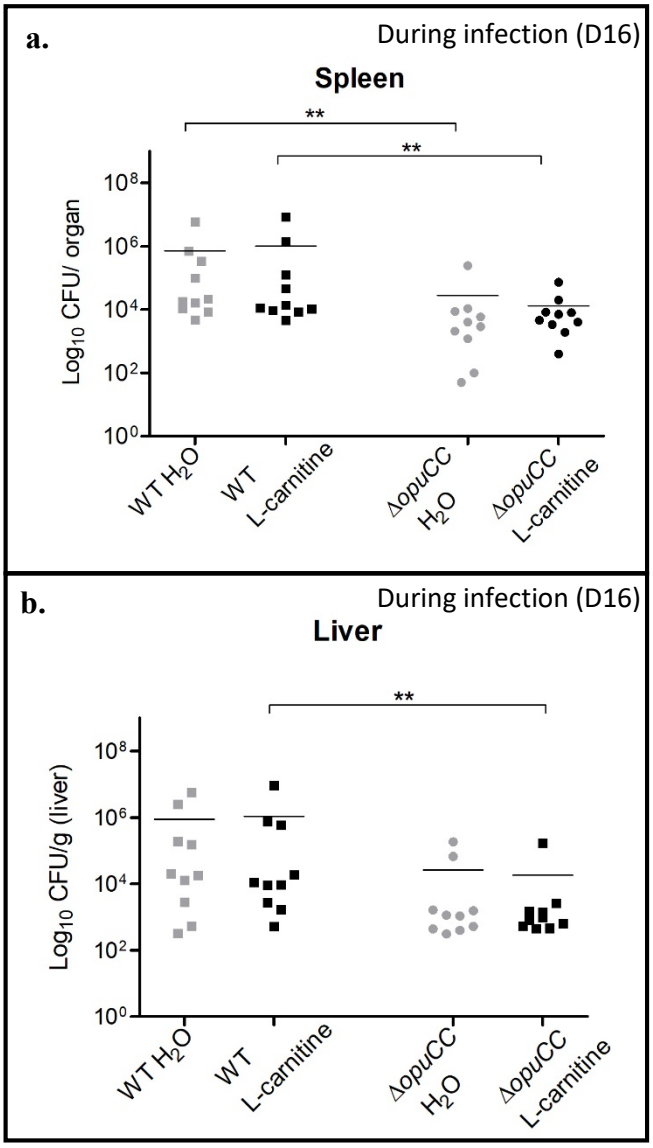



**Figure 9.** Impact of host dietary L-carnitine intake in host susceptibility to oral infection with either wild type *L. monocytogenes* EGDe InlA$^{m}$ strain (WT) or the *L. monocytogenes* EGDe InlA$^{m}$Δ*opuCC* strain (Δ*opuCC*). *Listeria* burden in the **a.** spleen and **b.** liver of C57BL/6 mice administrated a dietary supplement of L-carnitine through drinking water for 13 days' prior infection. Statistical analysis was conducted using Mann-Whitney nonparametric test; **, P <0.01.

Interestingly, the deletion of *opuCC* in *L. monocytogenes* EGDe $InlA^m$ significantly impairs the pathogens ability to cause infection in our murine model (**Fig. 9**). These results are in agreement with previous data that show a defect in virulence of *L. monocytogenes* LO28 *in vivo* in the mouse model, caused by the disruption of the *opuC* operon (Sleator et al., 2001). These results also correlate well with our previous *in vitro* work, where we demonstrate that *opuCC* is involved in the invasion of epithelial cell line C2BBe1, in response to adaptation to increased osmotic pressure (**Chapter III**). Together our findings indicate that L-carnitine uptake through OpuC is involved in the establishment of intestinal infection of *L. monocytogenes in vivo,* though supplementing the diet with L-carnitine does not influence this process.

Our data also suggest that *opuCC* does not have a direct role in survival in the intestinal lumen, given that faecal shedding of the Δ*opuCC* is not significantly different from that of the WT strain without dietary L-carnitine supplementation (WT $H_2O$) throughout infection (**Fig. 10**). Faecal sampling at day 15 revealed a significantly higher shedding of WT *L. monocytogenes* on mice from the $H_2O$ group, when compared to the other 3 conditions (**Fig. 10b**). However, this difference was not observed on day 14 and 16 and that data set had an unusually higher data variance, which could be a result of uneven faecal collection.

Although OpuC has been demonstrated to be involved in *L. monocytogenes* survival and growth under osmotic stress (Fraser et al., 2000), others have also demonstrated that under osmotic stress different osmolyte molecules can induce the uptake of other osmolyte import systems (Verheul et al., 1997). The regulation of Gbu and OpuC in *L. monocytogenes* can be induced by the accumulation of either compatible solutes (glycine betaine or L-carnitine).

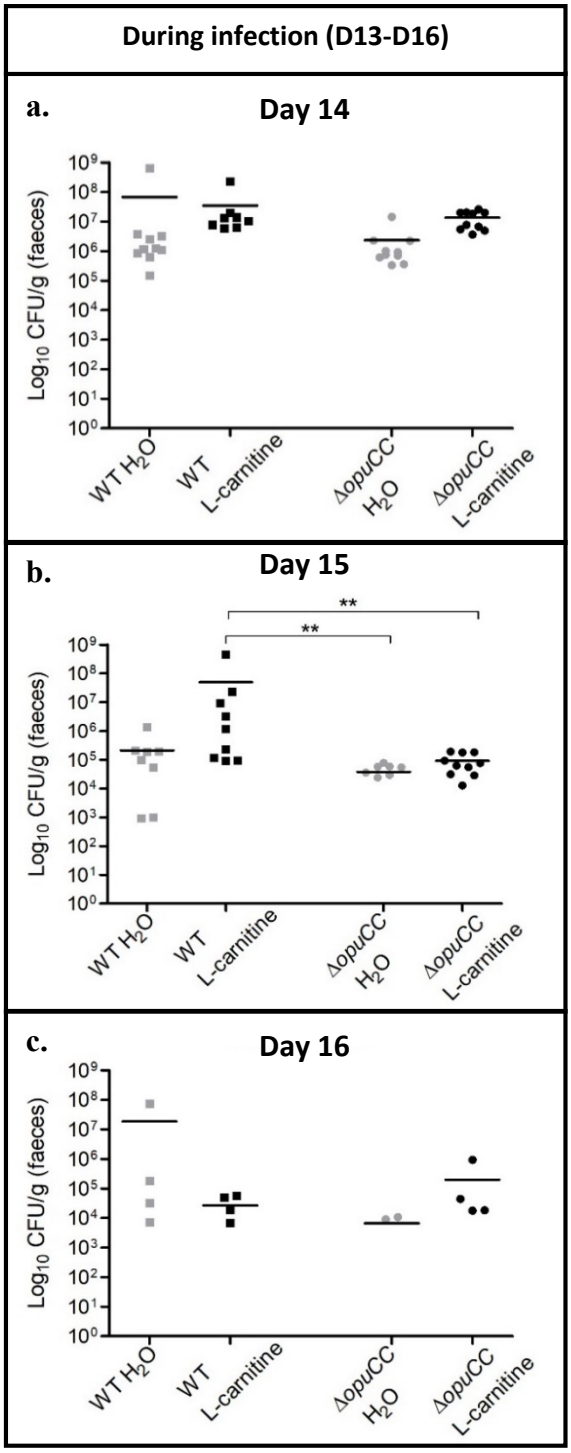



**Figure 10.** Impact of L-carnitine dietary supplementation in faecal bacterial shedding per gram of faecal sample during infection with either wild type *L. monocytogenes* EGDe InlA$^{m}$ strain (WT) or the *L. monocytogenes* EGDe InlA$^{m}$Δ*opuCC* strain (Δ*opuCC*). **a.** Day 14, 24h post infection. **b.** Day 15, 48h post-infection. **c.** Day 16, 72h post-infection. Statistical analysis was conducted using Mann-Whitney nonparametric test; **, P <0.01.

Together with our results these observations seem to indicate that the survival of *L. monocytogenes* Δ*opuCC* in our murine model could potentially be supported by the L-carnitine available in the gastrointestinal tract, through an induction of the Gbu glycine betaine uptake system. We therefore hypothesize that L-carnitine could be involved in the environmental signalling that triggers infection of *L. monocytogenes* in the gastrointestinal tract. Further studies would be necessary to fully unravel the exact mechanisms through which L-carnitine uptake by *L. monocytogenes* in the gut leads to increased pathogenicity. Attempts to monitor *in vivo* gene expression from *L. monocytogenes* virulence genes using the *lux* system were unsuccessful, potentially due to a lack of sensitivity (**Fig. S9**). Future studies could attempt to optimise the *lux* system further for gastrointestinal detection or utilise RT-qPCR to monitor virulence gene expression in *ex vivo* samples.

The gut of mice contains a natural amount of L-carnitine, available either though biosynthesis by the host cells or through dietary intake, that is relevant for the establishment of *L. monocytogenes* infection, as it was demonstrated by an impairment of virulence of a Δ*opuCC* mutant *in vivo* (**Fig. 9b**). It seems, however, that increased L-carnitine availability above such levels does not have a positive correlation with changes in intestine physiology or gut barrier function and consequently does not have an impact on the outcome of *L. monocytogenes* infection in the mouse.

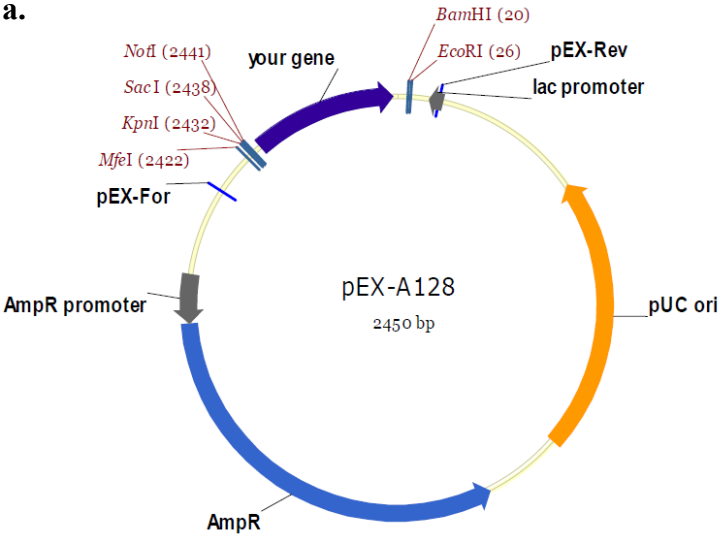
# 5. Conclusions

Considering the global increase in consumption of meat products, associated with the so-called Western-diet, surprisingly little is known regarding the influence of increased L-carnitine intake upon host gastrointestinal homeostasis. In addition, given the role of L-carnitine in *L. monocytogenes* survival and virulence, there is a lack of information regarding how increased intestinal availability of this solute may influence the progression of infection. In the current study, increased dietary L-carnitine in mice did not affect the number of goblet cells, villus height or immune cell infiltration in the ileum. However, our results show that dietary supplementation with L-carnitine alters the intestinal environment by influencing the composition of the microbiota and consequently SCFA levels in the cecum. Given that L-carnitine conversion occurs mainly in the cecum, it is possible to conclude that the L-carnitine concentration administrated in this study is modulating bacterial metabolism. Here we demonstrate for the first time that increased dietary L-carnitine does not induce host susceptibility to oral infection with *L. monocytogenes*. However, L-carnitine uptake through OpuC is involved in the establishment of infection. Given that numbers of both the OpuC mutant and the wild-type remain similar in the faeces we hypothesize that the OpuC system does not play a role in stress survival in this environment. Rather L-carnitine may act as a signalling molecule to induce epithelial cell invasion in the gastrointestinal tract (**Chapter III**). Future studies will analyse virulence gene expression *in situ* in this environment in both the wild-type and mutant in order to test this hypothesis. However, the current study provides key insights into the influence of dietary L-carnitine upon host physiology and gut microbial community structure and suggests that these effects do not directly alter susceptibility to *Listeria* infection.

# 6. Supplementary files

**Figure S1**

**a.**




**b.**

***opuCA* 5'-3'**

GGATCCCCAAAATAACCAAAAGGCCCAGAAGCATTTGCTTTTGGGCCTTTTTAAGTGTGTTATA
ATTAAATAAATACTATATTGCAGCTTATTACAATGCTCATTTTCACGATTACAAGTTTAGCATCG
ATGTCACACGTGCCACAAGTACGAAATCATCTTGTTTAGTAACTTCCGTTTGAATGTAACACTGT
CTTTAAGCTGTTTTTTTGTATTTAAAAAGTTTAAATCTATACTAGTTAGGGAAATTAGTTATCGA
TTGCCAATTTTTTGGTAGTGGTAAAGAAAAAATGGAGGTGTGTAGGCGTGTTAGGATAAGCTA
TGGACGCAATTGTTACATTTTTTCAAGAAAACGGCCATAACTTGCTTGTTCAAACATGGCAACA
TTTATTTATCTCCCTATCTGCTGTTATTTTAGGTATTGCAGTTGCGGTACCAACTGGGATTTTACT
CACGCGTTCACCAAAAGTAGCGAACTTTGTTATTGGTGTTGTTAGTGTTTTGCAAACCGTTCCGT
CTCTTGCTATTTTGGCGTTTATTATTCCGTTTCTTGGCGTTGGAACGTTACCAGCGATTATTGCGC
TATTTATTTACGCGCTTTTACCAATTTGTCGAC

***opuCC* 5'-3'**

GGATCCTGACGAATTGGCAACTAATTGTTAATGTCGAAATTCCTAACTCCATCTCCGTTATTAT
GGCTGGTATTCGTTTATCTGCTGTATACGTTATCGCTTGGGCGACGCTTGCTTCTTACATTGGAG
CTGGTGGACTAGGAGACTTTATTTTTAATGGTTTAAACTTATACCGTCCAGACTTAATTCTTGGT
GGGGCAATTCCGGTTACCATTTTAGCCCTTGTAGTAGAATTCGCGCTTGGAAAATTAGAATATC
GCTTAACACCAAAAGCCATCCGTGAAGCTCGGGAAGGGGGAGAATAACATGAAGAACTAAGG
AGGTGCCAAAAGATGGACACATTAAAACAATTAATTGATTATTACCAAACAAATGGAAGTTAT
GTCATGGAAGAGTTCTGGCGGCATTTCTTAATGAGTGCTTACGGAGTTATCTTCGCAGCAATCA
TAGCGATACCGCTTGGAGTATATATTGCAAGAAAAAAACGCTTAGCTGGTTGGGTTATCCAAAT
CGCTAATATCATCCAAACAATTCCGGCACTAGCAATGTTAGCCGTACTTATGCTTATCATGGGC
TTAGGGACGAATACAGTCGTCTTGTCCTTGTTCCTATATTCTTTATTACCAATTCTGTCGAC

**Figure S1. a.** pEX-A128 map. **b.** DNA sequences of *opuCA* and *opuCC* loci synthesized in the pEX-A128 plasmid. Sequences in blue are restriction sites (BamHI), in green the gene start codon, in red the gene stop codon and in purple restriction sites (SalI).

**Figure S2**

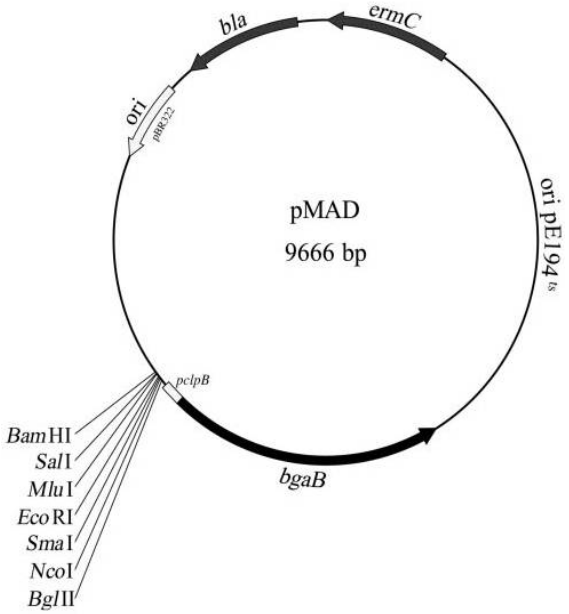



**Figure S2.** pMAD map.

**Figure S3**

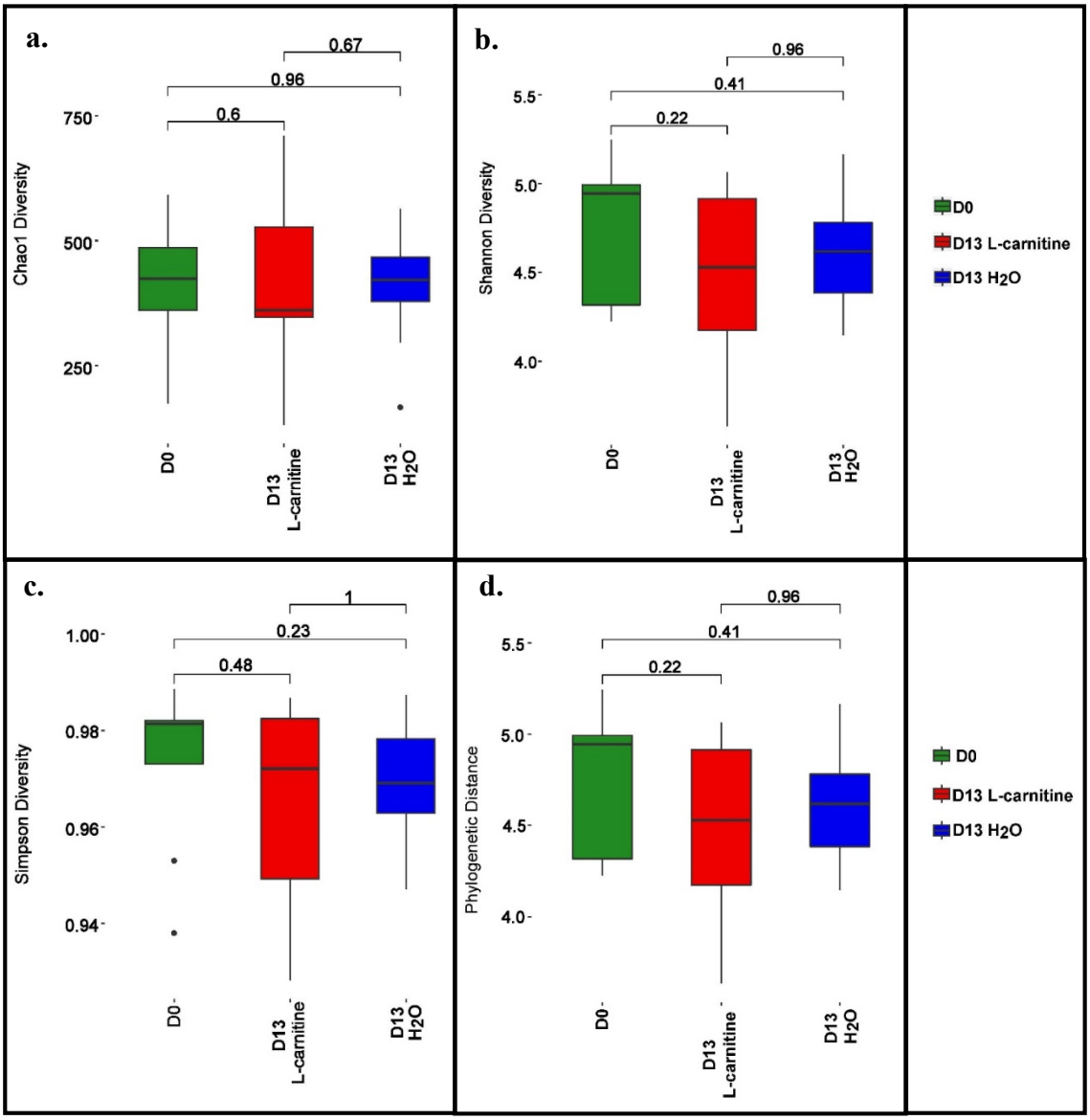



**Figure S3.** Assessment of diversity within the sample (Alpha diversity). Changes in intestinal microbiota communities caused by short-term increased dietary L-carnitine. Control group in D0 (in green), group with dietary L-carnitine supplementation for 13 days (D13 L-carnitine in red) and the control group at D13 (D13 $H_2O$, in blue). **a.** Chao1 as a measurement of sample coverage. **b.** Simpson and **c.** Shannon used as an estimation of richness and evenness respectively. **d.** PD (phylogenetic diversity) used to consider affiliation between taxa within diversity. Statistical analysis conducted using nonparametric Mann-Whitney test (P-values indicated in the figure).

**Figure S4**

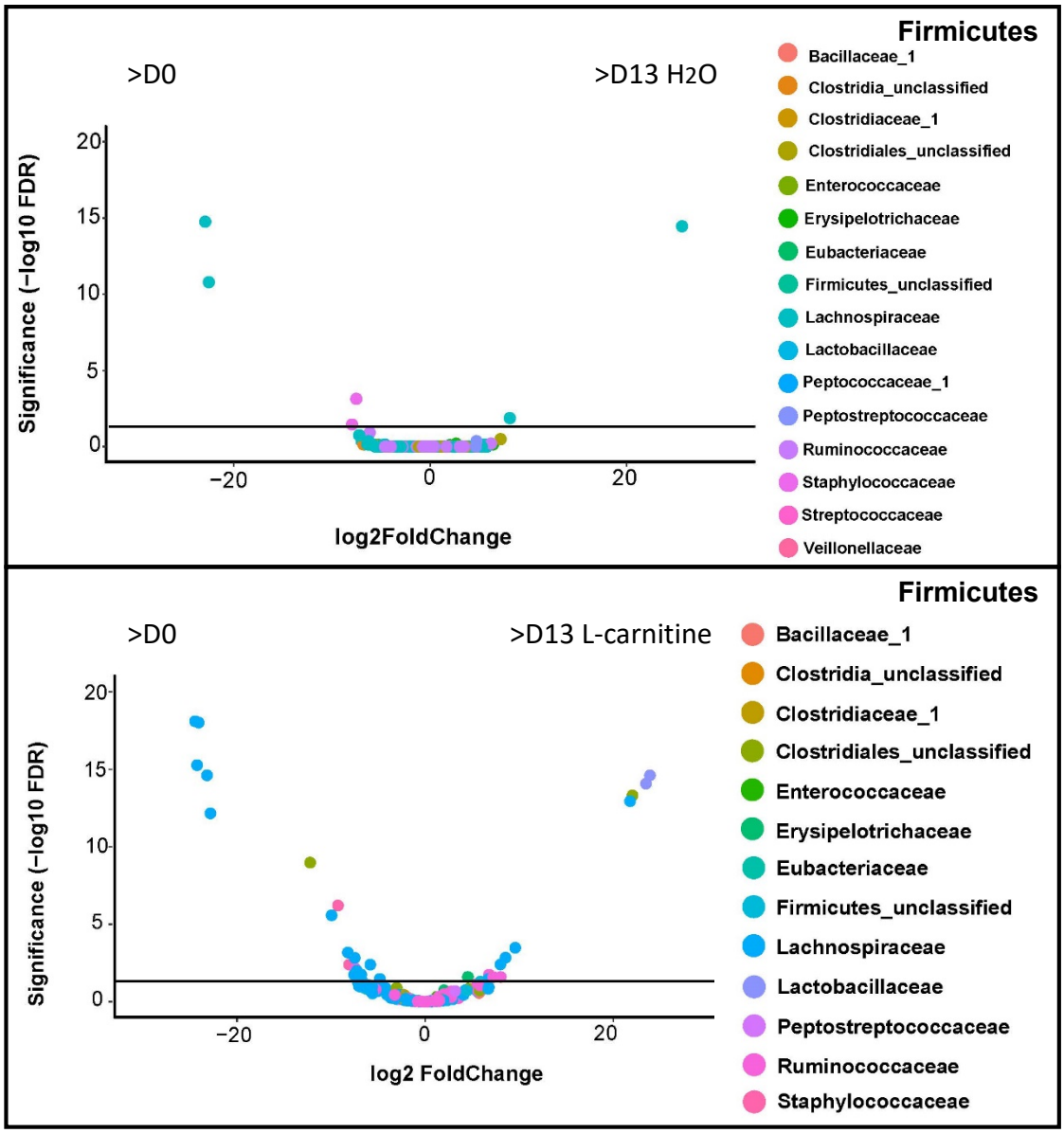



**Figure S4.** Changes in total Firmicutes abundancy in relation to the control (D0) at the family level. Volcano plot showing fold-change caused by the dietary intervention at D13. Each point represents an Operational Taxonomic Unit (OTU). The x-axis represents the log2 of the fold-change while the y-axis is the negative log10 determined by DESeq2. P-values adjusted for multiple testing using the False Discovery Rate method. The horizontal black line represents the cut off for statistical significance, an adjusted P-value of 0.05.

**Figure S5**

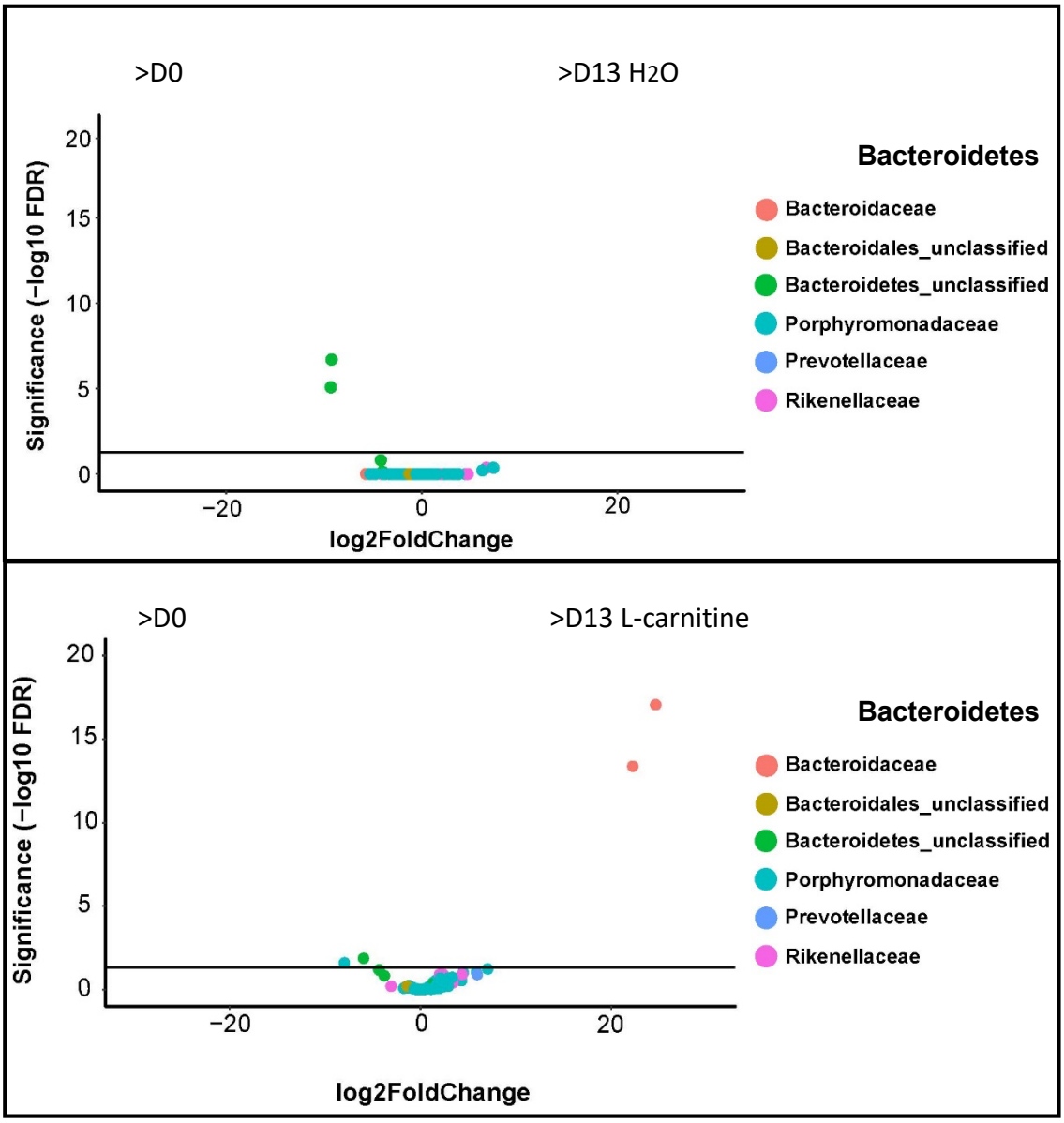



**Figure S5.** Changes in total Bacteroidetes abundancy in relation to the control (D0) at the family level. Volcano plot showing fold-change caused by the dietary intervention at D13. Each point represents an Operational Taxonomic Unit (OTU). The x-axis represents the log2 of the fold-change while the y-axis is the negative log10 determined by DESeq2. P-values adjusted for multiple testing using the False Discovery Rate method. The horizontal black line represents the cut off for statistical significance, an adjusted P-value of 0.05.

**Figure S6**

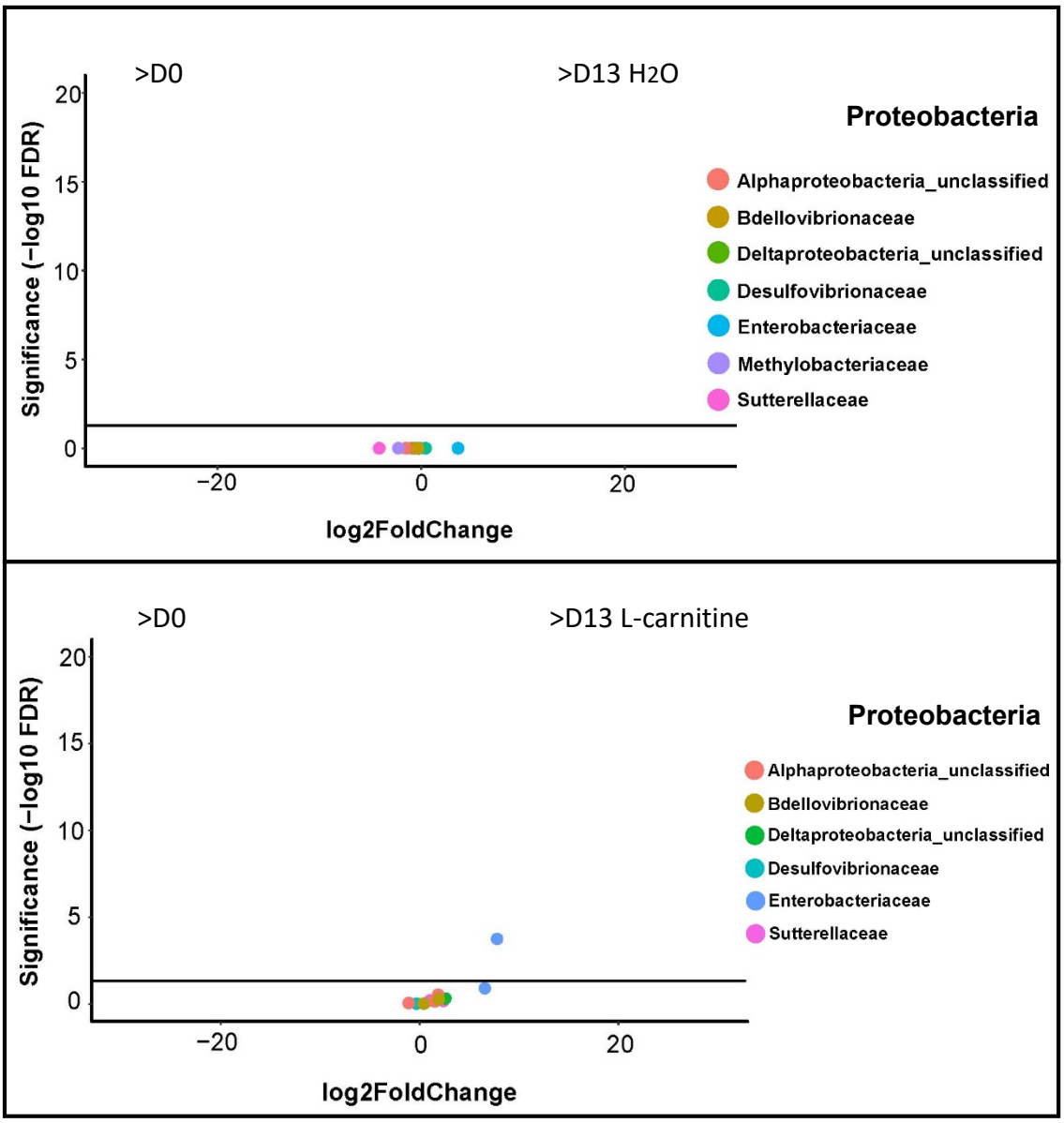



**Figure S6.** Changes in total Proteobacteria abundancy in relation to the control (D0) at the family level. Volcano plot showing fold-change caused by the dietary intervention at D13. Each point represents an Operational Taxonomic Unit (OTU). The x-axis represents the log2 of the fold-change while the y-axis is the negative log10 determined by DESeq2. P-values adjusted for multiple testing using the False Discovery Rate method. The horizontal black line represents the cut off for statistical significance, an adjusted P-value of 0.05.

**Figure S7**

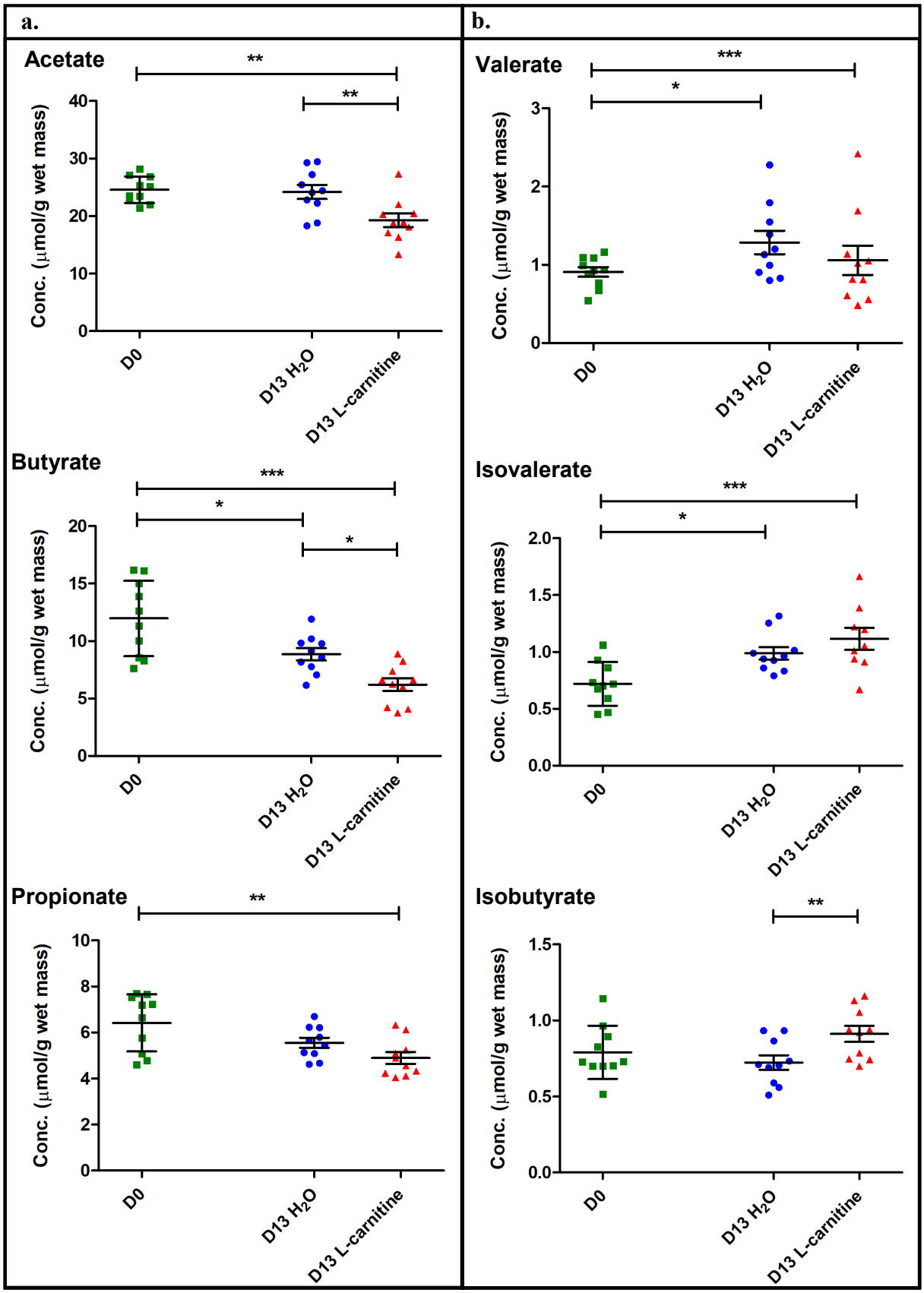

a.
b.
Acetate
Valerate
Butyrate
Isovalerate
Propionate
Isobutyrate
Conc. (µmol/g wet mass)
D0
D13 H2O
D13 L-carnitine

**Figure S7.** Composition of **a.** individual short-chain fatty acids and **b.** branched-short-chain fatty acids of the cecum content in the beginning of the dietary challenge (D0, n=10), control group (D13 $H_2O$, n=10) and fed the L-carnitine dietary supplement for 13 days (D13 L-carnitine, n=10), prior to infection with *L. monocytogenes*. Statistical analysis was conducted using One-way ANOVA and Dunnett's Multiple Comparison Test in relation to D0. Error bar represent ±SD. *, P <0.05; **, P <0.01; ***, P <0.001.

**Figure S8**

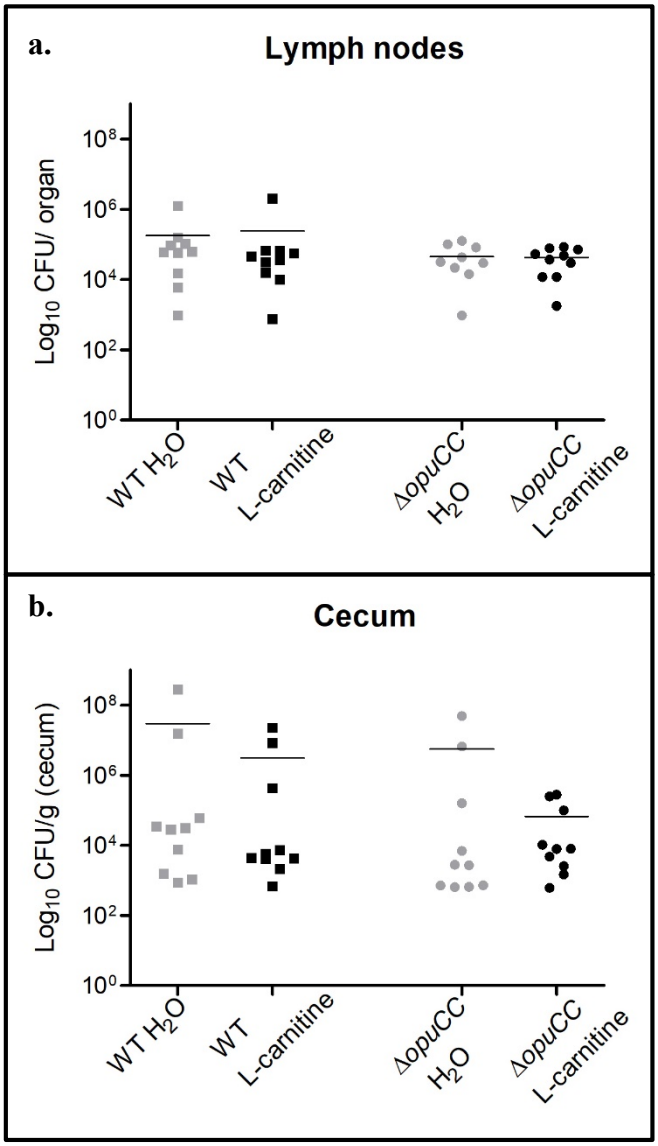



**Figure S8.** Increased dietary L-carnitine does not increase bacterial load in **a.** Lymph nodes and **b.** Cecum of seven-week-old female C57BL/6 mice orally inoculated either with *L. monocytogenes* EGDe InlA$^{m}$ nor *L. monocytogenes* EGDe InlA$^{m}$Δ*opuCC*. Statistical analysis was conducted using Mann Whitney Nonparametric Test.

**Figure S9**

Our previous *in vitro* work showed that L-carnitine induces the expression of virulence genes, including *hly* and *plcA*, in response to increased osmotic stress (**Chapter III**).

Here we attempted to perform a kinetic measurement of *L. monocytogenes* virulence gene expression during murine oral infection, in response to the increased intestinal osmotic pressure and L-carnitine dietary supplementation (**Fig. 11**). To do so, a bioluminescence luciferase-based vector, pPL2*lux*, was used as a tool to identify the expression of *hly*, *plcA* and *hpt*, genes involved in *L. monocytogenes* cell infection cycle (Joseph et al., 2006; Riedel et al., 2009).

In our experiments, it was not possible to successfully use this system for *in vivo* kinetic analysis of *L. monocytogenes* virulence gene expression, since it was only possible to detect light in the animals infected with the EGDe::pPL2*lux*$P_{help}$ immediately after inoculation (time 0 of the experiment) independently of the exposure time used (**Fig. 11**). We speculate that this result could be associated with the detection limit for measurements of light emission in internal organs, including lymph nodes, spleen, liver, stomach, small intestine and cecum. However, at 8h post-infection, no signal could be detected, despite the presence of CFU in the cecum and liver (data not shown).

It is possible to assume that further optimization of the protocol is required to use the *lux* mechanism as a real-time monitoring system for *L. monocytogenes* gene expression during *in vivo* infection. We suggest that it would be quite interesting to establish a correlation between gene expression level and signal detection for a large variety of genes in order to truly challenge and improve the limits and capabilities of these system.

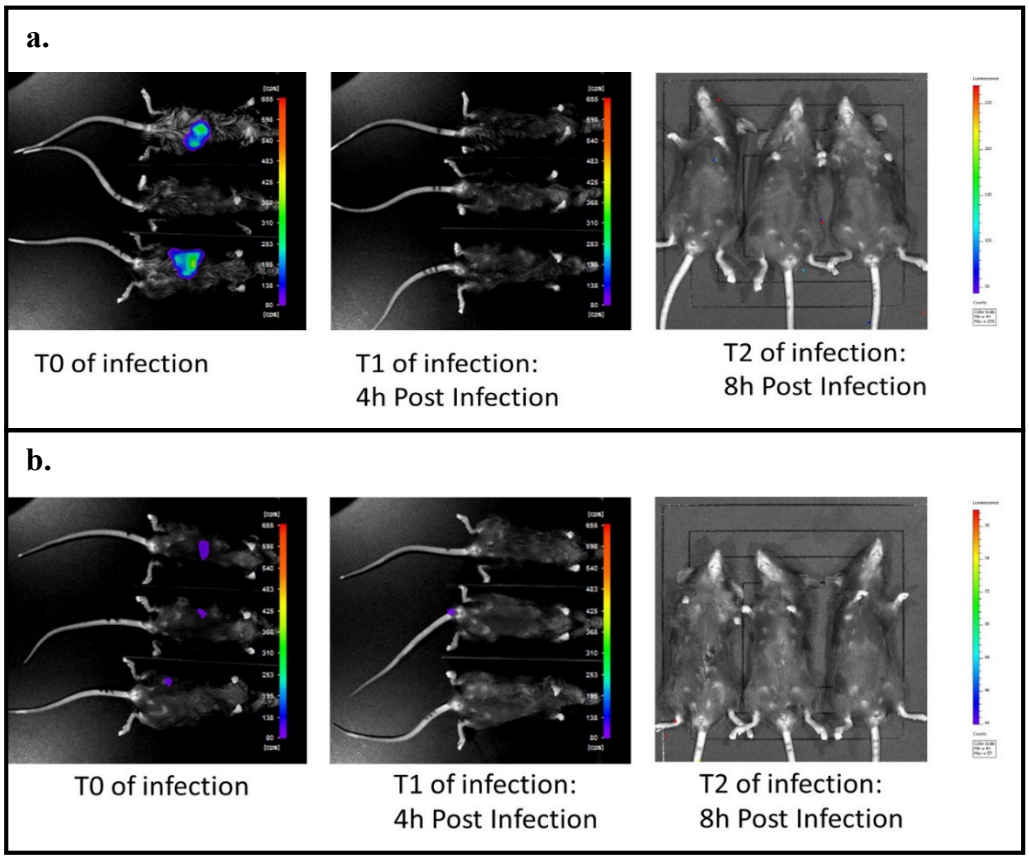



**Figure S9.** Role of L-carnitine dietary supplementation in *L. monocytogenes* virulence gene expression in the gastrointestinal tract. Mice (n=3) were submitted to a dietary supplementation of L-carnitine through the drinking water for 13 days, followed by oral infection with 5 x $10^9$ CFU in a 200 µl gavage. **a.** Analysis of organ-specific colonization of C57BL/6J mice by quantification of light emitted by the bioluminescent strain EGDe::pPL2*lux*$_{help}$ in the untreated control group immediately prior to infection (T0), 4h post-infection (T1) and at 8h post-infection (T2). **b.** Results comparison with group administrated the L-carnitine dietary supplementation in the same time points. Animals were euthanized 8h post-infection and bioluminescent imaging was performed for 5min, for both animals and individual organs (data not shown). The colour bar indicates the bioluminescence signal intensity (in photons $s^{-1}$ $cm^{-2}$) (minimum 50 and maximum 650).

**Table S1.** Plasmids and bacterial strains.

| Plasmid/Strain | Description | Source |
|---|---|---|
| *Escherichia coli* | | |
| DH5α | Cloning host strain; $F^-$ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17*($r_k^-$, $m_k^+$) *phoA supE44 thi-1 gyrA96 relA1* $\lambda^-$ | Life Technologies |
| *Listeria monocytogenes* | | |
| EGDe | Wild type; serotype 1/2a | (Glaser et al., 2001) |
| EGDe $InlA^m$ | EGDe with mutations S192N and Y369S in InlA. | (Monk et al., 2010) |
| EGDe $InlA^m$ Δ*opuCC* | EGDe $InlA^m$ *opuCC* deletion mutant | This study |
| EGDe::pPL2*lux*$P_{help}$ | EGDe with chromosomal integration of pPL2*lux*$P_{help}$ at the $tRNA^{Arg}$ locus; $Cm^r$ | (Bron et al., 2006) |
| EGDe::pPL2*lux*$P_{hly}$ | EGDe with chromosomal integration of pPL2*lux*$P_{hly}$ at the $tRNA^{Arg}$ locus; $Cm^r$ | (Bron et al., 2006) |
| EGDe::pPL2*lux*$P_{hpt}$ | EGDe with chromosomal integration of pPL2*lux*$P_{hpt}$ at the $tRNA^{Arg}$ locus; $Cm^r$ | (Joyce and Gahan, 2010) |
| EGDe::pPL2*lux*$P_{plcA}$ | EGDe with chromosomal integration of pPL2*lux*$P_{plcA}$ at the $tRNA^{Arg}$ locus; $Cm^r$ | (Joyce and Gahan, 2010) |
| Plasmids | | |
| pEX-A128 | Standard cubcloning vector; $Amp^r$ | Eurofins Genomics |
| pEX-A128(*opuCC*) | pEX-A128 with 5'- and 3'-flanking regions of *opuCC* locus; $Amp^r$ | This study |
| pMAD | Gram-negative/Gram-positive shuttle vector; thermosensitive replication; $Amp^r$ $Ery^r$ | (Arnaud et al., 2004) |
| pMAD(*opuCC*) | pMAD with 5'- and 3'-flanking regions of *opuCC* locus; $Amp^r$ $Ery^r$ | This study |

**Table S2.** List of primers.

| **Name** | **Sequence (5' - 3')** |
|---|---|
| *Mutant construction* | |
| *opuCA*-SeqF | TTTCTATCGTGATGAATGGG |
| *opuCA*-SeqR | ATGTAAGAAGCAAGCGTCG |
| *opuCC*-SeqF | ACCGTTCCGTCTCTTGCT |
| *opuCC*-SeqR | ATAACAGATAATGCGAGTGGC |
| pMAD-SeqF | TGATGGTCGTCATCTACCTGCC |
| pMAD-SeqR | CCTACGTAGGATCGATCCGACC |

**Table S3.** Primers for host transcriptome analysis. HKG (Housekeeping gene); I: Immune response; II: Tight-junction proteins; III: Host-cell invasion; IV: Liver metabolism. Designed in the Assay Design Center from Roche *Universal ProbeLibrary* (version 2.52, 2016). Two housekeeping genes calculated using a normalization algorithm ($V_{(n/n+1)}$) with cut-off value of 0.15. Calibrator calculation using pooling system, using average of the control for each gene in each specific organ. Genes with Ct values superior to 40 were not considered reliable and therefore not calculated.

| **Gene** | | | **Forward primer (5'-3')** | **Reverse primer (5'-3')** | **Probe** |
|---|---|---|---|---|---|
| **HKG Ileum** | B-actin | *actb* | aaggccaaccgtgaaaagat | gtggtacgaccagaggcatac | #56 |
| | Rib-S18 | *rps18* | aagcagacatcgacctcacc | ggaaccagtctgggatcttg | #88 |
| **HKG Liver** | Trypsin inhibitor | *itih4* | cccctggacctcctcttg | agcaggatattgtgctgcaa | #21 |
| | Macroglobulin /bikunin | *ambp* | ccactaataactgggaccctca | ggggccttctgagtaattga | #51 |
| **I** | TNF-α | *tnfg* | tcttctcattcctgcttgtgg | ggtctgggccatagaactga | #49 |
| | IL1-β | *il1b* | ttgacggaccccaaaagat | agctggatgctctcatcagg | #38 |
| | INF-ʊ | *infg* | atctggaggaactggcaaaa | ttcaagacttcaaagagtctgaggta | #21 |
| | IL10 | *il10* | cagagccacatgctcctaga | gtccagctggtccttttgttt | #41 |
| | IL6 | *il6* | gctaccaaactggatataatcagga | ccaggtagctatggtactccagaa | #6 |
| | Reg3-ʊ | *reg3g* | accatcaccatcatgtcctg | ggcatctttcttggcaactt | #108 |
| **II** | Ecad | *cdh1* | atcctcgccctgctgatt | accaccgttctcctccgta | #18 |
| | *gC1qR* | *c1qbp* | aggaggagccctcacagg | cacaaagttgggagttgatgtc | #92 |
| **III** | ZO-1 | *tjp1* | gagcctgctaagccagtcc | cagcatcagtttcgggtttt | #21 |
| | Occludin | *ocln* | ggtctctacgtggatcaatatttgta | aaccccaggacaatggcta | #79 |
| **IV** | Cyp7a1 | *cyp7a1* | ggagcttatttcaaatgatcagg | ttggccagcactctgtaatg | #110 |
| | Abcc2 | *mrp2* | caaatccaattctctacctatgcac | gcctgcagtgttggatca | #92 |
| | Slc10a1 | *ntcp* | tgaaaatcaaacctcagaaggac | tgggtacctttttccagagc | #66 |

**Table S4.** Classification of OTUs in the Firmicutes phylum with significant increased abundancy in the group administrated the L-carnitine dietary supplementation (D13 L-carnitine) in relation to the control group (D13 $H_2O$).

| Higher abundancy in D13 L-carnitine | | | | | |
|---|---|---|---|---|---|
| **OTU** | **Phylum** | **Class** | **Order** | **Family** | **Genus** |
| Seq 000401 | Firmicutes | Clostridia | Clostridiales | Clostridiales_ unclassified | Clostridiales_ unclassified |
| Seq 000443 | Firmicutes | Clostridia | Clostridiales | Clostridiales_ unclassified | Clostridiales_ unclassified |
| Seq 000455 | Firmicutes | Firmicutes_ unclassified | Firmicutes_ unclassified | Firmicutes_ unclassified | Firmicutes_ unclassified |
| Seq 000487 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Lachnospiraceae_ unclassified |
| Seq 000134 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Lachnospiraceae_ unclassified |
| Seq 000296 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Lachnospiraceae_ unclassified |
| Seq 000292 | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Oscillibacter |

**Table S5.** Classification of OTUs in the Firmicutes phylum with significant increased abundancy in the control group (D13 $H_2O$) in relation to the group administrated the L-carnitine dietary supplementation (D13 L-carnitine).

| Higher abundancy in D13 $H_2O$ | | | | | |
|---|---|---|---|---|---|
| **OTU** | **Phylum** | **Class** | **Order** | **Family** | **Genus** |
| Seq 000446 | Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | Allobaculum |
| Seq 000545 | Firmicutes | Clostridia | Clostridiales | Clostridiales_ unclassified | Clostridiales_ unclassified |
| Seq 000303 | Firmicutes | Clostridia | Clostridiales | Clostridiales_ unclassified | Clostridiales_ unclassified |
| Seq 000018 | Firmicutes | Clostridia | Clostridiales | Clostridiales_ unclassified | Clostridiales_ unclassified |
| Seq 000280 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Clostridium_XlVa |
| Seq 000083 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Lachnospiraceae_ unclassified |
| Seq 000419 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Lachnospiraceae_ unclassified |
| Seq 000080 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Lachnospiraceae_ unclassified |
| Seq 000159 | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Lachnospiraceae_ unclassified |

**Table S6.** Classification of OTUs in the Bacteroidetes phylum with significant increased abundancy in the group administrated the L-carnitine dietary supplementation (D13 L-carnitine) in relation to the control group (D13 $H_2O$).

| Higher abundancy in D13 L-carnitine | | | | | |
|---|---|---|---|---|---|
| **OTU** | **Phylum** | **Class** | **Order** | **Family** | **Genus** |
| Seq 000066 | Bacteroidetes | Bacteroidia | Bacteroidales | Bacteroidaceae | Bacteroides |
| Seq 000183 | Bacteroidetes | Bacteroidia | Bacteroidales | Bacteroidaceae | Bacteroides |
| Seq 000237 | Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Barnesiella |
| Seq 000222 | Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Barnesiella |

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# Thesis Summary

In this thesis, we reported novel advancements that contribute to a better understanding of the role of host nutrition in susceptibility to *Listeria monocytogenes* infection.

In the present work we present a comprehensive review of studies on the role of nutrition in host susceptibility to infection with foodborne pathogens. Currently the world is facing a nutritional transition towards increased consumption of ready-to-eat food products characterized by high levels of fat from animal sources (the so-called Western-diet). There is, however, a surprising gap in our knowledge of how this dietary change affects the progression of infectious disease. Whilst it is clear that individual food components (specific dietary fats and other food constituents) can modulate host physiology and immune function (Statovci et al., 2017) their impact upon resistance to pathogens is less clear. Through modulation of numerous physiological factors in the gastrointestinal landscape, including epithelial physiology, barrier function (González-Mariscal et al., 2014; Lee, 2015) and microbiota composition (Valdes et al., 2018), diet represents a relevant lifestyle factor in determining intestinal health and disease (O'Neill and O'Driscoll, 2015).

Dietary-driven modulation of nutrient availability in the intestinal lumen regulates the metabolism of the resident commensal bacteria (Everard and Cani, 2013). This is of particular concern in the context of infection with foodborne pathogens, such as *L. monocytogenes*. *L. monocytogenes* has the ability to constantly adapt its physiology in response to environmental changes in both saprophytic and gastrointestinal niches (Gahan and Hill, 2014). This adaptation is achieved by the regulation of complex networks of genes, which includes the expression of virulence genes (Cossart, 2011).

Understanding how the combination of bacterial, host and environmental factors contribute to the pathophysiology of *L. monocytogenes in vivo*, is essential in order to better understand the factors contributing to disease incidence and in particular to understand factors contributing to the development of single source outbreaks of listeriosis.

As a result of an increased dietary fat intake, we demonstrated a set of changes in host physiology prior to the onset of obesity, resulting in a significant and reproducible increase in susceptibility to infection with *L. monocytogenes*. We observed that a high-fat (HF) diet influenced physiological parameters involved in the establishment of both intestinal and systemic infection in a murine model. It is our understanding that these alterations in the host response to infection were a result of a diet-induced increase in number of goblet cells and modulation of the microbiota composition and the inflammatory response, associated with an immunosuppressive effect of the HF diet upon infection with *L. monocytogenes*. In particular, we noted a profound down-regulation of the host immune response associated with high-fat diet in mice undergoing a *Listeria* infection that was not seen in mice fed a control matched low-fat (LF) diet. Our novel results are indeed in accordance with previous reports in which dietary modulation is a factor affecting infection with other pathogens such as *Borrelia burgdorferi* (Zlotnikov et al., 2017), *Escherichia coli* (Martinez-Medina et al., 2014) and *Salmonella typhimurium* (Brugiroux et al., 2016). Our work, however, presents an innovative approach to this pathophysiologic effect of diet by incorporating a system biology approach in which we simultaneously analyse multiple parameters involved in the pathogens infection mechanisms.

Since diet plays an important role in modulating parameters involved in the host response to pathogens, we appreciate that it is critical to consider novel factors in the

gastrointestinal landscape in the context of infection. In that sense, our study steps away from the conventional parameters studied in *in vivo L. monocytogenes* infection, such as immune response, and contextualizes the relevance of microbiota composition and metabolism in crucial host response mechanism, including intestinal physiology, barrier function and host pathogen interaction genes. Notably, we report here for the first time that host diet plays a role in the regulation of genes encoding proteins involved in the listerial host-pathogen interaction, which may have an important role in the progression of intestinal infection.

To better unravel the physiological impact of diet it is crucial to understand the role of specific dietary components. The Western-diet is rich in meat products and ready-to-eat meat products that have been linked to numerous recent outbreaks of listeriosis. Red meat is rich in L-carnitine, a compatible solute involved in *L. monocytogenes* adaptation in response to osmotic stress. The gastrointestinal tract constitutes a niche environment where the levels of salt pose a threatening stress to *L. monocytogenes*. Our study constitutes a novel approach to the adaptation and response of *L. monocytogenes* to specific gastrointestinal niche environment and to determine how salt and L-carnitine may be involved in pathogenicity. We show here the first functional evidence that L-carnitine plays a critical role in *L. monocytogenes* fitness and cellular homeostasis in response to osmotic stress, by enabling the recovery of the pathogen from the physiological impact of increased osmotic pressure. We have also unravelled how L-carnitine promotes virulence gene expression during adaptation to the increased extracellular salt concentration and that the uptake of this compatible solute through the ATP-binding cassette transporter OpuC is essential for invasion of epithelial cells once the pathogen is under osmotic stress.

Importantly, we demonstrate that increased dietary L-carnitine intake modulates microbiota composition, with a prominent effect on the abundance of bacteria belonging to the Clostridiales family. Microbial metabolites were also influenced by L-carnitine supplementation, towards a decrease in SCFA availability in the cecum. While this dietary challenge did not increase host susceptibility to oral infection with *L. monocytogenes* we successfully validated that this dietary intervention significantly modulates the composition and metabolism of the intestinal flora, affecting fitness and survival of commensal bacteria. Furthermore, our studies revealed novel data indicating that while L-carnitine uptake through OpuC is involved in the establishment of infection, it is not required for intestinal survival. These discoveries provide new data on the role of dietary L-carnitine in *L. monocytogenes* gut infection but also unravel new insights of the complexity of osmoregulatory systems such as OpuC in the context of gut adaptation and infection. Based on our observations we hypothesize that L-carnitine could act as a signalling molecule in the gastrointestinal tract, being involved in the induction of *L. monocytogenes* virulence mechanisms. Further studies are essential to help decipher the intricate links between dietary compounds and pathogen gene expression *in vivo*. We believe that the luciferase system is a valid approach to the analysis of *L. monocytogenes* virulence gene expression *in vivo*, however we propose that further optimization of the protocol is required and that a correlation between gene expression levels and signal detection could be performed in order to make the system more quantitative.

In conclusion, this work demonstrates that the nutritional status of the host plays a critical role in the development of infection with *L. monocytogenes* and that diet should be considered as a fundamental factor in future host-pathogen interaction studies.

The insights provided in our study also reveal the different levels of relevance of diet in human health (Bortolin et al., 2017; Cordain et al., 2005). While diet is traditionally heavily associated with weight gain and associated metabolic disorders, such as obesity, we demonstrate a potential immunosuppressive effect of diet and consequent impairment of the host response against infection. Given the impact of diet in the modulation of the immune response against *L. monocytogenes*, future studies should include epidemiologic analysis of possible links between metabolic disorders and infection with foodborne pathogens.

Notably, in accordance with previous studies, we show that diet can also have a critical impact on the gut microbiota and helps demonstrating how such impact can affect human health and the outcome of death threatening infections (Brown et al., 2012). We believe this work constitutes new grounds to create a better awareness for the general population to the important need to have healthier lifestyles. Currently there is an increased interest in the mechanisms behind colonization resistance and how this may directly or indirectly determine the fate of gastrointestinal infections (Keith and Pamer, 2019; Libertucci and Young, 2019). Our study establishes a bridge between dietary modulation of microbiota composition, contributing to changes in host susceptibility to infection. Future studies are now necessary to further unravel the impact of different diet compositions in combination with other lifestyle factors, such as exercise, in the human health, microbiota composition and metabolism and susceptibility to infection with other foodborne pathogens.

We believe that the work presented in this thesis constitutes a relevant contribution to the existing knowledge of the influence of diet on host physiology and consequent alteration in susceptibility to infection with *L. monocytogenes*. Our new insights to the link between dietary composition and pathology of listeriosis identify a new

therapeutic opportunity based on dietary modulation, which may represent an economical benefit to the public health sector.

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