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BIOFUNCTIONALITY OF SOURDOUGH METABOLITES IN HEALTHY AND INFLAMED GASTROINTESTINAL TRACT

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1. ABSTRACT

Human health is significantly modulated by diet via a highly multifactorial interaction of diet, gut microbiota and host genetic factors. Alterations of the intestinal microbiota may be induced by dietary patterns and subsequently trigger pathological responses in susceptible individuals leading to inflammatory bowel diseases (IBD) and functional disorders. Metabolomics, a popular high-throughput analytical method, has been used to characterize diet-induced shifts in metabolic profile of host and bacteria and correlate them to disease risk. Sourdough bread, a common fermented cereal product, was selected as a matrix that may contain bioactive compounds produced by lactic acid bacteria (LAB). Effects of sourdough fermentation were investigated in the context of intestinal inflammation as well as by employing metabolic profiling in order to identify bioactive compounds.

IBD-related mouse models $TNF^{\Delta ARE}$ (ileitis model) and IL-$10^{-/-}$ (colitis model) in addition to healthy wild-type mice were fed sourdough bread over 12 and 20 weeks period respectively. Effect of sourdough feeding on inflammation scores and changes in metabolites profiles were analyzed. Furthermore, sourdough metabolites and sourdough LAB were evaluated for their effects in isolated gastrointestinal tissue and in *in vitro* cell cultures.

Feeding of $TNF^{\Delta ARE}$ mice with sourdough bread revealed no effect on the pathology of Crohn's disease-like ileitis. However, the development of chronic inflammation in IL-$10^{-/-}$ mice was significantly enhanced in caecum, but not in the colon. Subsequent metabolic profiling of caecal content and plasma of inflamed IL-$10^{-/-}$ showed no differentiation in metabolites upon sourdough bread feeding as compared to control group and could not be related to observed changes in histopathology. Furthermore, feeding of sourdough bread to wild-type mice likewise did not reveal any significant shifts in metabolite profiles of caecal content and plasma.

Metabolites in sourdough and analog bread also revealed surprisingly low differentiation, what may explain the lack of observed effects in metabolic profile in mice fed sourdough bread. However a notable exception in the sourdough bread are high amounts of common neurotransmitter acetylcholine (Ach), produced by LAB. Sourdough Ach induced motility and mucosal chloride secretion *ex vivo* in isolated tissue. Moreover, sourdough LAB and their metabolites inhibited secretion of pro-inflammatory chemokine IP-10 by intestinal epithelial cells.

In summary, sourdough bread contains bioactive compounds that can affect inflammatory processes in the *in vivo* model of experimental colitis but not ileitis. However, isolated sourdough LABs may have mitigating effects on intestinal disorders via production of intestinal function-stimulating acetylcholine and bacterial factors that inhibit IP-10 secretion, a chemokine relevant for IBD pathogenesis.

2. INTRODUCTION

In the nineteenth century Ludwig Andreas Feuerbach wrote an essay entitled "Der Mensch ist, was er ißt." The phrase is indirectly translated into English as “We are what we eat”. This statement is widely circulating now in the nutrition-related field. Although oversimplified this statement does hold true. It is well accepted that dietary factors significantly influence physiological and pathological processes in the gastro-intestinal tract including immune-related and functional mechanisms, and the extensive scientific research is starting to shed light on the mechanisms of this influence. Dietary components directly interact with the complete cellular network of the gut but also have significant indirect impact via microbiota residing in the intestine. The impact of diet can be both beneficial and harmful and is furthermore complicated by host-derived factors such as genetic susceptibility for certain pathologies. Predisposed individuals may develop inflammation to the commonly harmless food components or commensal bacteria that are well tolerated in others.

In this work we will take a closer look at a common fermented food, sourdough bread, and its impact at the physiological and molecular level in healthy and inflamed gut.

2.1. Fermented foods

Food fermentation has been used for thousands of years for both preservation and organoleptic purposes. Nowadays, fermented products comprise about a third of our daily diet with the most common examples being bread, yoghurt, soy sauce, sauerkraut, cheese, coffee, and kefir. In the early nineteenth century, a biologist and Nobel Prize winner, Ilya Illich Mechnikov, made an observation that consumption of fermented milk products was linked to the increased lifespan of inhabitants in Bulgarian mountains and attributed it to lactic acid bacteria (LAB) *Lactobacillus delbrueckii* sp. *bulgaricus* (Kaufmann, 2008). Since then, numerous microorganisms have been identified and investigated for their effects on the human health.

Fermentation of food with LAB was shown to increase its nutritional value through production of bioactive secondary metabolites, vitamins, and antioxidants. Fermentation also increases bioavailability of minerals, like iron and zinc. The impact of food fermentation, in particular with LAB, is not limited to only improving nutritional value but may impact human health. Fermented foods are an important vehicle for the delivery to the human gut

of live LAB which can have significant health impact, termed as “probiotics” by WHO (Borresen et al., 2012; Di Cagno et al., 2013; Korhonen and Pihlanto, 2003; Leroy and De Vuyst, 2004). Furthermore, prebiotic oligosaccharides and polyphenols, produced during fermentation, may be beneficial to the host via direct effects on host functions or via stimulation of the activity of certain intestinal bacteria (Bosscher et al., 2009; Clarke and Mullin, 2008).

2.2. Sourdough bread - general information

Cereal sourdough fermentation is one of the oldest biotechnological processes and is a common practice in regions where cereals are consumed as staple foods. Rye-flour based sourdough products are popular in the Northern and Central Europe while wheat-flour based sourdough is mainly consumed in the Mediterranean countries. Advantages of sourdough fermentation in bread production have been discussed at length in the context of increased shelf-life, anti-mold and anti-bacterial effects, as well as increased organoleptic properties (Arendt et al., 2007; Brandt, 2007).

Sourdough is a basic mixture of water and flour that is fermented by a heterogeneous microbial community, which includes both bacteria and yeasts. Based on the production process, sourdoughs have been grouped into three different types: type I, type II and type III. Type I sourdoughs are characterized by continuous, daily refreshments with flour of starter culture to keep the microorganisms in an active state. Type II sourdoughs, often used to acidify the dough, are semi-fluid silo preparations with long fermentation periods (from 2 up to 5 days) and fermentation temperature more than 30°C to speed up the process. Type III sourdoughs are dried preparations containing LAB resistant to freeze-drying. Unlike type I, sourdoughs of types II and III require the addition of baker’s yeast (*Saccharomyces cerevisiae*) as a leavening agent (Corsetti and Settanni, 2007).

Type I sourdough exemplifies the traditional type of sourdough, where initial fermentation is spontaneously initiated by endogenous bacteria present in the cereal flour as well as surrounding environment. Sourdough microorganisms mainly consist of acid-tolerant bacteria, belonging to the genera *Lactobacillus*, and yeast species of the genera *Saccharomyces* and *Candida.* The typical ratio of LAB to yeasts in sourdough is 100 to one, however the size of yeast cells is about 20 times that of the bacterial cell resulting in similar metabolic rates in the sourdough starter (Gobbetti, 1998).

During cereal fermentation sourdough microorganisms metabolize flour starch and non-starch polysaccharides, such as β-glycan, polyfructan, xylose and arabinoxylan. Heterofermentative LAB dominate the traditional sourdough, and utilize maltose and sucrose as main carbon source to produce carbon dioxide in addition to lactic and acetic acid responsible for the average dough acidity of pH 5. Homofermentative LAB mainly produce lactic acid through glycolysis via homolactic fermentation. Main metabolites of yeast are in turn carbon dioxide and ethanol (Candela et al., 2010b; Ganzle et al., 2007; Katina et al., 2009; Vernocchi et al., 2004).

Production of acid by LAB during sourdough fermentation reduces pH and consequently activates endogenous flour proteinases that hydrolyze large proteins to smaller peptides. Smaller peptides are then utilized by LAB to support their growth. LAB transport the peptides through the cell wall and hydrolyze the peptides internally, secreting excess free amino acids (Ganzle et al., 2007). During baking free amino acids react with carbohydrates via Maillard reaction and form flavor and odor compounds characteristic of sourdough bread (Silván et al., 2006).

The role of bacteria in sourdough is not limited to organoleptic properties. LAB also act as natural food bio-preservatives through production of organic acids, carbon dioxide, ethanol, hydrogen peroxide and diacetyl as well as anti-bacterial peptides such as bacteriocins (Corsetti et al., 1996; Messens and De, 2002). Furthermore, there are a number of functional compounds produced during sourdough fermentation that may have implications for human health, for example prebiotic exopolysaccharides (EPS), antioxidant peptides, GABA (γ-amino-butyric acid) (Katina et al., 2005; Poutanen et al., 2009).

2.3. Sourdough fermentation and implications for health

Sourdough fermentation enhances nutritional value of cereals by increasing or decreasing bioavailability of as well as production and degradation of certain compounds. However the health impact of sourdough bread is mainly hypothesized based on the *in vitro* studies, and the clinical relevance remains to be investigated.

Cereal grains are important sources of minerals such as iron, calcium, potassium, magnesium and zinc, but also contain myoinositol hexakiphosphate (phytic acid), which is considered to be an anti-nutritional factor for humans and animals. Its anti-nutritional property is due to the ability to chelate dietary minerals preventing their absorption and reducing their bioavailability. Phytic acid additionally complexes with the basic amino acid

groups of proteins, resulting in reduced bioavailability of proteins. Phytases are enzymes that hydrolyze phytic acid, and thus improve the nutritional quality of phytate-rich food and feed. Sourdough fermentation contributes to the reduction of phytic acid by reducing the pH of dough and subsequently activating endogenous phytase activity and by additional production of the enzyme by LAB (De Angelis et al., 2003;Leenhardt et al., 2005). Physiological relevance of phytase degradation during sourdough fermentation was supported by a study where rats fed sourdough bread and showed improved absorption of basic minerals like zinc, iron and magnesium (Lopez et al., 2003).

During cereal fermentation with yeast the amounts of folate, a critical vitamin for reproductive period, more than doubles (Kariluoto et al., 2006). Yeast fermentation also increases the content of riboflavin in bread by an estimated 30% and improves the retention of thiamin during baking process (Kariluoto et al., 2006).

Retardation of starch digestibility during sourdough fermentation with LAB may have an effect on glycemic index. Sourdough bread consumption has been reported to improve glucose metabolism in healthy subjects as well as postprandial glycaemic and insulinaemic responses in subjects with impaired glucose tolerance compared to bread leavened with baker yeast. The observed effect is probably due to high lactic acid levels as well as lower starch gelatinization in sourdough bread (Liljeberg and Bjorck, 1994; Maioli et al., 2008).

EPS are produced in large variety from sucrose by extracellular glycan-producing glucosyltransferases or fructan-producing fructosyltransferases present in sourdough LAB. Examples of sourdough EPS are reuteran, dextran and mutan (grouped as glycans) and levan, inulin and erlose (grouped as fructans) (Bello et al., 2001; Ganzle et al., 2007; Ganzle et al., 2009; Tieking and Gänzle, 2005; Tieking et al., 2003). EPS, such as inulin, is a well-established prebiotic with demonstrated ability to promote the growth of intestinal *Bifidobacteria* in the gut (Meyer and Stasse-Wolthuis, 2009).

Fermentation of common cereals produces GABA a chief inhibitory neurotransmitter of the central nervous system. GABA plays a role in induction of antihypertensive, prevention of diabetes, diuretic and tranquilizer effects (Coda et al., 2010). Additionally, recent publications indicated the production of antioxidant and chemopreventive peptides (lunasin) during sourdough fermentation with selected LAB (Coda et al., 2012; Rizzello et al., 2012).

Sourdough LAB actively hydrolyze proline-rich peptides present in wheat and rye consequently decreasing gluten toxicity to the susceptible population. This finding instigated

production of sourdough bread that contains wheat flour but is still well tolerated by gluten-intolerant individuals (Di Cagno et al., 2004; Di Cagno et al., 2008; Zannini et al., 2012).

In the context of gut health sourdough fermentation may have an influence via production of EPS with prebiotic properties, modification of dietary fiber available for intestinal fermentation and potentially bioactive metabolites from sourdough LAB with direct influence on the host or indirect, via host microbiota (Poutanen et al., 2009).

2.4. Interface function of gastrointestinal tract

The most intense interaction between external environment and human body is taking place in the gut via a thin layer of highly complex and multifaceted mucosa. The functions of the gut mucosa cover food digestion and nutrient absorption, barrier maintenance as well as immune surveillance with a variety of cellular actors. The resident microbiota contributes to most of these functions. A substantial amount of research is taking place to unravel the mechanisms of action related to each of those factors in order to understand how intestinal homeostasis in maintained.

The upper GI tract consists of mouth, pharynx, esophagus and stomach, while lower includes small and large intestine. The small intestine, in turn, is divided into duodenum, jejunum, and ileum, followed by large intestine, colon, and rectum. From the luminal side small and large intestines are lined with mucosa comprising of intestinal epithelial cells (IECs), lamina propria and muscularis (Fig. 1). The intermediate layer of submucosa contains blood vessels, neuronal cells and lymphatic vessels, while the outer layer contains muscles and serosa. The epithelium layer is folded into crypts and villus and functions as a thin highly selective barrier between the host and the luminal content. However the role of epithelial cell layer expands far beyond a simple physical barrier. It includes sensing of intestinal microbiota, sampling intestinal content, secretion of compounds that influence the microbiota as well as modulation of immune responses to luminal content.

The integrity of the IEC layer is critical for intestinal homeostasis (Garrett et al., 2010). Epithelial cells form a connected layer via adherent and tight junction proteins, gap junctions and desmosomes. The transfer of the nutrients and solutes occurs through paracellular space via pores formed by paracellular junction proteins and through transcellular channels in the apical plasma membrane (Anderson and Van Itallie, 2009).

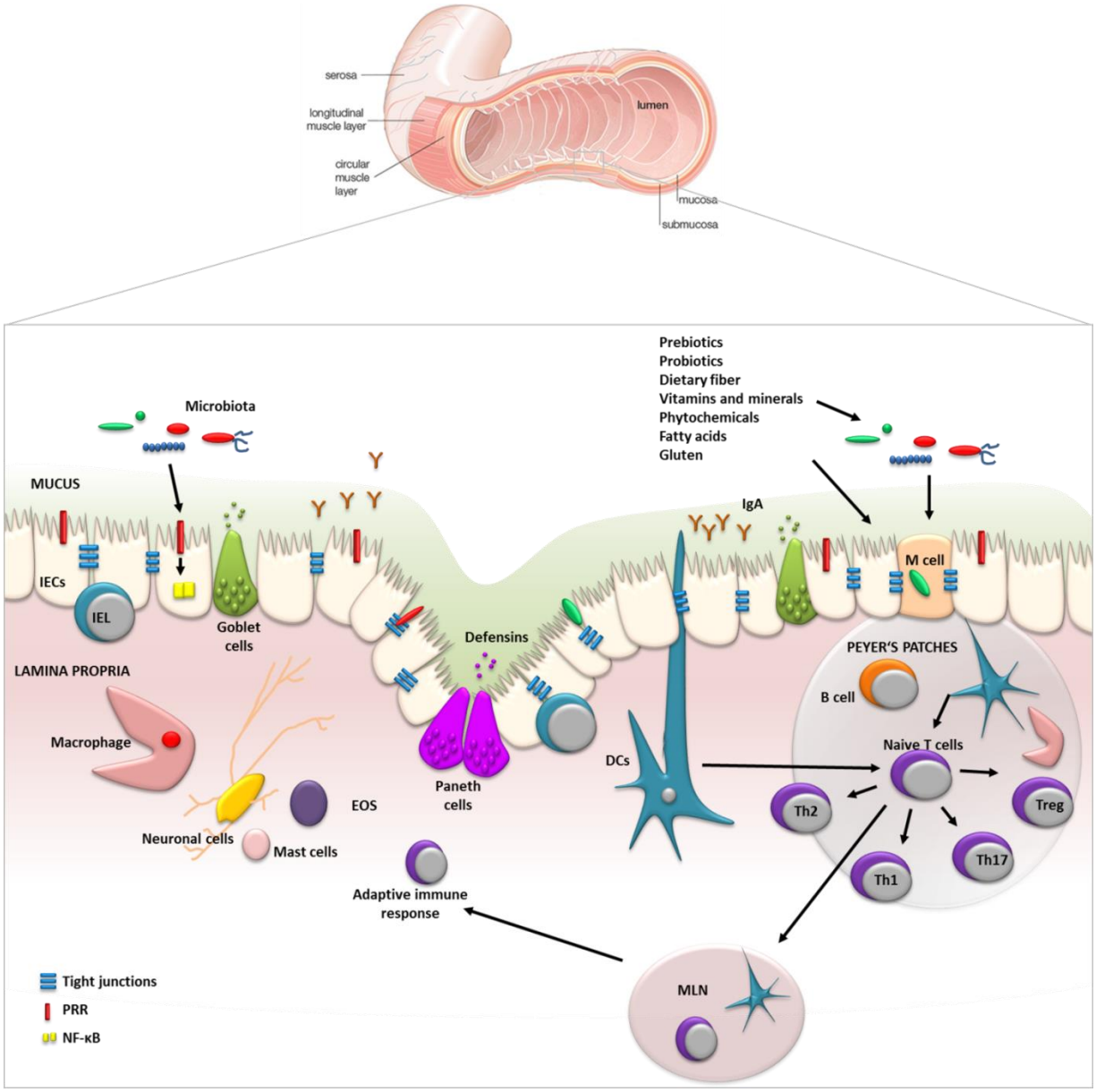


Figure 1. General anatomy of gastrointestinal tract adopted from Encyclopedia Britannica (2003) and the intestinal mucosa section with its major cellular actors in the intestinal immune homeostasis.
Mucosa is composed of structural intestinal epithelial cells (IECs) connected via tight junctions responsible primarily for nutrient absorption but also antigen sensing (via pattern recognition receptors (PRR)) and immune response. Antigen sensing by PRRs triggers downstream activation of cytokine and chemokine production mainly through nuclear factor (NF-κB) activation. Paneth cells secrete antimacrobial peptides and Goblet cells secrete mucins that form a thick mucus layer with a gradient of antimicrobial factors on top of IECs. Immune response are launched by immune cells that are represented in the mucosa by T cells, B cells, DCs, macrophages residing in the IECs-underlying lamina propria. Microbial or dietary antigens cross epithelium through M cells and dendritic cells (DCs). T cells, B cells and DCs are aggregated under M cells into Peyers Patches (PPs). B cells secrete IgA as one of the protective mechanisms against luminal antigens. Antigen presentation primes naïve T cells to either launch or suppress immune responses. Primed T cells will develop into pro-inflammatory T helper cells (Th1, Th2, Th17) and/or protective T regulators cells (T reg) based on surrounding cytokine environment. DCs with antigens also migrate to mesenteric lymph nodes (MLNs) from where mature T cells travel to the lamina propria and start adaptive immune response towards antigens. Other crucial actors of the mucosa are intraepithelial lymphocytes (IEL), mast cells, neuronal cells of enteric nervous system and eosinophils. Luminal nutrients modulate the immune responses via direct action on the cellular actors or via action on the intestinal microbiota.

IECs are subdivided into enterocytes, goblet cells, Paneth cells, enteroendocrine cells that arise from epithelial stem cells. Enterocytes are absorptive cells responsible for the transport of nutrients and constitute about 80% of all epithelial cells. Paneth cells, located at the base of the crypts, produce antimicrobial peptides (i.e. defensins, lysozymes, cathelicidins) that protect against pathogens and regulate commensal microbiota. Goblet cells are scattered throughout the mucosa and secrete gel-forming mucins that form a thick mucus layer to lubricate and protect the epithelia from luminal content actively forming a physical barrier. An extensively glycosylated mucus layer slows down nutrient transport to the absorptive microvillus brush-border and decelerates the diffusion of the secreted compounds (Garrett et al., 2010; Turner, 2009).

The mucosal immune system is represented by GALT (gut associated lymphoid tissue) and includes a wide variety of immune cell types that form the largest immune “organ” in the body (Fig. 1). GALT structures include aggregated Peyer’s patches (PPs), crypt patches (CPs) and isolated lymphoid follicles (ILFs), scattered intraepithelial lymphocytes (IELs), and recently identified innate lymphoid cells (ILCs), and mesenteric lymph nodes (MLNs) (Wershil and Furuta, 2008). ILCs play a role in generation of the lymphoid structures and also produce significant amounts of immune mediators such as IL (interleukin)-22. PPs are specialized lymphoid follicles in the small intestinal wall that contain compartmentalized naïve B cells and T cell population plus macrophages and dendritic cells (DCs). Specialized epithelial M (microfold) cells and DCs sample luminal antigens and present them to the immune cell population of the PPs, thus called antigen presenting cells (APCs). CPs and ILFs are additional lymphoid aggregates with B and T cell populations (Spahn and Kucharzik, 2004). IECs can also be considered a part of GALT as they can directly modulate neighboring immune cells and act as non-professional APCs (Artis, 2008).

Antigen presentation primes naïve T-cells in the PPs that consequently develop into effector T helper cells (Th1, Th2, Th17) and/or regulatory T (Treg) cells. The differentiation of naïve T-cells into specific effector types is influenced to a large degree by the cytokine present in the immediate vicinity (Zygmunt and Veldhoen, 2011). DCs can also travel to distant MLNs and prime local naïve T cells to differentiate into Forkhead box P3 (FOXP3)+ Treg cells that promote oral tolerance and protective immune responses via secretion of tumor growth factor (TGF) and IL-10 (Kamada et al., 2013).

Subsequent to antigen-specific activation B cells in the PPs, similar to T cells, migrate to MLNs and then eventually back home to the lamina propria. Effector immune cells

population in the lamina propria is thus composed of immunoglobulin (Ig) A secreting B cells, plasma cells and effector T cells matured to respond to the luminal antigens (Wershil and Furuta, 2008). Mast cells and eisonophils likewise reside in the lamina propria and perform effector functions during infections, allergic and autoimmune reactions (Atkins and Furuta, 2010).

IELs combine a heterogeneous population of mainly effector T cells with CD8 phenotype that develop independent of antigen presentation and play a role in the maintenance of intestinal homeostasis. IELs are scattered amongst IECs and contribute to barrier function by stimulation of epithelial cell turnover. Moreover IELs mediate host-microorganism interaction by secreting antimicrobial factors, such as REG3γ and lysing agents such as granzymes and perforin (Ismail et al., 2011).

Each type of helper T cells secretes a distinct set of cytokines and chemokines that regulate cytotoxic T cells, B cells differentiation and antibody production. Generally, Th1 cells specialize in defense against intracellular pathogens via production of pro-inflammatory IL-12, interferon (IFN-γ) and tumor necrosis factor (TNF). Th2 cells specialize in defense against helminthes and secrete IL-4, IL-5, IL-13 and IL-10. Th17 cells defend against extracellular pathogens and promote inflammatory and autoimmune response with IL-21, IL-22 and IL-17 (Atkins and Furuta, 2010; Garrett et al., 2010). Secreted cytokines in addition to bacterial antigens activate macrophages that consequently acquire either pro- or anti-inflammatory phenotype. Inflammatory phenotype M1 activated with bacterial lipopolysaccharide (LPS, a membrane component of Gram-negative bacteria) and IFN-γ have high microbicidall activity and increased production of reactive oxygen species (ROS) and pro-inflammatory cytokines. M2 macrophages, activated with Il-4 and IL-13, on the other hand, are associated with tissue repair, production of anti-inflammatory cytokines and reduced antigen presentation (Rodriguez-Prados et al., 2010). All those cell subtypes are present in healthy state and are in dynamic balance with the T regulatory cells assuring adequate response to the foreign antigens from the lumen.

The intestine is populated with over 2000 species of microorganisms, including fungi, archae, viruses and bacteria. The population is dominated with bacteria that count up to 10^{14} cells, tenfold that of the host, and are largely represented by four major phyla *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria* (Ley et al., 2008). The bacterial cell density increases down the GI tract and the majority of bacterial population resides in the colon. Commensal gut microbiota metabolize non-absorbed dietary factors subsequently

producing functional nutrients and supplying energy to the host, as well as protects host from pathogenic bacteria by competitive colonization and modulation of host immune responses. The immune-modulatory role of microbiota is evident from studies involving germ-free mice models that have deficient local and systemic lymphoid structure (Littman and Rudensky, 2010; Sekirov et al., 2010; Young, 2012). This suggests that microbiota is crucial in instructing and guiding immune cells on their development path. From the other side, intestinal mucosa provides the environment that may promote commensal colonization over other potentially harmful species. Such selective environment may be formed by fucosylation of glycans that are preferred by certain commensals (Umesaki et al., 1995). Commensal bacteria play an active role in maintaining host barrier function, by regulating epithelial cell renewal, promoting damage repair of the epithelium, and reorganizing the tight junctions (Chervonsky, 2010). Furthermore commensal bacteria produces metabolites and stimulates immune cell to secrete antimicrobial compounds thus promoting barrier function via creating unfavorable environment for potentially harmful bacteria (Kamada et al., 2013).

Lumenal bacteria are sensed by host via pattern recognition receptors (PRRs) expressed on both epithelial and immune cells. PPRs recognize specific pathogen-associated molecular patterns (PAMPs), such as LPS, flagellin, lipoteichoic acids, bacterial DNA and RNA, peptidoglycans, as well as commensals PAMPs. Three main families of PRRs are Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). Surface bound TLRs stimulated with bacterial factors activate downstream pro-inflammatory responses, while NLRs are located in cytoplasm and, in addition to inflammatory responses, regulate apoptosis (Kamada et al., 2013).

The intestinal function is complemented by the neuronal component. The enteric nervous system (ENS) modulates amongst other functions peristalsis, fluid secretion, and the digestive processes in the gut. The ENS is composed of three ganglionated plexuses: the myenteric Aurebach plexus in the muscular layer, the submucosal Meissner plexus, and the mucus plexus (Ben-Horin and Chowers, 2008). There is evidence for the role of ENS regulation in the gut barrier function via paracellular permeability modulation as well as vasoactive intestinal peptide (VIP) and tumor growth factor (TGF) secretion stimulation that effect intestinal epithelial proliferation and spatial organization (Cornet et al., 2001; Toumi et al., 2003). ENS also modulates intestinal motility contributing to regulation of microbiota

load on the mucosa. Moreover ENS can directly communicate with immune cells via nicotinic acetylcholine receptor alpha7 (α7 nAchR) subunit and Jak2-STAT3 transcription factor inhibiting macrophages activation and systemic inflammatory responses (de Jonge et al., 2005; Wang et al., 2003). Neuropeptides VIP, Y, calcitonin gene-related protein (CGRP), somatostatin, and substance P produced by ENS are collectively capable of modulating T cell migration and cytokine secretion (Ben-Horin and Chowers, 2008).

Host interaction with the luminal content is kept in homeostasis via intricate play of epithelial, immune and neuronal actors, which in healthy state efficiently differentiate between pathogenic antigens and commensal bacterial or food antigens. It is not yet clear the exact mechanisms by which mucosal immune system differentiates between benign and harmful antigens as the bacterial structures are rather similar. One possible approach may be the location of the antigen, where barriers is mainly breached by pathogens while commensals remain on the surface (Moens and Veldhoen, 2012).

2.5. Food components and intestinal homeostasis

Dietary habits on both short- and long-term basis modify host microbiota and its function, consequently altering host metabolism and the risk of disease development. In this context a number of food components and their impact on health are being investigated, such as dietary fiber, pre- and probiotics, polyunsaturated fatty acids, amino acids and polyphenols (Magrone and Jirillo, 2013).

Dietary components in fruits and vegetables (i.e. vitamins, phytochemicals and unsaturated fatty acids) may influence intestinal immune homeostasis via action on aryl hydrocarbon receptor (AHR). AHR is directly involved in IELs function maintenance and differentiation of T cells, evident from AHR-deficient animal models that also show IELs deficiency (Li et al., 2011). β-carotene, a precursor of vitamin A, is present in yellow vegetables such as carrots and sweet potatoes and also in animal livers. Adequate levels of vitamin A are critical for balancing T cells responses to infections, as in the conditions with vitamin A deficiency immune responses are tipped towards Th1 and Th17 induction (Veldhoen and Brucklacher-Waldert, 2012).

Immune responses may be regulated by the intake of certain fatty acids. Polyunsaturated fatty acids (PUFAs), derived from the plant-based products and fish, are precursors of steroid hormones. Omega-3 and omega-6 PUFAs are precursors of prostaglandins, potent regulators of inflammation. Omega-3 PUFAs replace omega-6 PUFAs

in metabolic pathways and lead production of mediators with anti-inflammatory properties. Omega-3 PUFA may influence immune cells via G-protein coupled receptor (GPR120) that inhibits TLR signaling pathways (Hirasawa et al., 2005).

Studies are available about the role of amino acids, and specifically glutamine, glutamate, arginine, methionine, cysteine and threonine on the mucosal immune function (Ruth and Field, 2013). The roles are diverse and include support of intestinal growth, signal transduction and provision of oxidative substrate for mucosal cells (Law et al., 2007; Tsuchioka et al., 2006; Wu et al., 2004; Zhu et al., 2013).

High-throughput sequencing demonstrated correlations between diets of varying fat and fiber content on the major enterotypes of the gut (*Bacteroides, Prevotella*, and *Ruminococcus*). *Bacteroidetes* and *Actinobacteria* Phyla positively associated with fat, whereas *Firmicutes* and *Proteobacteria* positively associated with fiber (Wu et al., 2011). Similar findings were observed in a study comparing European children, consuming typical Western diet high in animal protein and fat, to children in Burkina Faso, who eat high-carbohydrate/low-protein diets. *Bacteroides* enterotype dominated the European microbiota, whereas the African microbiota was dominated by the *Prevotella* enterotype (De Filippo et al., 2010). The observation might be relevant for the higher prevalence (detailed later) of intestinal disorders in developed vs. developing countries.

Breast-milk fed infants have higher levels of *Bifidobacteria* compared to infants fed cow or goat milk, or formula (Granier et al., 2013). This affect may be attributed to the fucosylated oligosaccharides present in human milk and favored by the selective bacteria (Yu et al., 2013). Intestinal bacterial fermentation of complex carbohydrates leads to the production of short-chain fatty acids (SCFA) such as butyrate, acetate and propionate, that are major sources of energy for intestinal cells, and also participate in gluconeogenesis and lipogenisis (Scott et al., 2013). Microbial transformation of plant cell wall during food or intestinal fermentation releases phytochemicals that may have anti-oxidant and anti-inflammatory effects in the intestine (Russell et al., 2008).

Probiotics, defined as “live microorganisms, which, when administered in sufficient amounts, confer health benefits to the host” belong mainly to the species *Lactobacilli* and *Bifidobacteria*. Their mechanism of action on the intestinal function is strain-specific and includes modification of endogenous microbiota, epithelial barrier function, and immune system. Release of antibacterial factors, SCFA, and reduction of luminal pH modulates the composition of intestinal microbiota (Calder et al., 2009). Induction of mucin secretion and

regulation of tight junction proteins are some of the mechanisms that support barrier function. Direct interaction of probiotics with IECs may inhibit activation of pro-inflammatory factors (i.e. NF-κB) and consequently lower secretion of cytokines such as TNF, IL-1 and IL-8. Heat-shock proteins defend cell from oxidative damage and may also be induced by direct interaction of IECs and probiotics (Petrof et al., 2004). Probiotics may directly interact with other mucosal actors, such as DCs, promoting secretion of IL-10, TGF-ß and maturation of regulatory T cells (O'Hara et al., 2006). Alternatively, probiotics may trigger pro-inflammatory signals by TLR2 activation via lipoteichoic acid (Matsuguchi et al., 2003).

The above examples of the influence of food components on the intestinal function are far from exhaustive and aim at exemplifying the potential of food to modulate intestinal immune responses.

2.6. Disorders of gastrointestinal tract

When the intestinal homeostasis is disturbed either by environmental factor or internal malfunctioning of host response systems an uncontrolled immune attack is triggered leading to numerous acute and chronic diseases. GI disorders span a wide range of conditions and effect over 30% of European population at least once during their lifetime and are on the rise in the developing world too (www.ueg.eu). The economic and social burden of this is enormous.

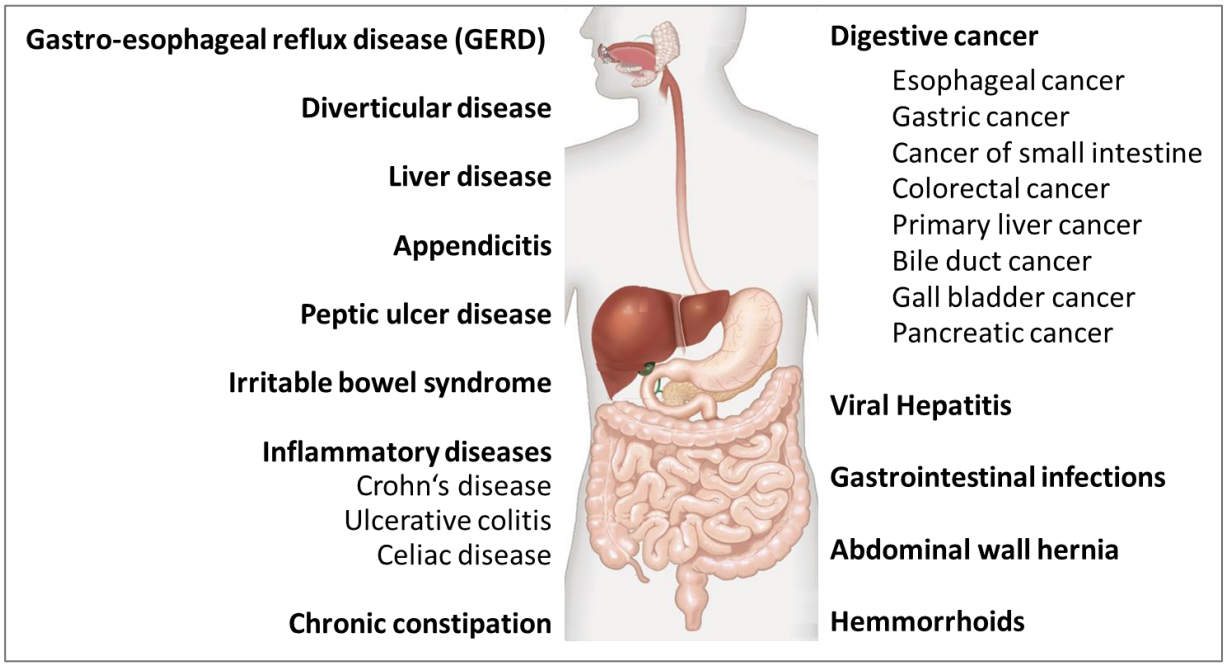


Figure 2. Overview of gastro-intestinal tract disorders *(picture adopted from Encyclopedia Britannica, 2003).*

Gastro-intestinal infections, chronic and acute inflammatory diseases, functional bowel disorders, neoplastic conditions are just some examples of current GI disorders (Fig. 2). It is not feasible to give a synopsis of all the disorders in this work but 2005 review by J.E. Everhart gives a detailed and comprehensive overview of all the digestive diseases (Everhart and Ruhl, 2009). The widespread and characteristic examples of inflammatory and functional disorders are inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS).

2.6.1. Inflammatory Bowel Diseases

IBD prevalence and incidence is on the rise globally with highest numbers in Europe of over 800 cases per 100,000 persons (Molodecky et al., 2012). The disease incidence appear to be connected to industrialized and urbanized lifestyles, as it is highest in industrialized countries but remains low in developing countries. Although with industrialization of developing countries the incidence is also increasing. The etiology of IBD remains unclear, however many factors such as immune hyperactivity, breakdown in gut microbiota homeostasis, as well as immunodeficiency have been implicated in its pathophysiology (Calder et al., 2009).

Two main forms of IBD are ulcerative colitis (UC) and Crohn's disease (CD) with the incidence and prevalence of UC is approximately twice that of CD (Molodecky, Soon et al. 2012). There are many overlapping epidemiological, clinical and therapeutic characteristics. But there are some important pathological and clinical differences that support the diagnosis and treatment choices. Symptoms typical for CD are abdominal pain, diarrhea, and perianal disease, whereas gastrointestinal bleeding is more frequently observed in UC patients.

CD is characterized by patchy inflammation extending through mucosa and serosa and may affect any part of the GI tract. At least 50% of CD patients require surgical treatment in the first 10 years of disease and 70–80% will require surgery within their lifetime, however in CD surgery is not curative and cost-intensive management is directed to minimizing the impact of the disease. UC, on the other hand is characterized by diffuse mucosal inflammation limited to the colon with associated bloody diarrhea. About 50% of patients with UC have a relapse in any year while significant number has frequently relapsing or chronic and continuous disease. Furthermore both UC and CD are associated with an increased risk of colonic carcinoma (Mowat et al., 2011).

Malfunctioning of intestinal mucosa is one of the host factors that contribute to IBD development. Reduction in mucin secretion by goblet cell, impaired secretion by Paneth cells of antimicrobial peptides, endoplasmic reticulum (ER) stress with subsequent unfolded

protein response (UPR), aberrant PPR signaling, macrophages immunodeficiency, and deregulated cytokine secretion have all been associated with IBD pathogenesis (Glocker et al., 2009; Kaser et al., 2010; Maloy and Powrie, 2011; Rath and Haller, 2012; Shkoda et al., 2007; Siddiqui et al., 2010; Smith et al., 2009).

Both UC and CD are characterized with increased levels of CD4 positive neutrophils and lymphocytes in addition to increased numbers of B cells, macrophages, DCs, plasma cells, eosinophils and mast cells. Deregulated Th1 response with increased levels of TNF-α and IFN-γ is also present in both disorders, together with upregulation of IL-12, IL-23 and IL-17. The differentiating factors for the two disorders may be the expansion of phagocytic cells in CD and Th2 responses (IL-4 and IL-13) for UC. (Roberts-Thomson et al., 2011)

Over the course of last decade a number of genetic factors have been linked to IBD pathogenesis. Gene polymorphisms affecting bacterial recognition and autophagy (NOD2/CARD15, ATG16L1, TLR4, TLR9) and immune regulation (IL-23R, TNFSF15) have been associated with the diseases (Duerr et al., 2006; Franchimont et al., 2004; Hampe et al., 2007; McGovern et al., 2009; Ogura et al., 2001; Torok et al., 2004; Yamazaki et al., 2005). Twin studies conducted in Sweden and Denmark corroborate the role of genetic susceptibility. The disease concordance rate between monozygotic twins was significantly higher than between dizygotic twins especially in CD patients (Atkins and Furuta, 2010; Orholm et al., 2000; Tysk et al., 1988). The most recent meta-analysis of GWAS (genome-wide association studies) identified a total of 163 loci associated with IBD, 30 of those are solely associated with CD and 23 with UC (Jostins et al., 2012).

Although a large number of multiple susceptibility genes for IBD have been identified the contribution of each separate gene to disease heritability remains modest. For example NOD2 mutations are not implicated in UC pathogenesis and are present in only 25% of CD patients (Veldhoen and Brucklacher-Waldert, 2012). Furthermore, genetic clues do not explain the global distribution and incidence of the diseases, suggesting environmental factors still take dominance (Leone et al., 2013).

Both UC and CD exhibit loss of oral tolerance towards intestinal microbiota. Increased activation of effector immune cells of GALT leads to high levels of pro-inflammatory cytokines (such as TNF, IL-6, IFN-γ) and results in tissue damage and inflammation. The increased activation of immune cells may be linked to the increased levels of NF-κB in macrophages and epithelial cells in patients with IBD (Atreya et al., 2008). Tissue damage reinforces inflammatory responses due to increased antigen penetration through the

mucosal barrier. Increased number of mucosa-associated bacteria compared to healthy individuals was observed in IBD patients in addition to altered gut microbiota composition (Sartor, 2008). Antibiotic treatment and diversification of fecal stream mitigate IBD, suggesting, together with earlier mentioned observation, that gut microbiota is fundamentally implicated in the disease etiology (Flanagan et al., 2012). Furthermore the influence of microbiota on inflammation is strain dependent, with certain commensals protecting host against inflammation, while other normally harmless bacteria promoting development of IBD (Kamada et al., 2013).

Environmental factors such as smoking and diet may also be associated with IBD pathogenesis. In CD patients smoking had detrimental effects while in UC patients the effect was opposite. The mechanisms behind this observation are unclear with hypoxia, carbon monoxide and nicotine implicated as possible mediators (Birrenbach and Bocker, 2004). High-fat, high-refined sugar and high-animal protein diets may promote while PUFA-3 mitigate the disease development (Ananthakrishnan, 2013; Gruber et al., 2013). Environmental influences during the development can cause epigenetic changes, such as DNA methylation, and may also lead to pathogenesis of IBD (Kellermayer, 2012).

ENS also plays role in IBD. Activation of ENS reduces intestinal epithelial permeability, via several mediators such as S-nitrosoglutathione and VIP. Both VIP and TGFβ secretion affect colonic epithelial proliferation and spatial organization (Neunlist et al., 2003). This effect is important in the context of CD where defect in intestinal barrier is implicated in driving immune hyperreactivity and inflammation. GDNP protein, produced by enteric glial cells, activates MAPK and Akt signalling pathways that can protect colonic epithelial cells from apoptosis (Leung et al., 2012; McLarnon, 2012; Neunlist et al., 2007; Steinkamp et al., 2003; Toumi et al., 2003). Parasympathetic nicotinic signaling, primarily via nAchRs has immune-modulatory and may also effect disease pathogenesis. It was shown that signaling through these receptors can inhibit the activation of NF-κB signaling by directly mediating inflammatory responses (de Jonge and Ulloa, 2007; Kikuchi et al., 2008).

IBD is generally incurable, conventional maintenance treatments involve the use of immune-suppressant and anti-inflammatory drugs, antibiotics, biologicals as well as surgery. First-line therapy for patients with UC is anti-inflammatory drug mesalazine (5-aminosalicylic acid), that is also used to prevent recurrence of CD (Ford et al., 2011). Corticosteroids (i.e. hydrocortisone, budesonide, prednisone and beclomethasone) are effective in inducing remission in active IBD although their use is limited by steroid-related side effects.

Immunosuppressive thiopurines (azathioprine, 6-mercaptopurine or methotrexate) are prescribed for corticosteroid-resistance disease but again have to be used cautiously as many patients develop myelo- and hepatotoxicity (Neurath, 2010; Taylor and Irving, 2011). Ciclosporin is an inhibitor of calcineurin, preventing clonal expansion of T-cell subsets. It has a rapid onset of action and demonstrated effectiveness in the management of severe UC. Antibiotics, such as ciprofloxacin, metronidazole and rifaximin, are used to mainly treat the infectious complications of IBD, such as abscesses and wound infections. The use of antibiotics as primary treatments has modest effects in both UC and CD (Baumgart and Sandborn, 2007; Mowat et al., 2011; Taylor and Irving, 2011). Recently developed biological drugs are mainly anti-TNF antibodies (infliximab, adalimumab or certolizumab pegol) and are administered for induction and maintenance of remission with or without an immunomodulators. TNF antibodies have potent anti-inflammatory effects and although initially their effect was attributed solely to neutralization of TNF, evidence is emerging of other mechanisms of actions that remain to be clarified (Girardin et al., 2012; Oikonomopoulos et al., 2013).

2.6.2. Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is another widespread disorder of gastrointestinal tract effecting up to 20% of population in the Western countries. It is a functional disorder characterized by presence of symptoms in the absence of demonstrable pathological abnormalities. Common patients' complaints are recurrent abdominal pain and discomfort, visceral hypersensitivity, bloating and disturbances associated with bowel movements (Camilleri and Andresen, 2009; Longstreth et al., 2006). IBS does not represent a severe illness however the quality of life of IBS patients is gravely reduced, comparable to diabetes as well as chronic renal failure (Gralnek et al., 2000).

IBS patients are categorized according to the bowel movement alterations into diarrhea-predominant (IBS-D), constipation-predominant (IBD-C) and mixed subtype with altering episodes of diarrhea and constipation. Post-infectious IBS (PI-IBS) diagnosis is another category of IBS given to patients that develop IBS-like symptoms in consequence of acute bacterial gastroenteritis (Salonen et al., 2010; Thompson, 1999). Similarly to IBD the etiology and pathogenesis of IBS is multifactorial but clear triggers remain unknown. Motor and sensory dysfunction, neuro-immune mechanisms, psychological factors, diet, changes in

intestinal microbiota all play a determinate role in the IBS pathophysiology (Camilleri, 2005; Drossman et al., 2002; Simren et al., 2007).

Serotonin is one of the major regulators of ENS that mediates GI motility and chloride secretion. Increased levels of serotonin as well as serotonin enteroendocrine cells were encountered in the small and large intestine of both IBS-D and IBS-C patients, however the opposite was observed in the PI-IBS patients, suggesting different pathophysiology of PI-IBS compared to sporadic IBS (El-Salhy et al., 2012; Wang et al., 2007). Anti-enteric autoimmune antibodies detected in IBS patients might also be responsible for enteric neuronal damage and subsequent symptoms (Wood et al., 2012).

Increased mast cell infiltration to the mucosa as well as increased mucosal and systemic cytokines levels indicate presence of low-grade inflammation in IBS patients (Ohman and Simren, 2010; Philpott et al., 2011). Abdominal pain severity is also associated with increased number of colonic mast cell infiltration and mediator release in proximity to the mucosal nerves in the lamina propria (Barbara et al., 2004). The role of mast cells is further corroborated by the increased mast cell derived serine protease activity in stool of IBS-D patients (Roka et al., 2007). Furthermore, decreased ratio of IL-10 to IL-12 cytokine and changes in local defense mechanisms in the sigmoid and colonic mucosa were observed in IBS patients (Aerssens et al., 2008; Borman, 2001; Camilleri and Andresen, 2009).

The role of host microbiota in IBS has been confirmed by several observations. PI-IBS develops in up to 32% of patients with acute bacterial gastroenteritis caused by *Campylobacter*, *Salmonella*, diarrheagenic strains of *Escherichia coli*, and *Shigella* species (Spiller and Garsed, 2009). There is evidence of altered host microbiota characterized by increase in pseudomonas and E*nterobacteria* and decrease in *Coliforms*, *Lactobacilli*, and *Bifidobacteria* in the IBS patients compared to healthy subjects. Small intestine bacterial overgrowth (SIBO) is frequently observed in IBS patients suffering from diarrhea, bloating, and pain. Furthermore use of antibiotics has been repeatedly associated with increasing the odds ratio of developing IBS to over 3 (Dahlqvist and Piessevaux, 2011; Kassinen et al., 2007; Salonen et al., 2010).

Role of diet in IBS can be exemplified with the case of gluten, which can generate IBS symptoms in the absence of transglutaminase antibodies or histological markers of coeliac disease (Wahnschaffe et al., 2001). Several mouse models also implicated the role of gluten proteins, without autoimmune response, in activation of innate immunity, increased small intestinal permeability, neuro-muscular dysfunction and dysbiosis (Simren et al., 2013).

Some evidence is accumulating regarding the genetic factors associated with IBS. Polymorphisms connected to increased TNF and decreased IL-10 production have been detected in IBS patients with higher frequency than in healthy individuals (van der Veek et al., 2005). Patients that went on to develop PI-IBS had higher frequency of polymorphisms in CDH1 coding for E-cadherin, a tight junction protein, TLR9 – mediator of the cellular response to bacterial DNA and IL-6 (Villani et al., 2010).

Traditional treatment options for IBS include agents targeting various symptoms associated with the disorder (i.e. abdominal pain, nausea, constipation, bloating and diarrhea) but have limited efficacy in address the overall symptoms complex (Lacy and Weiser, 2006). Common medications are antiemetic agents, antihistamines, phenothiazines, benzodiazepines, opioid agents, antispasmodics, and tricyclic antidepressants. Among the new agents currently in the development are guanylate cyclase-c agonist, cholecystokinin antagonists, melatonin as well as serotonin (5-hydroxytryptamine) receptor modulators (Camilleri and Andresen, 2009). Antibiotics, although being a possible trigger for IBS, are used for treatment of SIBO (i.e. tetracycline, doxycycline, rifaximin) (Salonen et al., 2010; Schey and Rao, 2011; Simren et al., 2007). Additional alternative treatments include fiber supplementation, polyethylene glycol, and fatty acid lubiprostone (Andresen et al., 2011; Salonen et al., 2010; Schey and Rao, 2011; Simren et al., 2007).

2.7. IBD, IBS and dietary interventions

Dietary interventions present a promising tool to address alterations of intestinal microbiota and host metabolism in IBD and IBS. Despite contradictory reports, the evidence is accumulating about the evidence of probiotics, prebiotics, their combination (synbiotics), as well dietary fiber and phytochemicals to modulate microbiota and its function with subsequent beneficial effects to the host (Fig. 3). A strong plus of the dietary modulation is the possibility of long-term interventions as well as low risk of adverse effects, as opposed to antibiotics or immune-suppressive conventional treatments (van Hylckama Vlieg et al., 2011).

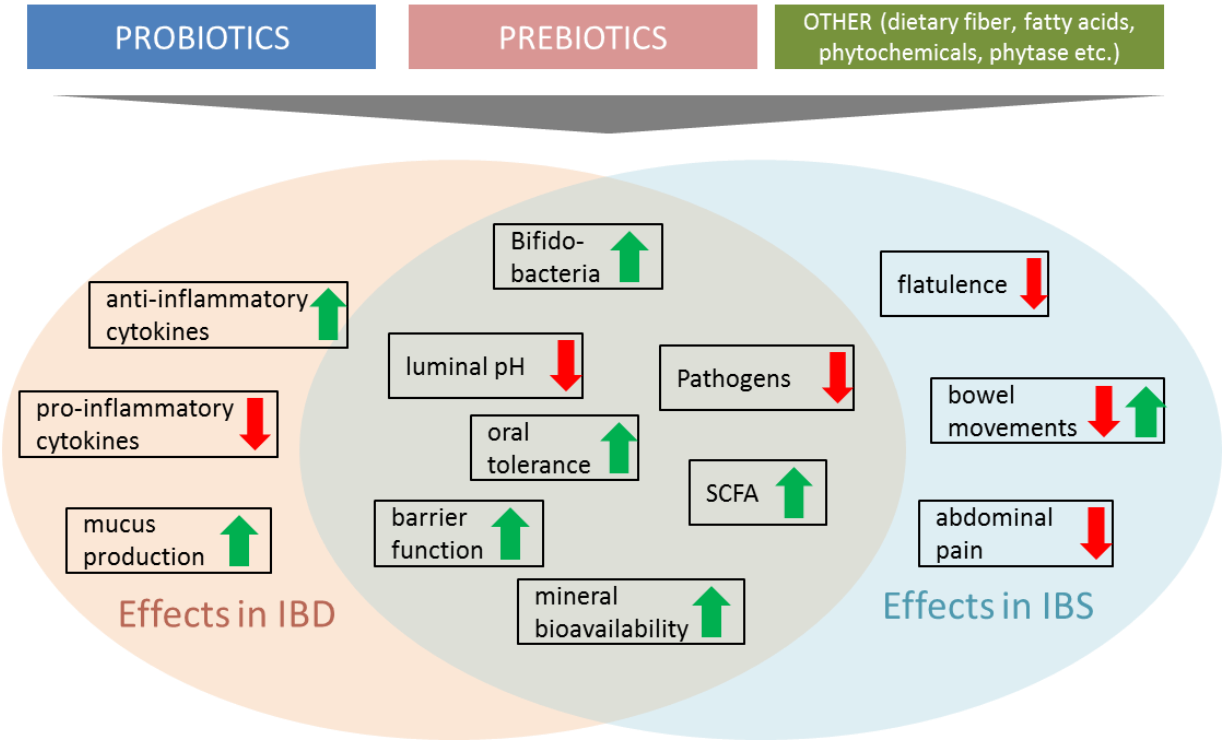


Figure 3. Dietary intervention effects with probiotics, prebiotics and other functional foods in IBS and IBD.

2.7.1. Probiotics in *in vitro* and *in vivo* experimental models

Effectiveness of probiotics in modulation of intestinal function depends on the individual probiotic strain, as well as host's genetics and endogenous microbiota (Geier et al., 2007; Shanahan, 2004). Common mechanisms of probiotics action characterized up to date include barrier function to pathogens by adherence and production of substances that have an antibiotic effect, stabilization of indigenous microbiota, stimulation of immune processes and reduction of excessive pro-inflammatory factors production, enhancement of epithelial cell proliferation and consequently barrier function, inhibition of apoptosis and provision of metabolic energy for enterocytes through fermentation of non-soluble and soluble dietary fiber (Geier et al., 2007; Hoveyda et al., 2009; Ritchie and Romanuk, 2012).

VSL#3 is one of the most studied probiotics up to date. Its disease modulating activity *in vitro* has been attributed to the stimulation of TGF-β and IL-10 secretion while decreasing IL-8 secretion by the gut mucosa and induction of mucin production in the colon via up-regulation of the gene MUC2. Trans-epithelial resistance decrease following incubation with pathogenic *E. coli* and *Salmonella* strains could also be prevented with VSL#3. Furthermore, the probiotic mix induces delay in inflammatory response through inhibition of NF-κB activation and stabilization of IκB, NF-κB inhibitor, levels (Caballero-Franco et al., 2007; Calcinaro et al., 2005; Jijon et al., 2004; Lammers et al., 2003; Madsen et al., 2001; Otte and

Podolsky, 2004; Zeng et al., 2008). *L. paracasei*, one of VSL#3 strains, stands out due its capacity in down-regulation of pro-inflammatory cytokines and chemokines. This strain expresses serine protease Prtp-encoded lactocepin that mediates loss of IEC-derived IP-10 chemokine both *in vitro* and *in vivo*. IP-10 is a major pro-inflammatory chemokine in IBD and its reduction in IL-$10^{-/-}$ mice upon VSL#3 feeding was associated with attenuated intestinal inflammation (Hoermannsperger et al., 2009; Tien et al., 2006; von Schillde et al., 2012).

L. acidophilus can also reduce secretion of pro-inflammatory cytokines, such as IL-12 and IFN-γ (Chen et al., 2005; Rousseaux et al., 2007). Downstream production of IL-8, induced by TNF, could be attenuated by another set of probiotic bacteria: *L. rhamnosus GG* and *L. reuteri* that additionally up-regulated anti-inflammatory nerve growth factor (NGF) (Ma et al., 2004; Zhang et al., 2005). Further immunomodulatory action of probiotics was observed upon administration of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 that leads to increase in $CD4^{+}$ $CD25^{+high}$ T cells proportion and decrease in IL-2^{+} and $CD69^{+}$ T cells (Lorea Baroja et al., 2007).

Modulation of cell proliferation and apoptosis in the gut by *L. rhamnosus* GG and *Clostridium butyricum* is suggested to be due to the ability of the strains to produce SCFA, to activate the anti-apoptotic Akt/protein kinase B pathway and also to inhibit the pro-apoptotic p38/MAPK pathway (Ichikawa et al., 1999; Yan and Polk, 2010).

Administration of *B. animalis subspecies lactis* fermented milk significantly reduced intestinal inflammation in T-$bet^{-/-}$/$Rag2^{-/-}$ mice model of UC. The reduction in inflammation is probably due to observed increase in the population of bacteria that produce butyrate from lactate and reduced pH. Low pH inhibits *Enterobacteriaceae* species colitogenic to the same mice model (Veiga et al., 2010).

Protection against pathogens by probiotics may involve several other mechanisms. Probiotics release antimicrobial factors, such as defensins (i.e. *E. coli Nissle 1917)*, bacteriocins, hydrogen peroxide, nitric oxide and SCFA that are unfavorable to pathogens. In addition, probiotics adhere to the intestinal mucosal surface preventing colonization with pathogenic bacteria and block their actions on TLRs in the innate arm of the immune system (Dieleman et al., 2003; Guarner and Malagelada, 2003; Parkes et al., 2008; Penner et al., 2005; Wehkamp et al., 2004).

L. acidophilus may be applicable for pain management associated with GI disorders as it was shown to up-regulate μ-opioid and cannaboid receptors in colonic epithelium in *in vitro* as well as *in vivo* (Chen et al., 2005; Rousseaux et al., 2007). In an antibiotic-induced murine

model of visceral hypersensitivity *L. paracasei* attenuated abdominal pain and mucosal inflammation (Verdu et al., 2006). In a rat model of stress and hypersensitivity milk fermented with a mix of *Lactococcus lactis CNCM I-1631, Lactobacillus bulgaricus, Streptococcus thermophiles* and *B. animalis subspecies lactis* reduced *v*isceral hypersensitivity, stress-induced blood endotoxin levels and reversed stress-induced downregulation of TJ proteins (Agostini et al., 2012). A commercial probiotic modulates degranulation of mast cells *in vitro*, an effect that could be relevant in the context of IBS (Femke Lutgendorff, 2009).

Caution has to be taken when administering probiotics in inflamed situations, as even probiotics strains may induce rather than mitigate inflammation. IL-$10^{-/-}$ mice monoassociated with *B. animalis* developed extensive duodenitis and mild colonic inflammation (Kim et al., 2005; Moran et al., 2009). Later observation suggests that the probiotic effect is multifactorial and depends on the host genetic factors, specific strain administered as well as luminal environment.

2.7.2. Probiotics and the clinical evidence

Clinical evidence of probiotic effectiveness in disease modulation is limited compared to the studies with *in vitro* and *in vivo* models (Sanders et al., 2013). According to a recent meta-analysis beneficial effects of probiotics were significant in UC patients but could not be substantiated in CD (Jonkers et al., 2012).

Probiotic treatments with proven beneficial effects are so far VSL#3, a proprietary mixture of *Lactobacillus, Bifidobacterium* and *Streptococcus* strains, and *Escherichia coli Nissle* strain that induce and maintain remission of disease activity in mild to moderately severe UC (Adam et al., 2006; Tursi et al., 2010)**.** Supplementation of *B. longum* in combination with inulin to UC patients increased the bacterial numbers to the level observed in healthy individuals as well as mitigated inflammation, demonstrated by reduction in sigmoidoscopy scores, TNF and IL-1 and regeneration of epithelial tissue (Furrie et al., 2005).

Meta-analysis of clinical trials with administration of probiotics to IBS patients have demonstrated largely positive, although strain-specific effects (Whelan and Quigley, 2013). Probiotics alleviated symptoms of abdominal pain, flatulence and bloating but had no

significant effect on functional symptoms of constipation or diarrhea in IBS patients (Haller et al., 2010; Hoveyda et al., 2009; Ritchie and Romanuk, 2012).

Despite limited effectiveness in clinical trials probiotics remain a focus of intense research. Lack of significant effects in meta-analysis may be also due to low comparability of studies with varying experimental settings. A set of recommendations has been developed to guide the future studies aimed at demonstrating probiotic benefit and to allow for better comparability across studies (Rijkers et al., 2010). Furthermore, although adverse effects reporting of probiotics is rare, administration of probiotics in patients with compromised health carries higher risk of adverse effects and needs to be considered with caution (Hempel et al., 2011).

2.7.3. Prebiotics in *in vitro* and *in vivo* experimental models

Prebiotics are a category of functional foods defined as "a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the GI microbiota, thus conferring benefit(s) upon host health" (Gibson and Roberfroid, 1995). To be termed as prebiotics, the dietary component has to meet several criteria: resistance to digestion and absorption in the upper GI tract by gastric acid and host enzymes, fermentation by selective bacteria in the lower GI tract, stimulation of growth and activity of selective host colonic bacteria beneficial to the host (Gibson et al., 2004).

Two best studied types of prebiotics with demonstrated health benefits are inulin-type fructans and the galactooligosaccharides (GOS). Effects of prebiotics comprise stimulation of beneficial bacterial growth and subsequent prevention of pathogenic adherence, decrease in luminal pH, production of anti-bacterial substances and SCFA (Calder et al., 2009; Candela et al., 2010a; Looijer-van Langen and Dieleman, 2009; van Hylckama Vlieg et al., 2011).

Fructooligosaccharides (FOS), GOS, xylooligosaccarides, isomalto-oliggisaccradies and inulin promote *Lactobacilli* and *Bifidobactereria* growth while inhibiting intestinal colonization by disease-inducing bacteria like *Clostridia* and *Enterobacteria* (Bomba et al., 2002; Hoentjen et al., 2005; Howard et al., 1995; Kaneko et al., 1995). One mechanism for preventing pathogen colonization may be the interference of terminal sugars of prebiotic oligosaccharides with pathogens' receptors (Zopf and Roth, 1996).

A commercial mixture of long-chain inulin and oligosaccharides demonstrated immune-modulatory effect *in vivo* via decreased levels of the IL-1β and increased levels of TGF-β (Hoentjen et al., 2005). Similar mixture with inulin-enriched oligofructose enhanced the

production of IL-10 in Peyer's patches and increased the secretion of IgA in the ileum (Roller et al., 2004). Increased IgA secretion was also observed in gut-associated lymphoid tissue in rats consequent to lactulose consumption (Schley and Field, 2002).

Inulin-type fructans reduced inflammatory processes in experimental models of inflammation by chemical agents such as dextran sodium sulphate (DSS) and trinitrobenzenesulfonic acid (TNBS) (Cherbut et al., 2003; Videla et al., 2001). Oral administration of oligofructose-enriched inulin to the transgenic HLA-B27 rat model that spontaneously develops colitis also decreased inflammatory histological scores and altered mucosal cytokine profiles (decreased IL-1ß and increased TGF-ß levels) (Hoentjen et al., 2005).

Germinated barley foodstuff (GBF) is a dietary component high in glutamine-rich protein and hemi-cellulose-rich dietary fiber. GBF supplementation lead to decreased incidence of bloody diarhoea and mucosal injury in the DSS model of rat colitis as well as increased number of commensals *Bifidobacteria* and *E. limosum* (Kanauchi et al., 2005; Kanauchi et al., 1998). Lactulose and goat's milk oligosaccharides ameliorated DSS colitis in a dose-dependent manner in all parameters (Lara-Villoslada et al., 2006; Rumi et al., 2004).

2.7.4. Prebiotics and the clinical evidence

Similar to probiotics the clinical evidence of prebiotics efficacy is limited when compared to experimental models of diseases.

Clinical trials with UC patients did corroborate *in vitro* effectiveness of FOS to stimulate *Lactobacilli* and *Bifidobacteria* and increase SCFAs in the large bowel (Lewis et al., 2005). Four weeks supplementation of UC patients with GBF resulted in a significant clinical and endoscopic improvement associated with an increase in stool butyrate concentrations (Bamba et al., 2002). In clinical trial with oligofructose-enriched inulin the levels of calprotectin in the faeces was lowered and thus improved UC patients' response to therapy (Casellas et al., 2007). Furthermore combination of commercial prebiotics mix (synergy 1) and *B. longum* improved sigmoidoscopy scores, decreased β-defensin mRNA, TNF and IL-1α and reduced inflammation seen in biopsies from UC patients (Furrie et al., 2005).

In CD patients oligofructose-enriched inulin shows variable effect on disease activity and fecal *Bifidobacteria* counts (Benjamin et al., 2011; Joossens et al., 2012)**.**

IBS symptoms were significantly alleviated with administration of synbiotics. *B. longum* W11, and the short chain oligosaccharide prebiotic Fos Actilight increased stool frequency in

patients with IBS-C and reduce abdominal pain and bloating in those with moderate-severe symptoms (Colecchia et al., 2006; Gorissen et al., 2010). Synbiotic Flortec with *L. paracasei* B21060 significantly reduced bowel movements, pain, and IBS scores in IBS-D patients (Andriulli et al., 2008). Trans-GOS was also effective in stimulating fecal *Bifidobacteria* growth and mitigating IBS symptoms in humans in double-blind, placebo-controlled trial (Silk et al., 2009).

2.7.5. Other dietary interventions

A number of dietary compounds fall outside probiotic and prebiotic classification but also demonstrated some effectiveness in modulating disease activity. Those are minerals, vitamins, unsaturated fatty acids as well as dietary fiber supplementations.

A wide array of vitamin and mineral deficiencies exist in patients with IBD, with varying degrees of clinical significance. Diminished levels of calcium, vitamin D, folate, vitamin B12 and zinc have been recorded in IBD, leading to the development of individually tailored supplementary nutrition to counterbalance those deficiencies (Goh and O'Morain, 2003). Dietary fiber, non-fermentable or bulking agents are used in the management of patients with IBS-C to modulate stool frequency and have demonstrated some success (Park and Floch, 2007; Zuckerman, 2006). On the other hand reduction of overall fiber intake can improve bloating and diarrhea in IBS patients (Dear et al., 2005).

Fatty acids, such as Omega-3 PUFA and conjugated linoleic acid (CLA) may have immune-modulatory effects in IBD. Omega-3 PUFA and CLA are natural ligands of PPARγ (peroxisome proliferator-activated receptor gamma), a nuclear receptor and transcription factor involved in lipid metabolism and glucose homeostasis (Xu et al., 1999). PPARγ also plays anti-inflammatory role via modulation of Treg cells (Hontecillas and Bassaganya-Riera, 2007). CLA administration to CD patients with mild to moderate CD decreased disease activity, probably via suppression of IFN-γ, TNF-α and IL-17 production by T cells (Bassaganya-Riera et al., 2012; Viladomiu et al., 2013). VSL#3 bacterial mixture is also able to produce CLA, that may result in PPARγ-dependent anti-inflammatory effects in different murine models of experimental colitis (Hormannsperger and Haller, 2010).

2.8. Experimental models of IBD

Experimental models of intestinal inflammation are a useful tool for the initial assessment of treatment efficacy and for the clarification of environment and host factors in disease pathogenesis. There are over 60 animal models that mimic pathophysiology of IBD and are in detail described in the review by A. Mizoguchi (2012) (Mizoguchi, 2012).

$TNF^{\Delta ARE}$ mice develop a severe CD8+ T cell-dependent ileitis that closely resembles manifestation of human CD together with arthritis. The mice develop trans-mural lesions and granulomatosis due to constant overproduction of TNF in the terminal ileum (Kontoyiannis et al., 1999). The overproduction of TNF is caused by deletion of repeated AU-rich element (ARE) in the untranslated region of the TNF encoding gene $TNF^{\Delta ARE}$. This genetic manipulation results in increased stability of TNF mRNA and consequently leads to overproduction of TNF (Apostolaki et al., 2008). Similar to CD, disease development in $TNF^{\Delta ARE}$ mice is characterized by Th1 response. Intestinal inflammation in $TNF^{\Delta ARE}$ mice is associated with increased Th17 and decreased Th1 responses, increased production of TNF and IFNγ by CD8αβ IELs and decrease in CDββ IELs (Apostolaki et al., 2008). Genetic ablation of β2 microglobulin or β7 integrin prevents pathogenesis of chronic ileitis in the $TNF^{\Delta ARE}$ mice while CD4 deletion aggravates the diseases indicating the critical role of CD8 T cell in disease development (Kontoyiannis et al., 2002). The metabolomics analysis in $TNF^{\Delta ARE}$ mice intestine further demonstrated significant shifts in metabolism of cholesterol, triglycerides, phospholipids, plasmalogens, and sphingomyelins correlated to tissue inflammation (Baur et al., 2011).

Interleukin 10-deficient ($IL\text{-}10^{-/-}$) mice resemble on the other hand a colitic UC-related disease phenotype with epithelial cell hyperplasia and immune cell infiltration into the lamina propria of the cecum and colon (Kuhn et al., 1993). In the early stages of inflammation the immune response is characterized by Th1 response and shifts to Th2 response, due to lack of IL-10, in the later stages. The inflammation in $IL\text{-}10^{-/-}$ mice does not develop in germ-free, emphasizing the role of host microbiota in the induction of inflammation (Strober et al., 2002). $IL\text{-}10^{-/-}$ mice have increased intestinal permeability even before disease manifestation what might contribute to bacteria crossing intestinal barrier and thus facilitating the development of inflammation (Madsen et al., 1999).

Probiotics administration has not shown significant effects in $TNF^{\Delta ARE}$ mice, while in $IL\text{-}10^{-/-}$ mice VSL#3, *L. casei*, *L. reuteri, L. salivarius* and *B. infantis* demonstrated ability to reduce mucosal inflammation with proposed mechanisms being normalization of colonic

microbiota, enhanced barrier function and normalization of cytokine production (Hoermannsperger et al., 2009; Madsen et al., 2001; Madsen* et al., 1999; McCarthy et al., 2003). Dietary fiber also demonstrated inflammation mitigating potential in IL-$10^{-/-}$ mice (Bassaganya-Riera et al., 2011). Interestingly, experimental elemental diet demonstrated preventive effects in the development of inflammation in $TNF^{\Delta ARE}$ mice when administered in the early age, where gluten was identified as the antigen-independent trigger of pathogenesis (Wagner et al., 2013). Moreover high-fat diet also accelerates pathogenesis of ileitis in $TNF^{\Delta ARE}$ mice without weight-gain with likely mechanisms being increased intestinal permeability, altered luminal factors, and enhanced Th17 responses (Gruber et al., 2013).

$TNF^{\Delta ARE}$ mice model proved useful to demonstrate the role of dietary iron in disease development. The mice did not develop intestinal inflammation when kept on iron-free diet what correlated with reduced cellular markers of ER stress responses and pro-apoptotic mechanisms in the ileal epithelium. The iron depletion in diet induced some compositional changes in microbiota but not overall diversity (Werner et al., 2011).

Models of experimental IBS are scarce as no evident pathology is available and diagnosis is based on patients' descriptions of symptoms. Mice models with separate symptoms of IBS have been used exhibiting acute or chronic visceral hyperalgesia, stress hyper-responsiveness, intestinal transit, and altered fecal pellet output (Holschneider et al., 2011). However the predictive value of these models for the *in vivo* efficacy of therapeutic intervention is limited and clinical trials remain the main source of evaluation.

3. AIMS OF THE WORK

Alterations of the intestinal microbiota may be induced by dietary patterns and subsequently trigger pathological responses in susceptible individuals leading to IBD and functional disorders. Furthermore, diet-induced shifts in metabolic profile of host and microbota can be correlated to the disease risk. Hence the present work was aimed at investigating the effect of a wide-spread LAB-fermented product, sourdough, on intestinal inflammation and metabolic activity. IBD-related mouse models $TNF^{\Delta ARE}$ and $IL\text{-}10^{-/-}$ were used in order to assess bioactivity of sourdough bread in the context of inflammatory processeces. Furthermore, sourdough metabolites and sourdough LAB were evaluated for bioactive compounds *in vitro* using whole tissue and cell cultures.

4. MATERIALS AND METHODS

Materials	Manufacturer
Acetone	Roth, Karlsruhe, Germany
Acetonitrile	Rathburn Chemicals, Walkerburn, Scotland
Acetylcholine	Sigma Aldrich, Steinheim, Germany
Agar	Roth, Karlsruhe, Germany
Agarose	Qbiogene, Heidelberg, Germany
Ammonium sulphate	AppliChem. Darmstdt, Germany
Antibiotic and antimycotic mixture	Gibco Invitrogen, Carlsbad, CA
Bradford protein assay	BioRad, Munich, Germany
CHAPS	Roth, Karlsruhe, Germany
Coomassie Brilliant Blue	AppliChem. Darmstadt, Germany
DTT	Roth, Karlsruhe, Germany
Dulbecco´s modified Eagle´s medium (DMEM)	Invitrogen, Carlsbad, USA
Dulbecco's Phosphate-Buffered Saline (PBS)	Invitrogen, Carlsbad, USA
EDTA	Roth, Karlsruhe, Germany
Ethanol	Roth, Karlsruhe, Germany
Fetal calf serum (FCS)	Biochrom, Berlin, Germany
Formalin	Sigma Aldrich, Steinheim, Germany
Formic acid	Aldrich, Steinheim, Germany
Glutamine	Invitrogen, Carlsbad, USA
Hematoxylin- and eosin (HE)	Sigma-Aldrich, Steinheim, Germany
HEPES	Invitrogen, Carlsbad, USA
Lactic acid	Roth, Karlsruhe, Germany
L-cystein	Roth, Karlsruhe, Germany
Lippopolisacharide (E.coli LPS)	Sigma-Aldrich, Steinheim, Germany
Methanol	Roth, Karlsruhe, Germany
MRS	Fluka, Sigma-Aldrich, Taufkirchen, Germany
Percoll gradient	Sigma-Aldrich, Steinheim, Germany
Polyacrylamide	Roth, Karlsruhe, Germany
Pharmalyte	Amersham Biosciences, Freiburg, Germany
Protease inhibitor cocktail	Roche Diagnostics, Mannheim, Germany
RPMI	Invitrogen, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe, Germany
Thiourea	Roth, Karlsruhe, Germany
TNF	R&D Europe, Abington, England
Triethylammonium bicarbonate (TEAB)	Sigma Aldrich, Steinheim, Germany
trifluoroacetic acid	Roth, Karlsruhe, Germany
trizol	Qiagen, Maryland, USA
Trypsin	Promega, Madison, USA
Tween 80	Fluka, Sigma-Aldrich, Taufkirchen, Germany
Urea	Roth, Karlsruhe, Germany
Ssniff	Soest, Germany
Acetic acid	Fluka, Sigma Alrich, Taufkirchen
PCR	Fermentas, Germany
PCR Clean-up	Invitrogen, Carlsbad, USA

Sourdough and bread

Sourdough, sourdough bread and analog bread were provided by Ludwig Stocker Hofpfisterei GmbH (Munich, Germany). Sourdough (Vollsauer) was prepared by traditional propagation of type I sourdough rye starter. The composition of sourdough and sourdough bread is: 71% rye flour, 25% wheat flour, 1.8% salt and 2% bread crumbs (pH 4.5, acidity 9-10). The dough was baked at 298°C for 1.5 hours. Analog bread was identical to sourdough bread with substitution of sourdough starter with 2.5% sodium bicarbonate, 0.13% acetic acid and 1.2% lactic acid.

Animal models

Heterozygous TNFΔARE/WT and WT mice, gift of Kollias G., Institute for Immunology, Biomedical Sciences Research Center "Al. Fleming", Greece, on C57BL/6N background, were conventionally raised at constant room temperature (22 ± 2 °C), air humidity (55 ± 5%), and a light/dark cycle of 12/12 h. Upon weaning at the age of four weeks mice were provided ultrapure water, standard Chow diet (Ssniff R/M-H, Soest, Germany), dehydrated sourdough bread or dehydrated analog bread ad libitum. Mice were sacrificed by cervical dislocation at the age of 12 weeks.

IL-$10^{-/-}$ mice on 129/SvEv background were raised under specific pathogen-free (SPF) conditions. $IL10^{-/-}$ mice were moved to a conventional animal facility at four weeks of age and provided ultrapure water and standard Chow (Ssniff R/M-H, Soest, Germany) dehydrated sourdough bread or dehydrated analog bread ad libitum. Mice were sacrificed using carbon dioxide chamber at the age of 24 weeks.

The weight and feed consumption was monitored weekly. After sacrificing the mice, sampling of plasma, gut content, and gut sections was performed. Sections of the gut for the histopathology were fixed in 10% neutral buffered formalin. Fixed tissues were HE-stained and embedded in paraffin. Histology scoring was performed by an independent pathologist in a blinded way, assessing the degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion and architectural distortion, resulting in a score from 0 (not inflamed) to 12 (severely inflamed), as previously described (Katakura et al., 2005).

Isolation of primary mouse IEC

Primary IEC were purified as previously described (Ruiz et al., 2005). Briefly, mice were sacrificed and the ileum was removed. The ileum was cut open longitudinally and washed with wash buffer (HBSS, 2% FCS). The tissue was cut in 0.5 cm pieces and incubated with 5 ml digestion buffer (HBSS, 5mM EDTA, 1mM DTT, 10% FBS) in a horizontal shaker at 220 rpm, 37°C, 30 min. Undigested tissue residues were mashed through a 100 µm cell strainer (BD Falcon). Tissue cell suspensions were combined and centrifuged at 350 g, 4°C, 5 min. For density gradient centrifugation, the pellet was resuspended in 40% isotonic percoll and put beneath a 20% isotonic percoll layer. Another layer of 80% isotonic percoll was put beneath both layers. Density gradient centrifugation was performed at 720 g, 20 min, 4°C. The IEL/LPL fraction was collected at the interface of 80% and 40% percoll layers. Cells were washed in wash buffer, counted using a Neubauer counting chamber.

Protein isolation

Briefly, IEC or whole tissue sections were re-suspended in lysis buffer containing 7M urea, 2M thiourea, 2% CHAPS, 1% DTT and protease inhibitor cocktail and 2% Pharmalyte and homogenized by ultra-sonication. Total protein concentrations were determined using Roti-Quant protein assay (Roth, Karlsruhe, Germany) and used for further analysis or stored at -80°C.

Two-dimensional SDS-polyacrylamide gel electrophoresis

Proteins from purified primary IEC or whole tissue were subjected to isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the 2nd dimension as previously described (Werner et al., 2007). For IEF, 200 mg of solubilised total protein was cup-loaded onto rehydrated immobilized pH gradient strips (IPG) with pH 3-10 and 18 cm in length (Amersham Biosciences, Piscataway, USA). Subsequent to the first dimension, IPG stripes were loaded onto 12.5% SDS - polyacrylamide gels and electrophoresis was performed. Gels were next fixed (40% ethanol plus 10% acetic acid), stained in Coomassie Brilliant Blue solution (0.1% CBB, 25% methanol, 10% ammonium sulphate, 2% phosphoric acid). One mouse sample corresponded to one gel (n ≥3 for each group). Gels from each experimental setup were simultaneously submitted to all steps of 2-dimensional (2D)-gel electrophoresis to minimize variability between the samples. Coomassie stained gels were scanned by

ImageScanner (Amersham Biosciences, Piscataway, USA) and analyzed by ProteomWeaver software (BioRad, Munich, Germany). For each experiment, gels from control- and treatment groups were compared. Spots, which significantly differed at least 1.2-fold from the control group in the protein intensity according to the Mann-Whitney-test ($p < 0.05$) and were present in at least 6 of 9 gels, were picked for further matrix-assisted laser desorption/ionization time of flight (MALDI TOF/TOF) mass-spectrometric analysis.

Preparation of protein spots and MALDI-TOF-MS

Stained 2D gel spots were placed into polypropylen 96-well plates with drilled 0.4 mm holes in each well and subjected to in-gel trypsin digestion using triethylammonium bicarbonate (TEAB), formic acid (FA) and acetonitrile (ACN) according to the following procedure:

Stage	Vol., µL	Buffer	Time, min	Temp., C°	Spin 1 min. 1000 rpm	Keep Solution?
1. Destain	100	50% 5mM TEAB/50%EtOH	20	55	Yes	No
2. Destain	15	50% 5mM TEAB/50%EtOH	20	55	Yes	No
3. Dehydration	15	Absolute EtOH	10	RT	Yes	No
4. Wash	15	5mM TEAB	10	RT	Yes	No
5. Dehydration	15	Absolute EtOH	10	RT	Yes	No
6. Dry in speedvac	-/-	-/-	20	RT	No	No
7. Trypsin addition	5	5 ng/µL ice cold trypsin in 5mM TEAB	15	RT	No	Yes
8. Digestion buffer addition	5	5mM TEAB	-/-	RT	No	Yes
9. Place 96well-plate above collector plate	-/-	-/-	4h	37	No	Yes
10. Acidification	5	1% FA	0	RT	Yes	Yes
Option for MALDI: remove 2 µl of the acidified digest and spot on MALDI plate						
13. Extraction	20	60%ACN 40% 0.1% FA	20	RT	Yes	Yes
14. Speed vac	-/-	-/-	Until dry	RT	-/-	-/-

Protein digests were reconstituted with 5 µl of 30% acetonitrile in 0.1% trifluoroacetic acid and mixed with an equal volume of α-cyano-4-hydroxy-cinnamic acid solution (10 mg/ml in 30% acetonitrile, 0.1 % trifluoroacetic acid) on a stainless steel MALDI target (Bruker Daltonik, Bremen, Germany). MALDI MS and MS/MS spectra were acquired on an Ultraflex eXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with a 1 kHz Smartbeam II laser and controlled via FlexControl software (version 3.3.69.0). Protein Calibration standard I ranging from approx. 4-20 kDa (Bruker Daltonik, Bremen, Germany) was used to calibrate MALDI-TOF MS spectra. Briefly, a full MS spectrum (m/z 400

to 5500) of the protein spot was acquired in positive reflectron mode with 1000 laser shots randomly distributed over the MALDI spot. MS/MS spectra were acquired using LIFT fragmentation technique (Suckau et al., 2003) with 2000 shots per spot and a laser power boost of 40%. MS and MS/MS spectra were processed with FlexAnalysis version 3.3.65.0 (Bruker Daltonik, Bremen, Germany) using default parameters. Proteins were identified by using the Mascot Server 1.9 (Bruker Daltonics) based on peptide mass searches within murine sequences only. Mass accuracy was set to ±100 ppm. A significant Mascot score obtained from protein spots derived from at least 4 different gels was selected as criteria for positive identification of proteins.

Metabolite analysis

Plasma and ceacal content of mice, water extracts (<10 kDa) of sourdough, sourdough bread and analog bread as well as MRS growth media of LAB were subjected to LC-MS/MS analysis for metabolite quantification. Plasma was mixed with standards' solution for normalization after measurement. The plasma and standards mixture was precipitated with 1:1:1 acetonitril/methanol/aceton solution, centrifuged and supernatant completely evaporated. The remaining dry pellet was resuspened in 80 % methanol centrifuged again and supernatant frozen at -20 C° for further analysis. Ceacal content of mice was re-suspended in 1:5 in RPMI medium mixed with 250 mg of 0.1 mm glass beads (ROTH) and disrupted in a bead-beater (FastPrep-24, MP Biomedicals LLC) at 6.5 M/s, 0.5 min (four cycles interrupted by 3 min on ice). The glass beads and non-lyzed cells were removed by centrifugation at 350 g for 5 min. Supernatants were collected and centrifuged at 10000 g, 10 min. After centrifugation, the supernatant was filtered through 10 kDa Vivaspin 500 filters (Sartorius Stedim biotech, Goettingen, Germany). MRS media was filtered with 10 kDa Vivaspin 500 filters before analysis as well.

Samples were measured using:

Dionex Ultra High Performance Liquid Chromatography UltiMate® 3000 (Dionex, Idstein, Germany)

- Pump – HPG-3400SD
- Degasser – SRD-3400
- Autosampler – WPS – 3000TSL
- Column oven – TCC-3000SD

API 4000 QTRAP, Linear Ion Trap Quadrupole Mass Spectrometer (AB Sciex, Darmstadt, Germany):

- Ionization type – electrospray ionization (ESI)
- Instrument control – Analyst software (AbSciex, Darmstadt, Germany)

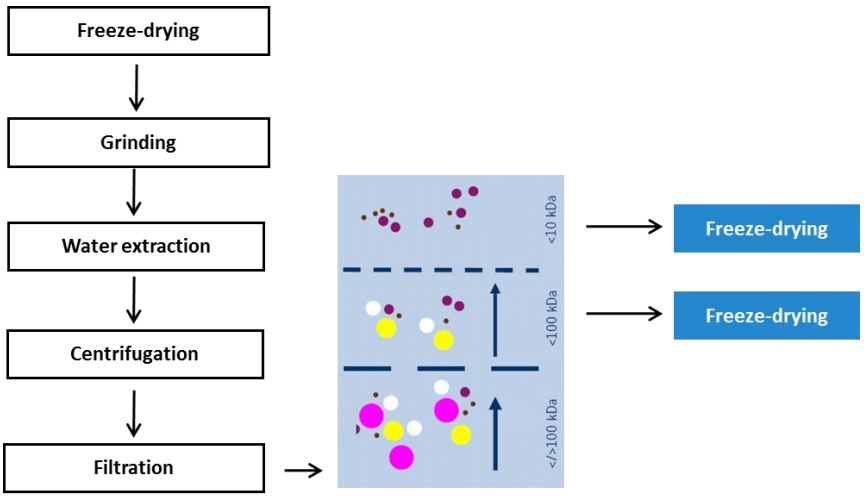
The MS spectra were analysed with Multiquant 2.0 (AB Sciex, Darmstadt, Germany) and concentrations in the samples were calculated according to the spectra of standards.

***In vitro* cell culture**

The murine small intestinal epithelial cell line Mode-K (Vidal et al., 1993) (passage 10-25) was grown in DMEM containing 10% FCS, 1,0% glutamine and 0,8% antibiotic/antimycotic in humidified 5% CO_2 atmosphere at 37°C. Murine macrophage cell line 264.7 (passage 5-15) were grown in RPMI medium 10% FCS, 1,0% glutamine and 0,8% antibiotic/antimycotic in humidified 5% CO_2 atmosphere at 37°C. Cells were grown to confluence in 24 or 48 well tissue culture plates (Greiner bio-one, Frickenhausen, Germany) prior to stimulation experiments. Confluent Mode-K or RAW 264.7 cell monolayers were stimulated with TNF (10 ng/ml), LPS (1 µg/mL), 0.1% water extracts of sourdough, sourdough and analog bread, 20 MOI (multiplicity of infection) of formalin fixed bacteria or concentrated conditioned media of bacteria. Water extracts concentrations of 0.01% - 1% were tested and the effect of extracts on cells' viability assessed using enzymatic activity of viable cells to reduce tetrazolium dye (MTT assay). Concentration of 0.1% was chosen since no impact on cells' viabilty was observed upon 24 hours of incubation.

Extraction

Sourdough, sourdough bread and analog bread were freeze-dried and grinded into powder (See the scheme below). The powder was extracted with distilled water 2x 3 hours and 1x overnight. The un-solubilized fraction was centrifuged at 9000 rpm for 20 min and the supernatants were step wise filtered using Vivaflow 200 cassettes of 0.2 µm, 100 kDa and 10 kDa exclusion thresholds (Sartorius, Goettingen, Germany). The filtrate was freeze dried and re-suspended in distilled water at 25% for in vitro assays.



Endotoxin measurement and clean-up

Endotoxin concentrations measurement in water extracts from sourdough, sourdough bread and analog bread were determined using Limulus Amebocyte Lysate (LAL) Chromogenic Endpoint Assay (Hycult biotech, Uden, Netherlands). The assay was performed according to the manufacturer's instructions. Endotoxin contamination in water extracts from sourdough, sourdough bread and analog bread was removed using Detoxi-Gel^{TM} Endotoxin Removing Columns (Thermo Scientific, Rockford, USA). The columns are filled with resin with immobilized polymyxin B to bind and remove pyrogens from solution. The removal of endotoxin was performed according to the manufacturer's instructions.

ELISA

IP-10 (murine/human) and TNF (murine) concentrations in cell culture supernatants were determined using the appropriate ELISA kits (R&D Europe, Abington, England) according to the manufacturer's instructions. The ELISA was performed using Nunc MaxiSorp® flat-bottom 96 well plates (Greiner Bio-One GmbH, Frickenhausen, Germany). Briefly 96-well plates were coated with the appropriate capture antibody overnight at RT. Plates were washed 3 times with PBS, blocked with 1% bovine serum albumin in PBS and incubated with cell culture supernatants for 1.5 h at RT. Plates were washed and incubated with the appropriate detection antibody for 1.5 h at RT. Plates were washed and incubated with a detection enzyme. Plates were washed and incubated with a substrate solution. Protein concentration was determined by photometrical analysis of the reaction of substrate and detection enzyme.

Isolation of sourdough bacteria

Sourdough bacteria (*L. sanfranciscensis* strains) were isolated by Prof. Micheal Gänzle (University of Alberta, Canada) using following procedure. Cooled or frozen sourdough (Hofpfisterei GmbH, Munich, Germany) was serially diluted in buffered peptone water and plated on mMRS4 (peptone, yeast and beef extract, K_2PO_4H, KH_2PO_4, NH_4Cl, cystein-HCl, tween 80, maltose, fructose and glucose, $MgSO_4x7H_2O$ and $MnSO_4xH_2O$, plus vitamin mix containing cobalamin, folic acid, riboflavin, pyridoxine, pantothenic acid, thiamine (Stolz et al., 1995)) agar with addition of 100 mg L-1-cycloheximide to suppress the growth of yeast and incubated anaerobically for 48 hours at 30°C. For strain identification a total of 77 morphologically different colonies were picked from the plates and incubated in liquid mMRS4 at 30°C for 24 hours. DNA from each colony was isolated using DNeasy Blood & Tissue Kit (Qiagen, Missiauga, Canada). Clonal isolates were eliminated by RepPCR analysis with two primer pairs (Box2AR and GTG5). The 16S rRNA gene of 25 remaining isolates was amplified with PCR primers 616F and 630R, PCR products (about 1500 bp) cleaned-up using the Qiagen PCR Purification Kit and sequenced by Macrogen (Rockville, MD, USA). For taxonomic identification of isolates the 16S rRNA gene sequences were compared with the type strains of the Ribosomal Database (http://rdp.cme.msu.edu/). *L. rossiae* E1 strain was isolated at Prof. Haller lab (TÜ München, Germany) from fresh sourdough provided by Hofpfisterei GmbH on MRS plates (1.5% agar, 0.15% L-cystein, pH 5.4) and identified by colony morphology and 16S DNA sequencing. For 16S DNA sequencing total bacterial DNA

was isolated using DNeasy Blood and Tissue Kit according to the manufacturer's protocol for Gram-positive bacteria. The 16S DNA was amplified using 27F (5'-AGAGTTTGATCMTGGCTCAG -3') and 1492R (5'-TACGGYTACCTTACGACTT-3') primers (Biomers, Ulm, Germany) and DreamTaqTM DNA polymerase (Fermentas GmbH, St. Leon-Rot, Germany), the PCR product (about 1000 bp) was cleaned-up using PCR Clean-up Kit and sequenced by GATC-Biotech (Constance, Germany).

Bacterial culture

L. sanfranciscensis strains (DSM 23090, DSM 23091, DSM 23092, DSM 23093, DSM 23174, DSM 23200, DSM 23201) and *L. rossiae* (E1) isolated from Hofpfisterei GmbH sourdough, *L. sanfranciscensis* type strain DSMZ 20451 (DSMZ GmbH, Braunschweig,Germany), *L. plantarum FUA 3038* and *L. brevis 3113* (provided by Prof. Gänzle from University of Alberta, Canada*), L. paracasei* VSL#3 (provided by Dr. DeSimone, L'Aquila, Italy) were grown at 30°C in MRS broth (pH 5.4) containing freshly added 0.15% L-cystein under anaerobic conditions using Anaerogen packages (Anaerogen, Basingstoke, Oxoid, UK). Fixed bacteria (5% formaldehyde, 4 hours, 4°C) were washed three times with sterile PBS before use. To generate concentrated conditioned media (CM), bacteria ($5x10^7$ cfu/ml) from an overnight culture were transferred to DMEM (1% glutamine, 20 mM HEPES) and cultivated anerobically overnight at 30°C. Bacteria and bacterial supernatant (CM) were separated after centrifugation (4500 g, 10 min, RT). CM was adjusted to pH 7.4, filter sterilized (0.22 µm), and concentrated (100x) using Vivacell filter systems with an exclusion size of 100 kDa (Satorius Stedim Biotech, Goettingen, Germany). Concentrated conditioned media was diluted to 1x in the cell culture stimulation experiments. Agar plates were obtained by adding 1.5% of agar to the above described respective medium.

Bacterial DNA isolation

Bacterial total DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol for Gram-positive bacteria. The total DNA was amplified with Lactocepin PrtP specific primers (1073 forward: 5'-aagacttcggcaaagatagtgtg-3', 1074 reverse: 5'-aaagccggcagtgacataac-3', (Biomers, Ulm, Germany)) and the PCR product (100 bp) was loaded onto 1.5% agarose gel for size separation. The amplified DNA bands were visualized with visualized by Geneflash Imager (VWR, Ismaning, Germany).

Motility

The motility measurements were performed with corpus circular muscle preparations from Dunkin Hardley guinea pigs (Sulzfeld and Harlan Winkelmann GmbH, Borchen, Germany) Contractile force of the muscle was measured using force transducer in organ bath using LabChart 5 software (ADInstruments, Spechbach, Germany). Briefly, stomach muscle tissue was dissected from mucosa layer in continuously perfused ice-cold preparation Krebs solution (pH 7,4) ($MgCl_2*6H_2O$ 1,2 mM, $CaCl_2*2H_2O$ 2,5 mM, NaH_2PO_4 1,2 mM, NaCl 117 mM, $NaHCO_3$ 25 mM, $C_6H_{12}O_6$ 11 mM, KCl 4,7 mM). A 1.5 cm^2 piece of corpus circular muscle was cut out and mounted from both ends with polyamide thread between two electrodes into organ bath in 20 mL experimental Krebs solution (identical to preparation Krebs except for $NaHCO_3$ 20 mM) at 37°C and aerated continuously with Carbogen (95% O_2 and 5% CO_2). After an equilibration period of 45 min muscle preparations were stimulated by electrical field stimulation (EFS) to test vitality. The change in contractile force during EFS as well experimental treatment was measured by the force transducer. The time lapse between any treatments was always 20 min.

Ussing Chamber

The ion movement across intestinal epithelia was measured with Ussing chamber technique (Easy mount chambers, Physiologic instruments, San Diego, USA) and LabChart 5 software (ADInstruments, Spechbach, Germany). Briefly segments, of the distal colon of Dunkin Hardley guinea pigs (Sulzfeld and Harlan Winkelmann GmbH, Borchen, Germany) were dissected, the muscle layers removed and mucosa/submucosa preparations were mounted into slider with a recording area 0.5 cm^2. Apical and basolateral sides were bathed separately in 5 mL Krebs solution. During experimental procedures, the bath was maintained at 37°C and aerated continuously with Carbogen (95% O2 and 5% CO2). After an equilibration period of 45 min tissue was electrically stimulated (Parameters: stimulus strength 6V, duration 10 sec, frequency 10Hz, single pulse duration 0,5ms) to assess tissue vitality. For assessment of active ion transport spontaneous occurring transepithelial voltage (V_{TE}) formed by passive ion transport across the tissue was set to 0 mV by applying short circuit current (I_{SC}). When the active chloride ion secretion is induced an increase in I_{SC} is observed necessary to keep V_{TE} at 0 mV. The change in I_{SC} is equivalent to the current generated by the anions secretion

or cation absorption. Transepithelial resistance (TER= V_{TE}/I_{SC}*1000/2) of tissue was measured at the beginning and at the end of each experiment to assess the tissue integrity.

Statistical analysis

Data are expressed as mean values ± standard deviation (SD). All statistical computations were performed using SigmaPlot 11.0 software from Systat Software Inc. Data comparing treatment vs. corresponding control group were analyzed using unpaired t-tests. Data comparing several treatments vs. corresponding control group were analyzed using One-Way ANOVA followed by an appropriate multiple comparison procedure. If data was not normally distributed or comprised discontinuous data, non-parametrical tests (Mann-Whitney/Rank sum test, ANOVA on ranks) were used. Differences were considered significant if p-values were < 0.05 (*) or < 0.01 (**).

5. RESULTS

5.1. Sourdough bread feeding has variable effect in experimental models of colitis and ileitis.

5.1.1. Impact of sourdough bread feeding on intestinal inflammation in IL-10$^{-/-}$ mice with acute form of colitis

IL-10$^{-/-}$ mice spontaneously develop experimental colits over time. To evaluate the role of sourdough bread in the development of chronic intestinal inflammation, IL-10$^{-/-}$ mice were fed ad libitum three different diets: standard chow diet, chow with addition of sourdough bread, and chow with addition of analog bread.

Both sourdough and analog bread were dehydrated at 50°C for 4 hours in order to quantify the amount of bread eaten without the bread weight change due to drying and/or staling. Analog bread was prepared with identical ingredients compared to traditional sourdough bread with the exception the sourdough starter culture. The analog bread was prepared with addition of lactic and acetic acid to concentration and subsequent pH equivalent to sourdough bread. Thus analog bread was used to differentiate between the specific effects of LAB fermentation in sourdough bread outside of effects from organic acid production as well as bread ingerdients such as flour. The feeding started after weaning at week 4 of age and lasted for 20 weeks. Animals were sacrificed at the age of 24 weeks, weighed and sections of ceacum and entire colon were collected for histopathological scoring.

The bread and chow consumption was monitored over the 20 weeks of feeding and the mean energy consumption per week was calculated from the weighed in chow and bread. Chow (Ssniff pellets) has 16.3 kJ/g energy content, while sourdough bread has 9.2 kJ/g (information provided by Hofpfisterei GmbH, Germany). Ten mice (5 male and 5 female) fed chow consumed total of 63048 kJ, ten mice (5 male and 5 female) on chow plus analog bread diet 47327 kJ (28297 kJ from chow and 19030 kJ from bread), while ten mice (5 male and 5 female) on chow plus sourdough bread diet had lower energy consumption of 45556 kJ (21630 kJ from chow and 23926 kJ from bread). The differences in the energy consumption could be partially explained by the inaccuracy of weighing the feed and by the loss of bread due to crumbling.

Despite significant differences between the energy consumption of mice no weight variances were observed in IL-10$^{-/-}$ mice on different diets with average weight of 25 grams at 24 weeks of age across all diet groups (Fig. 4B). It may be speculated that the absorbable energy from sourdough bread is higher than that from either chow or analog bread. However this needs to be investigated by measuring the secreted energy such as in feaces and urine of animals on sourdough bread. Such measurements were not performed during this study.

A

	Total kJ consumed by male mice (N=5)		Total kJ consumed by female mice (N=5)		
Diet	**Ssniff pellets, kJ**	**Bread, kJ**	**Ssniff pellets, kJ**	**Bread, kJ**	**Total, kJ**
Chow	29079	-	33969	-	**63048**
Sourdough bread	14116	12359	7514	11568	**45556**
Analog bread	13611	10169	14686	8861	**47327**

B

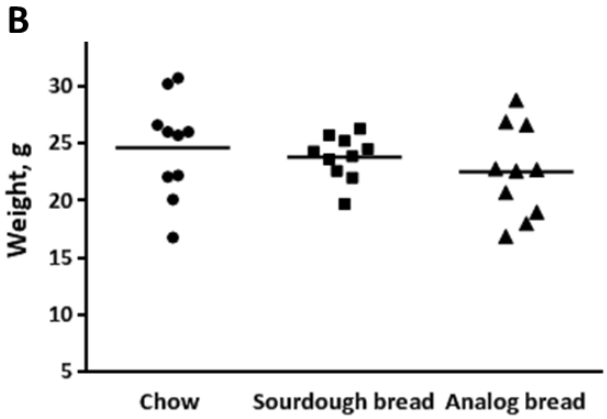


Figure 4: Weekly energy consumption (N=10/group) (A) and weight (B) at week 24 of age of IL-10$^{-/-}$ mice fed chow, chow plus sourdough bread and chow plus analog bread.
A. Total energy consumption by IL-10$^{-/-}$ mice fed chow, sourdough and analog bread was recorded over 20 weeks, and the energy consumption calculated. Each diet group had 10 animals with 5 male mice and 5 female mice. **B.** Weight of IL-10$^{-/-}$ mice mice (n=10 per group) at week 24 of age fed chow, chow plus sourdough bread and chow plus analog bread.

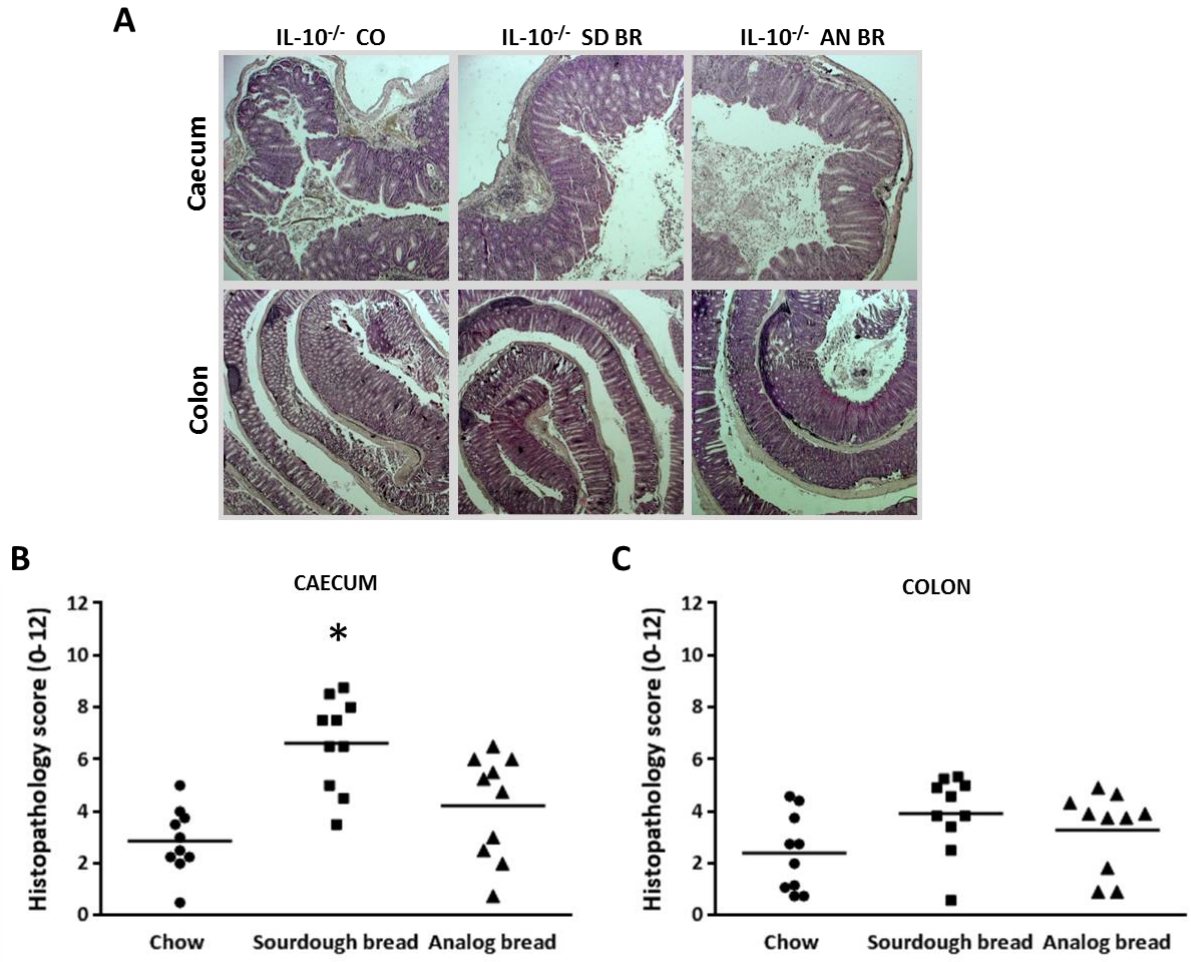


Figure 5: Sourdough bread feeding significantly increases the histopathological score in the ceacum but not in the colon of IL-10$^{-/-}$ mice.

The figure A shows representative H&E stained caecal tip tissue sections and entire colon (Swiss roll) with distal colon being on the outside and proximal colon on the inside of the tissue roll. The histopathology scores of differentially treated IL-10$^{-/-}$ mice (n=10 per group) in caecal tip (B) and colon (C) demonstrate that sourdough bread feeding has pro-inflammatory effect in the ceacum (*p<0.01) but not in the colon when compared to chow or chow plus analog bread feeding.

The histopathology score was significantly higher in the ceacum of IL-10$^{-/-}$ mice fed chow with addition of sourdough bread (average histopathology score of 3) compared to mice fed only chow (av. histopath. score of 4) and chow plus analog bread (av. histopath. score of 7) (Fig. 5B). However no differences were observed in the histopathology across the whole length of colon, with average score being between 2.5 and 4 (Fig. 5C).

5.1.2. Impact of sourdough bread feeding on the intestinal inflammation in TNF$^{\Delta ARE}$ mice with chronic form of ileitis

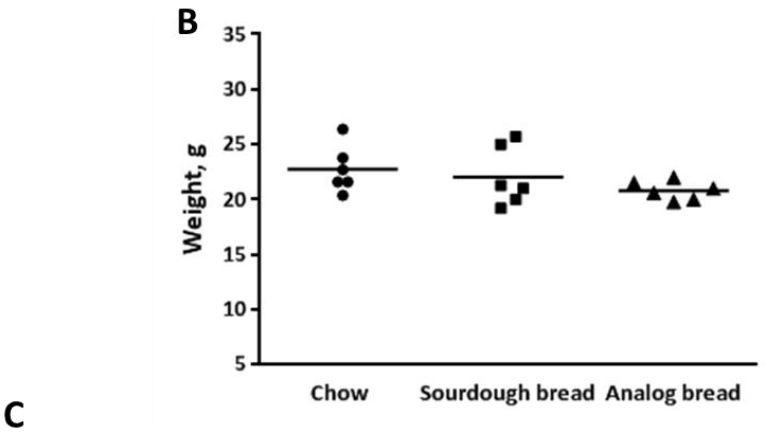
TNF$^{\Delta ARE}$ is another genetically engineered mouse model with a deletion of AU-rich elements (ARE) in the tumour necrosis factor (TNF) gene. These mice are characterized by elevated TNF levels and the development of spontaneous inflammation in the terminal ileum mimicking human CD. We fed these mice chow (Ssniff pellets), chow plus sourdough bread and chow plus analog bread for 8 weeks post-weaning. The feeding period, compared to IL-10$^{-/-}$ mice, was shorter before sacrifice since inflammation has earlier onset than in IL-10$^{-/-}$ mice. Animals were sacrificed at the age of 12 weeks, weighed and sections of ileum were collected for histopathology scoring.

The bread and chow consumption was monitored over the 8 weeks of feeding and the total energy consumption calculated form the weighed in chow and bread. TNF$^{\Delta ARE}$ and wild-type C57BL/6 mice were housed together the energy consumption is measured per diet group and includes for mice control and inflamed. Twelve mice (6 male and 6 female) fed chow consumed a total of 35640 kJ, twelve mice (6 male and 6 female) on chow plus analog bread diet 26512 (17340 kJ from chow and 9173 kJ from bread), while twelve mice (6 male and 6 female) chow plus sourdough bread diet had lower energy consumption of 23026 kJ (11516 kJ from chow and 11510 kJ from bread) (Fig. 6A).

TNF$^{\Delta ARE}$ mice had no differences in weight when fed different diets as is the case with IL-10$^{-/-}$ mice, with the average weight of all the mice on the different diets of 22.5 grams (Fig.6B). In addition, no change in histopathology was observed in the distal ileum of mice fed either sourdough or analog bread when compared to standard chow diet (Fig. 6C). The average histopathology score in all TNF$^{\Delta ARE}$ mice at 12 weeks was 5.5.

A

Diet	Total kJ consumed by male mice (N=6)		Total kJ consumed by female mice (N=6)		
	Ssniff pellets, kJ	Bread, kJ	Ssniff pellets, kJ	Bread, kJ	Total, kJ
Chow	18134	-	17506	-	**35640**
Sourdough bread	6326	6523	5190	4987	**23026**
Analog bread	9694	4771	7646	4402	**26512**



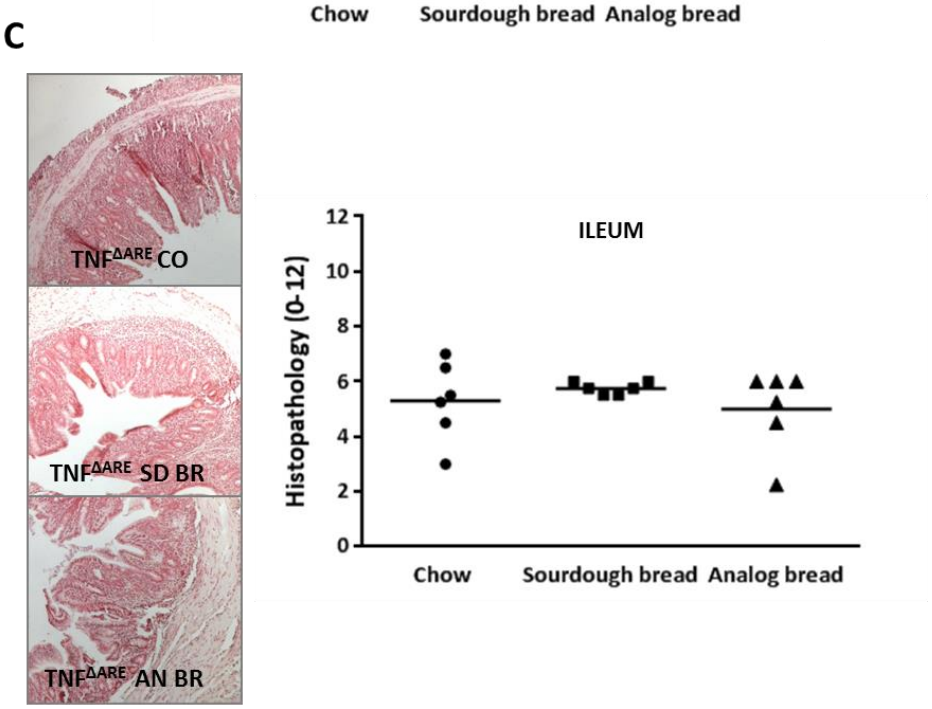


Figure 6: Average energy consumption per TNF$^{\Delta ARE}$ mice diet group (N=6/group) (A), weight of TNF$^{\Delta ARE}$ mice at 12 weekd (B) and ileum histopathology (C).

A. Total energy consumption by TNF$^{\Delta ARE}$ and wild-type C57BL/6 mice (housed together) over the course of 8 weeks feeding with chow, chow plus sourdough bread or chow plus analog bread. 6 male and 6 female mice per diet group. **B.** Weight of TNF$^{\Delta ARE}$ mice (n=6 per group with 3 males and 3 females) at week 12 of age. **C.** Representative H&E stained distal ileal tissue sections. TNF$^{\Delta ARE}$ CO - ileum tissue section of TNF$^{\Delta ARE}$ mouse on chow diet; TNF$^{\Delta ARE}$ SD BR - on chow plus sourdough bread; TNF$^{\Delta ARE}$ AN BR – on chow plus analog bread. Histopathology scores of distal ileum tissue sections from differentially treated TNF$^{\Delta ARE}$ mice, demonstrating that neither sourdough nor analog bread had protective or aggravating effect on ileal inflammation.

5.1.3. Sourdough bread feeding has no impact on weight and intestinal mucosa morphology of the wild-type mice.

C57Bl/6 and 129/Sv wild-type models served as healthy controls to $TNF^{\Delta ARE}$ and $IL\text{-}10^{-/-}$ mice, respectively, and were in fed ad libitum three different diets: standard chow diet, chow plus sourdough bread, and chow plus analog bread. The feeding started after weaning at week 4 of age. The C57Bl/6 and 129/Sv mice were sacrificed at week 12 and week 24 respectively. Animals weight was measured weekly over the course of feeding, sections of ileum, ceacum and colon were collected for evaluation of morphological changes.

A

	Total kJ consumed by male mice (N=5)		Total kJ consumed by female mice (N=5)		
Diet	Ssniff pellets, kJ	Bread, kJ	Ssniff pellets, kJ	Bread, kJ	Total, kJ
Chow	26916	-	20562	-	**47478**
Sourdough bread	15012	11597	15811	7300	**49720**
Analog bread	21206	7983	20196	6523	**55909**

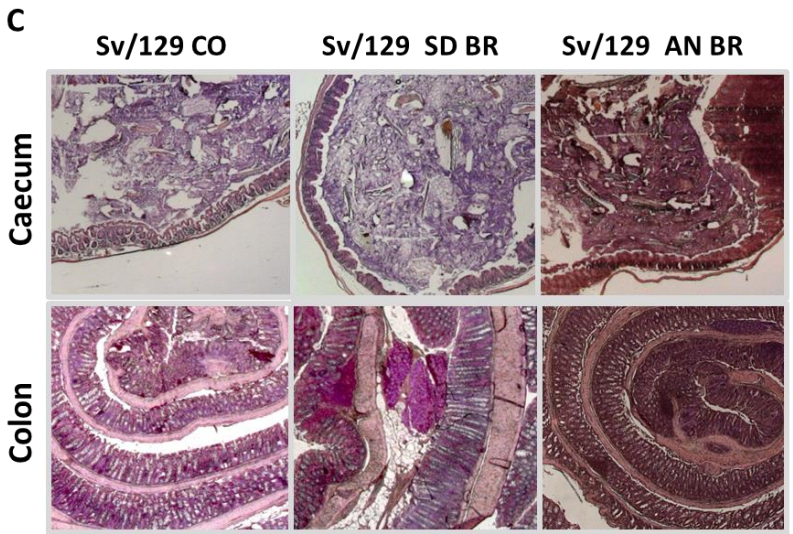


Figure 7: Addition of sourdough bread to the standard chow diet of wild-type 129/Sv mice has no impact on the weight and he morphology of the intestinal mucosa.
A. Total energy consumption by wild-type Sv/129 mice fed chow, sourdough bread and analog bread was recorded over the course of 20 weeks, and the energy consumption calculated. Each diet group had 10 animals with 5 male mice and 5 female mice. **B.** Weight of wild-type 129/Sv mice (n=10 per group) at week 24 of age fed chow, chow plus sourdough bread and chow plus analog bread. **C.** Representative tissue slides from the distal ileum of 129/Sv mice show no measurable changes between the groups of mice on different diets.

Energy consumption was measured and calculated according to the method used for IL-10$^{-/-}$ and TNF$^{\Delta ARE}$ mice. Similar to IBD-related models, on average 40% of total energy consumption was derived from sourdough bread in the both C57Bl/6 and 129/Sv mice groups fed chow and sourdough bread (Fig. 7A and 8A). 129/Sv animals on chow diet consumed a total of 47478 kJ, mice on chow plus analog bread diet 55909 kJ (41402 kJ from chow and 14507 kJ from bread), while mice on chow plus sourdough bread diet had energy consumption of 49720 kJ (30823 kJ from chow and 18897 kJ from bread) (Fig. 7A). C57Bl/6 mice energy consumption was measured together with TNFΔARE mice as the animals had common housing (Fig. 8A).

A

Diet	Total kJ consumed by male mice (N=6)		Total kJ consumed by female mice (N=6)		Total, kJ
	Ssniff pellets, kJ	Bread, kJ	Ssniff pellets, kJ	Bread, kJ	
Chow	18134	-	17506	-	**35640**
Sourdough bread	6326	6523	5190	4987	**23026**
Analog bread	9694	4771	7646	4402	**26512**

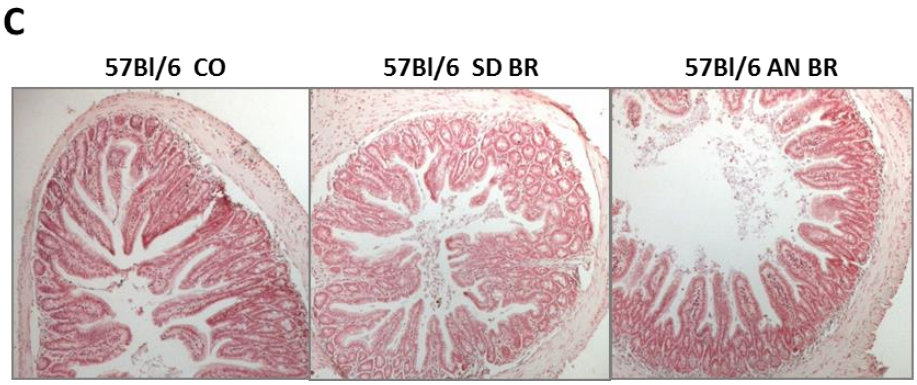


Figure 8: Addition of sourdough bread to the standard chow diet of wild-type C57Bl/6 mice has no impact on the weight and physiology of the intestinal lining.
A. Total energy consumption by wild-type C57Bl/6 mice and TNF$^{\Delta ARE}$ (mice were housed together) over the course of 8 weeks fed chow, chow plus sourdough bread or chow plus analog bread. 6 male and 6 female mice per diet group. **B.** Weight of wild-type C57Bl/6 mice (n=6 per group) at week 12 of age fed chow, chow plus sourdough bread and chow plus analog bread. **C.** Representative tissue slide from the distal ileum of wild-type C57Bl/6 mice show no detectable alterations between the groups of mice on different diets.

No significant changes in the weight of animals on different diets were recorded, with average weight of 129/Sv mice at 24 weeks of 27.5 grams and average weight of C57Bl/6 mice at 12 weeks of 25 grams (Fig. 7B and 8B). Furthermore no morphological changes were observed in the tissue slices of ileum mucosa of C57Bl/6 (Fig. 7C) and ceacum and colon mucosa of 129/Sv mice fed different diets (Fig. 8C).

5.2. LC-MS/MS analysis of metabolites in the sourdough bread and the metabolites in the intestine and blood of mice fed sourdough bread

5.2.1. LC-MS/MS analysis of metabolites in sourdough bread, analog bread, and raw sourdough

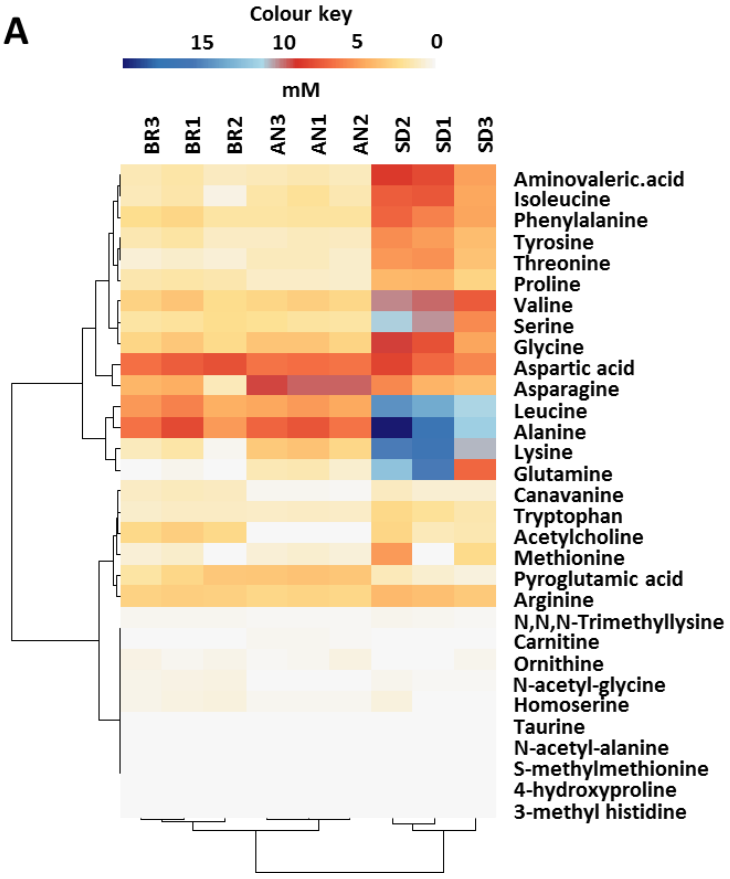
Sourdough fermented bread and analog as well as raw sourdough metabolites were analyzed using LC-MS/MS to elucidate the differences brought about by the fermentation process. Water soluble extracts (<10 kDa, triplicates) of raw sourdough, sourdough bread and analog bread were prepared from three different batches and subjected to LC-MS/MS analysis. Dry mass of freeze dryed water soluble extracts on average constituted 10% of the dry total bread mass. For the concentration of all analytes see Appendix. The concentration of metabolites in extracts was determined by comparison to the standard solution with known concentration of metabolites.

The concentration of metabolites in each sample was visualized using statistical freeware R with a heatmap (Fig. 9A). In the heatmap the maximum to minimum concentration range is depicted by the change from dark blue (highest – 20 mM) to light blue/red (middle 10 mM) to yellow (minimum 0.01 mM). The metabolites were also depicted in the relative concentration heatmap where red represents the highest concentration for the given metabolite and blue the lowest (Fig. 9B). Raw sourdough visualy stands out due to high concentration of free amino acids.

To estimate if there are significant differences in metabolites' concentrations between different extracts, multivariate analysis was performed using software R. PCA analysis substantiated significant differences in metabolites isolated from sourdough, sourdough bread and analog bread with clear separation of components on the PCA plot with 95% confidence eclipse (Fig. 9C and 9D). Raw sourdough has significantly higher amounts of free amino acids, depicted with arrows pointing to the right of the plot and

pulling the components of the raw sourdough samples to the right. High amino acids can be explained by the proteolitic acitivity of endogenous flour enzymes as well as LAB proteases. However this difference is diminished in baked sourdough bread. Before baking fresh unfermented flour is added to the sourdough what could explain lower relative amount of free amino acid comparable to that of unfermented analog bread. Only two metabolites, acetylcholine and canavanine, were found to be present in both sourdough and sourdough bread in significantly higher amounts than in analog bread (Fig. 10). The concentration of acetylcholine in sourdough bread extract was 2.6 mM, and 1.8 mM in raw sourdough while none was detected in analog bread extract. The differences in concentration between raw sourdough and sourdough bread may be due to extraction efficiencies. Canavanine concentration in analog bread, sourdough bread and raw sourdough was 0.2 mM, 1.2 mM and 1 mM respectively. Fermentation by sourdough is the only difference between sourdough bread and analog bread, suggesting the compounds are produced by microorganisms present in sourdough starter culture. In summary, metabolism of amino acid asparagine and carnitine, in combination with production of acetylcholine and canavanine have significant influence on separation of the sourdough and sourdough bread from analog bread components in PCA.

Canavanine was shown to be toxic to bacteria and yeast, as well as cancer cells *in vitro* (Ho Jang et al., 2002). If this could be one of the bioactive compounds secreted by sourdough microorganisms remains a speculation at this stage. Acetylcholine on the other hand is a well-known neurotransmitter abundant in most of the organisms and is discussed in more detail later.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BR2
AN3
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AN2
SD2
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SD3
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Phenylalanine
Tyrosine
Threonine
Proline
Valine
Serine
Glycine
Aspartic acid
Asparagine
Leucine
Alanine
Lysine
Glutamine
Canavanine
Tryptophan
Acetylcholine
Methionine
Pyroglutamic acid
Arginine
N,N,N-Trimethyllysine
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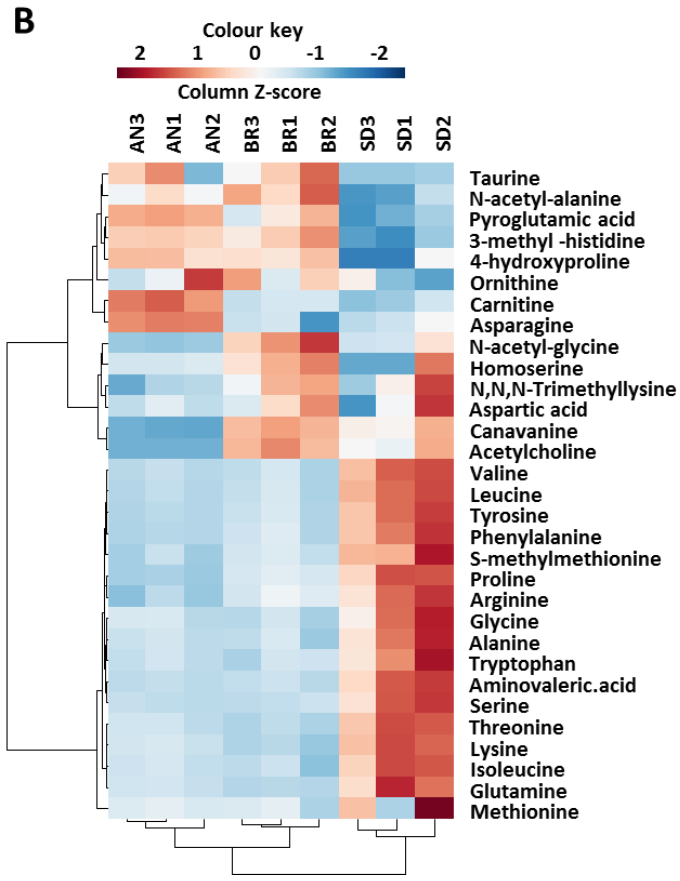
4-hydroxyproline
3-methyl histidine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AN2
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BR1
BR2
SD3
SD1
SD2
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N-acetyl-alanine
Pyroglutamic acid
3-methyl -histidine
4-hydroxyproline
Ornithine
Carnitine
Asparagine
N-acetyl-glycine
Homoserine
N,N,N-Trimethyllysine
Aspartic acid
Canavanine
Acetylcholine
Valine
Leucine
Tyrosine
Phenylalanine
S-methylmethionine
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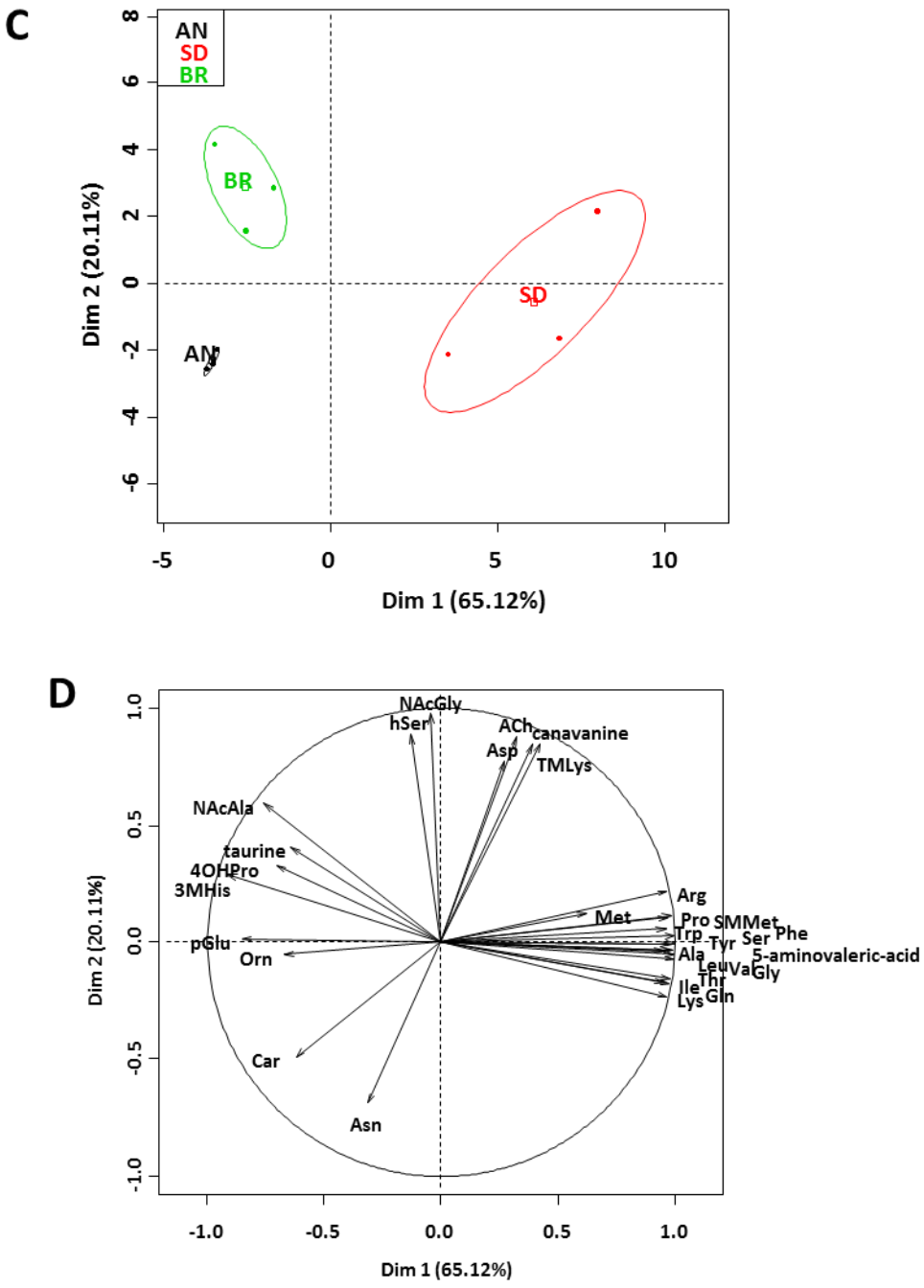


Figure 9: Significantly higher concentration of free amino acids was observed in the in water extracts of raw sourdough vs. sourdough bread and analog bread.
A. Heatmap calculated from absolute concentration of metabolites in water extracts of raw sourdough (SD), sourdough bread (BR) and analog bread (AN) in triplicates. Water extracts constitute 10% of total dough or bread dry mass. **B.** Scaled heatmap calculated from relative concentration of metabolites in each extract. **C and D.** PCA (principal component analysis) with confidence eclipse of 95% of the concentration of metabolites depicts significant differences in metabolites concentration between sourdough (SD), sourdough bread (BR) and analog bread (AN). **D.** The weight of each individual metabolite in the separation of the components of PCA plot. Arrow's direction indicates the course of metabolite's impact in the component distribution on the PCA plot.

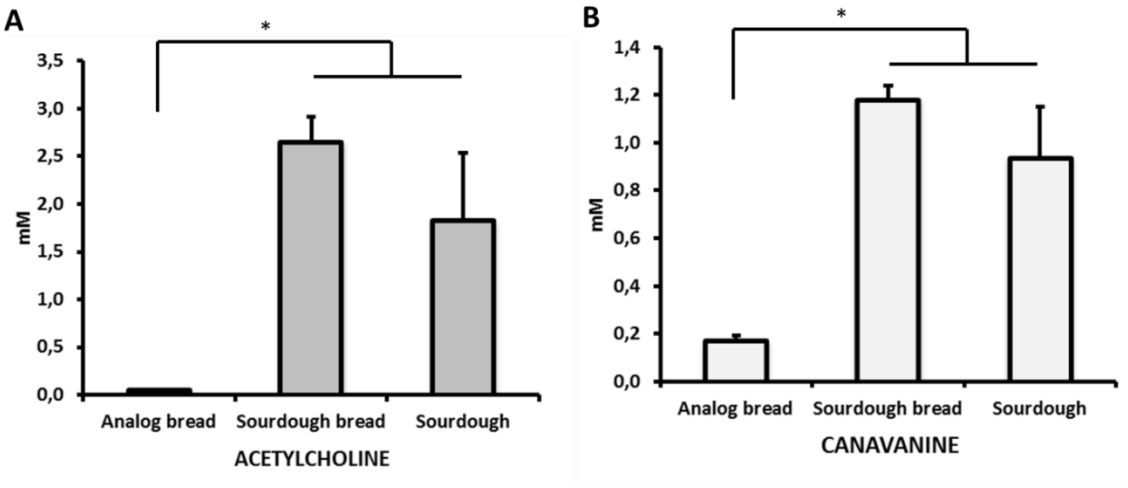
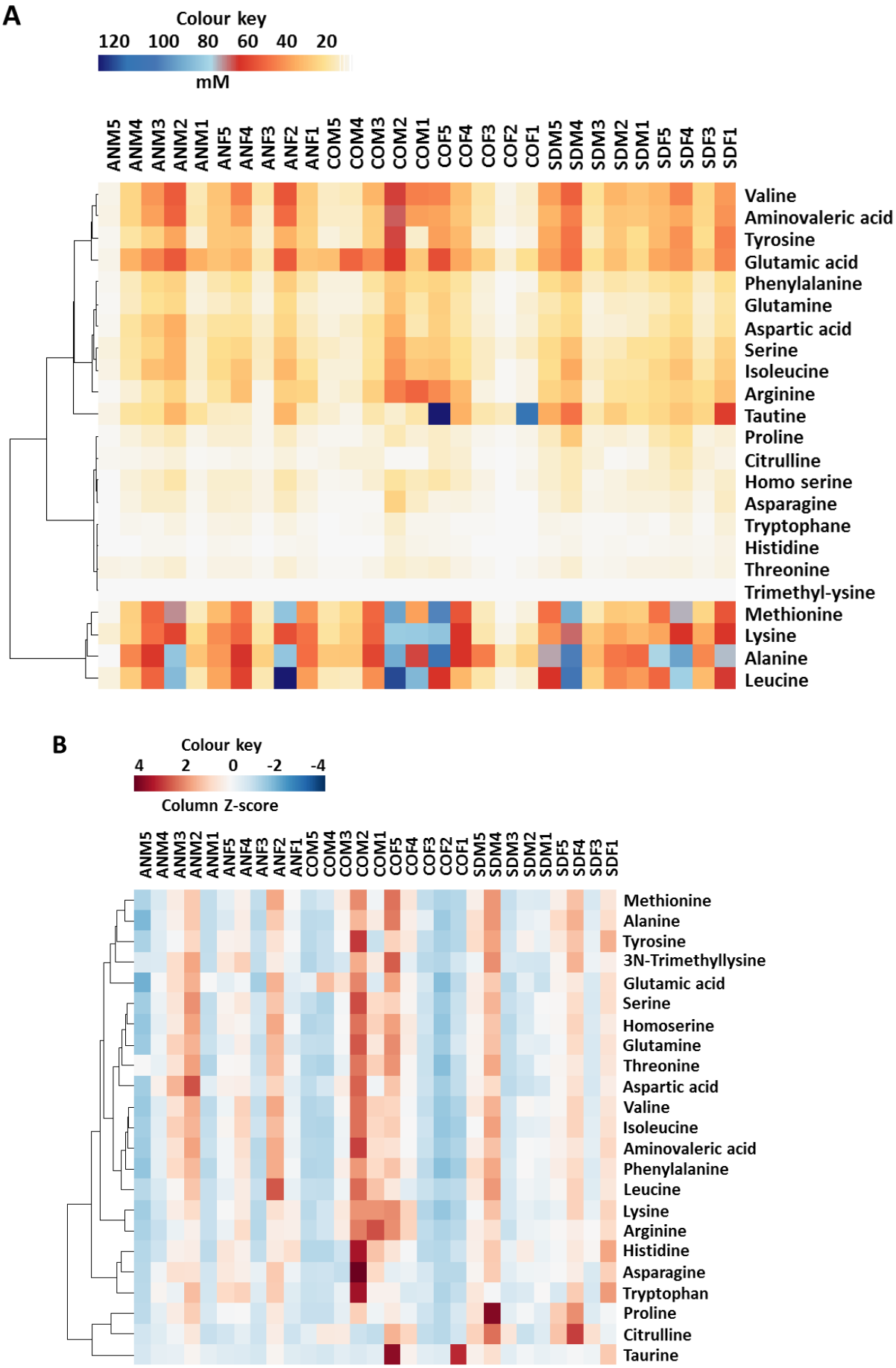


Figure 10: The levels of acetylcholine and canavanine metabolites are significantly higher in fermented sourdough bread and raw sourdough extracts when compared to unfermented analog bread.
A. Concentration of acetylcholine in sourdough, sourdough fermented bread and unfermented analog bread extracts**. B.** Concentration of canavanine in sourdough, sourdough fermented bread and unfermented analog bread extracts (* $p<0.01$).

5.2.2. LC-MS/MS analysis of metabolites in the ceacum of inflamed IL-10$^{-/-}$ mice.

The feeding of sourdough bread to IL-10$^{-/-}$ mice with acute form of colitis led to significantly increased histopathological scores in the ceacum of mice. In order to elucidate if the intestinal metabolic profile can be associated with augmented inflammation, the ceacal content of IL-10$^{-/-}$ mice was collected, lyzed and subjected to LC/MS-MS for identification and quantification of metabolites. The concentration of all analytes is given in the Appendix.

The concentration of metabolites in each mouse fed different diet was visualized using heatmap of absolute concentrations (Fig. 11A) and heatmap of scaled relative concentration of each metabolite (Fig. 11B). PCA analysis did not show any significant differences in the metabolites concentration in the ceacum of mice fed different diets depicted in the PCA plot where no group-wise component separation can be observed (Fig. 11C). Furthermore neither acetylcholine nor canavanine found in sourdough bread could be detected in the ceacal content of mice fed sourdough bread.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Valine
Aminovaleric acid
Tyrosine
Glutamic acid
Phenylalanine
Glutamine
Aspartic acid
Serine
Isoleucine
Arginine
Tautine
Proline
Citrulline
Homo serine
Asparagine
Tryptophane
Histidine
Threonine
Trimethyl-ysine
Methionine
Lysine
Alanine
Leucine
B
Colour key
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Alanine
Tyrosine
3N-Trimethyllysine
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Serine
Homoserine
Glutamine
Threonine
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Valine
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Taurine

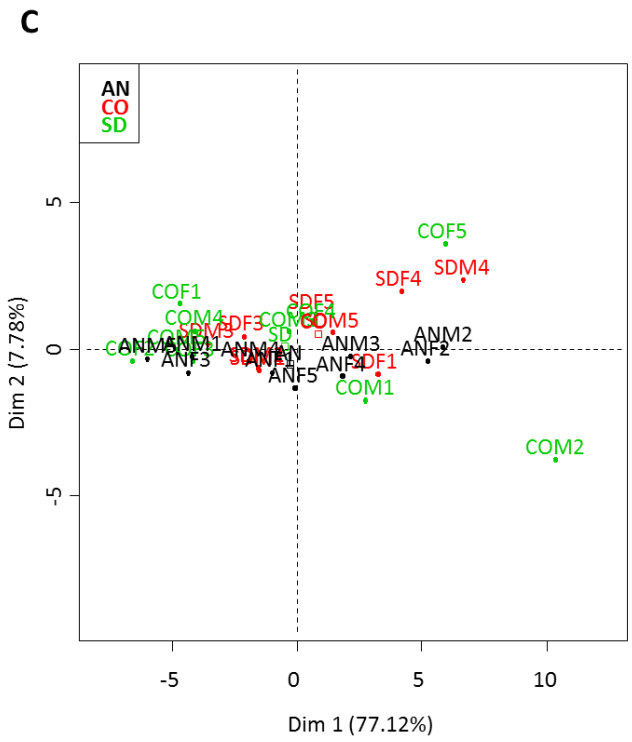
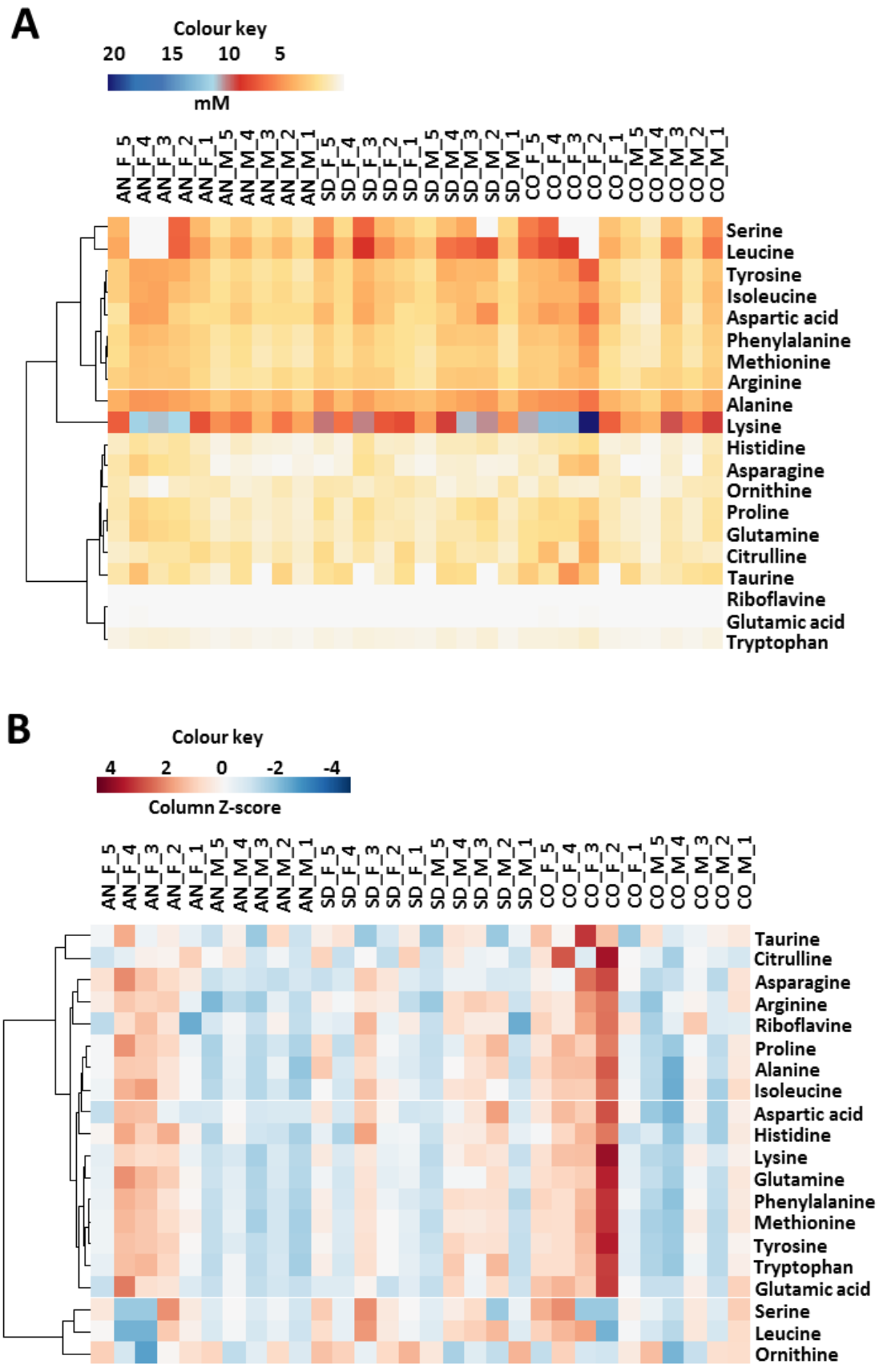


Figure 11: No difference was observed in the concentration of metabolites in the ceacum of IL-10$^{-/-}$ mice fed chow, chow plus sourdough bread and chow plus analog bread.
A. Heatmap of absolute metabolites concentration in the mouse ceacum. **B.** Scaled heatmap of relative concentration of metabolites in each animal. AN - analog bread and chow group. SD – sourdough bread and chow group, CO – chow group. M – male. F – female. **C.** PCA analysis of metabolites shows no significant differences in metabolites concentration between different diet groups.

5.2.3. LC-MS/MS analysis of metabolites in the ceacum of wild type mice.

Ceacal content of wild-type mice fed different diets was collected upon sacrifice, lyzed, and subjected to LC-MS/MS analysis to elucidate the impact of sourdough bread feeding on the digestive activity of host and its gut microbiota. The metabolites concentration were depicted using heatmaps and analysed with PCA as described earlier. The concentrations of all analytes are given in the Appendix. The metabolites were analyzed in the wild-type 129/Sv (Fig. 12) and C57Bl/6 mice (Fig. 13). The PCA analysis did not show any significant differences in the metabolites in the ceacum of mice fed sourdough bread as compared to analog bread and control chow group.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Serine
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Methionine
Arginine
Alanine
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Histidine
Asparagine
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Proline
Glutamine
Citrulline
Taurine
Riboflavine
Glutamic acid
Tryptophan
B
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Taurine
Citrulline
Asparagine
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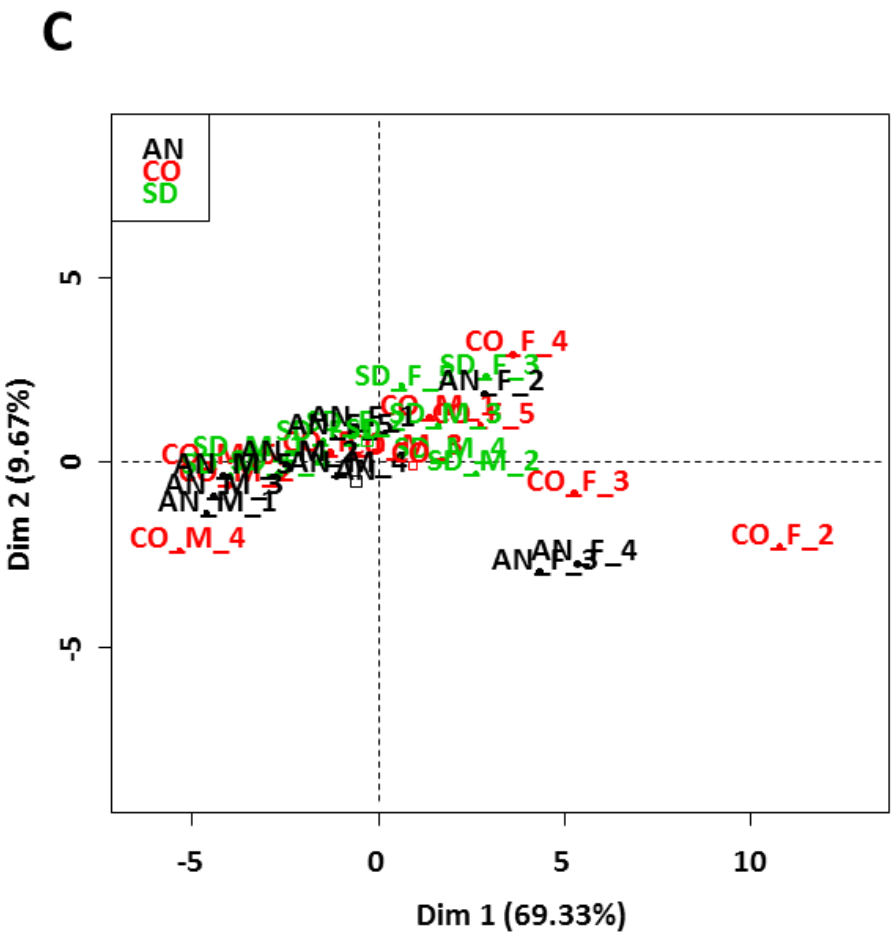
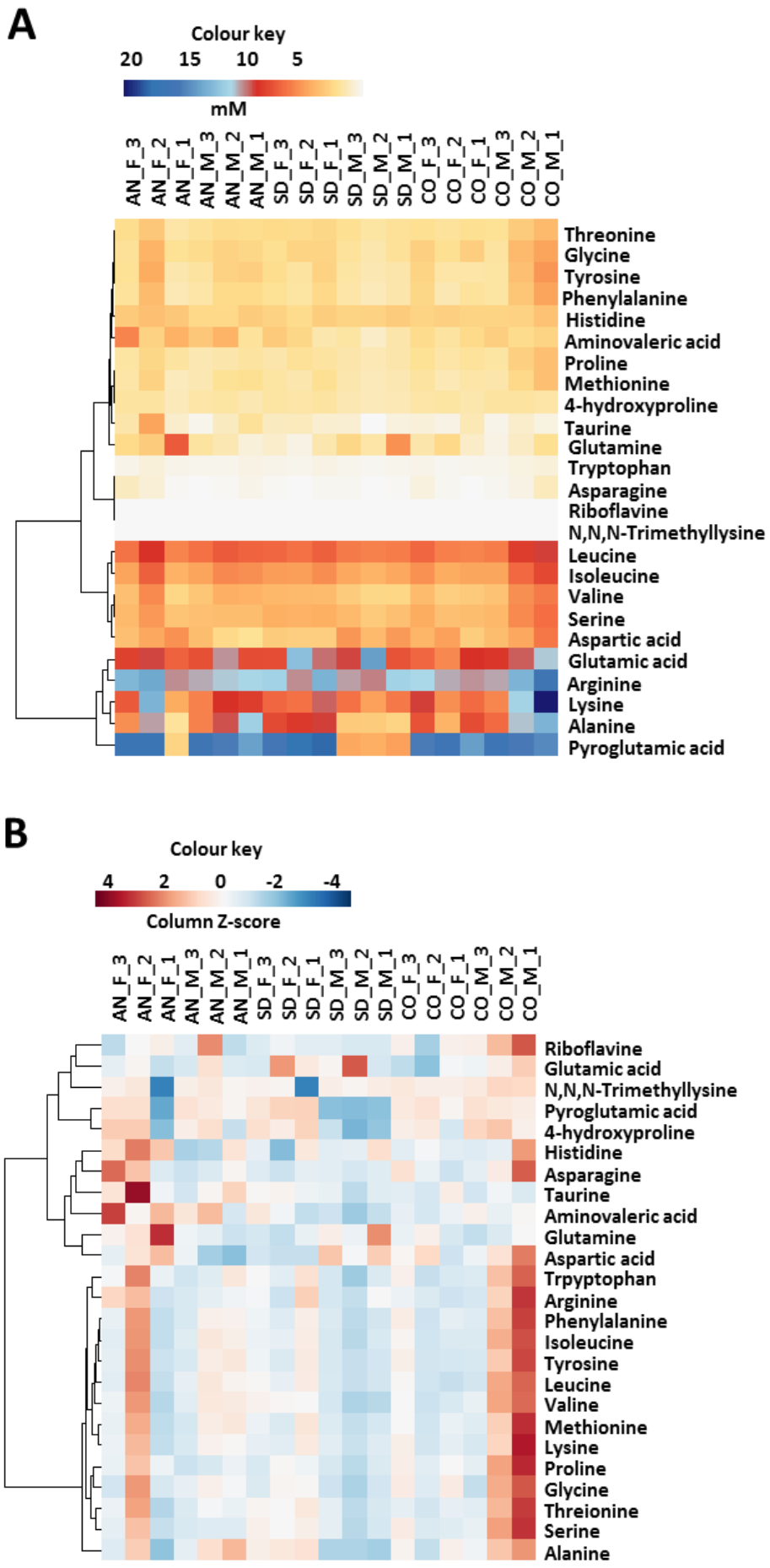


Figure 12: No difference was observed in the concentration of metabolites in the ceacum of wild-type 129/Sv mice fed chow, chow plus sourdough bread and chow plus analog bread.

A. Heatmap of absolute metabolites concentration in the mouse ceacum. **B.** Scaled heatmap of relative concentration of metabolites in each animal. AN - analog bread and chow group. SD – sourdough bread and chow group, CO – chow group. M – male. F – female. **C.** PCA analysis of metabolites shows no significant differences in metabolites concentration between different diet groups.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B
Colour key
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Glutamic acid
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Pyroglutamic acid
4-hydroxyproline
Histidine
Asparagine
Taurine
Aminovaleric acid
Glutamine
Aspartic acid
Trpyptophan
Arginine
Phenylalanine
Isoleucine
Tyrosine
Leucine
Valine
Methionine
Lysine
Proline
Glycine
Threionine
Serine
Alanine

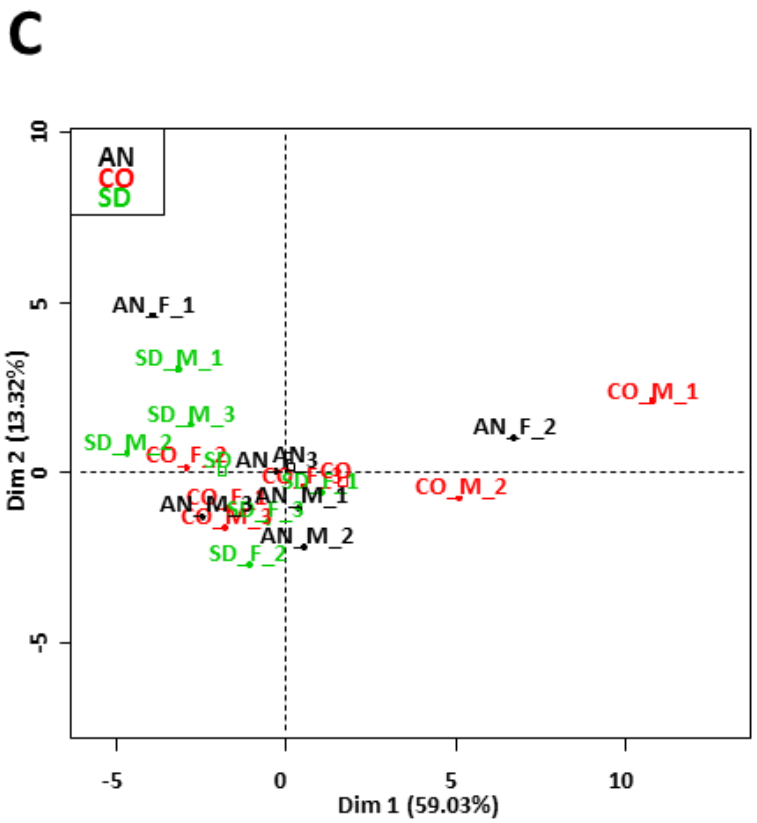
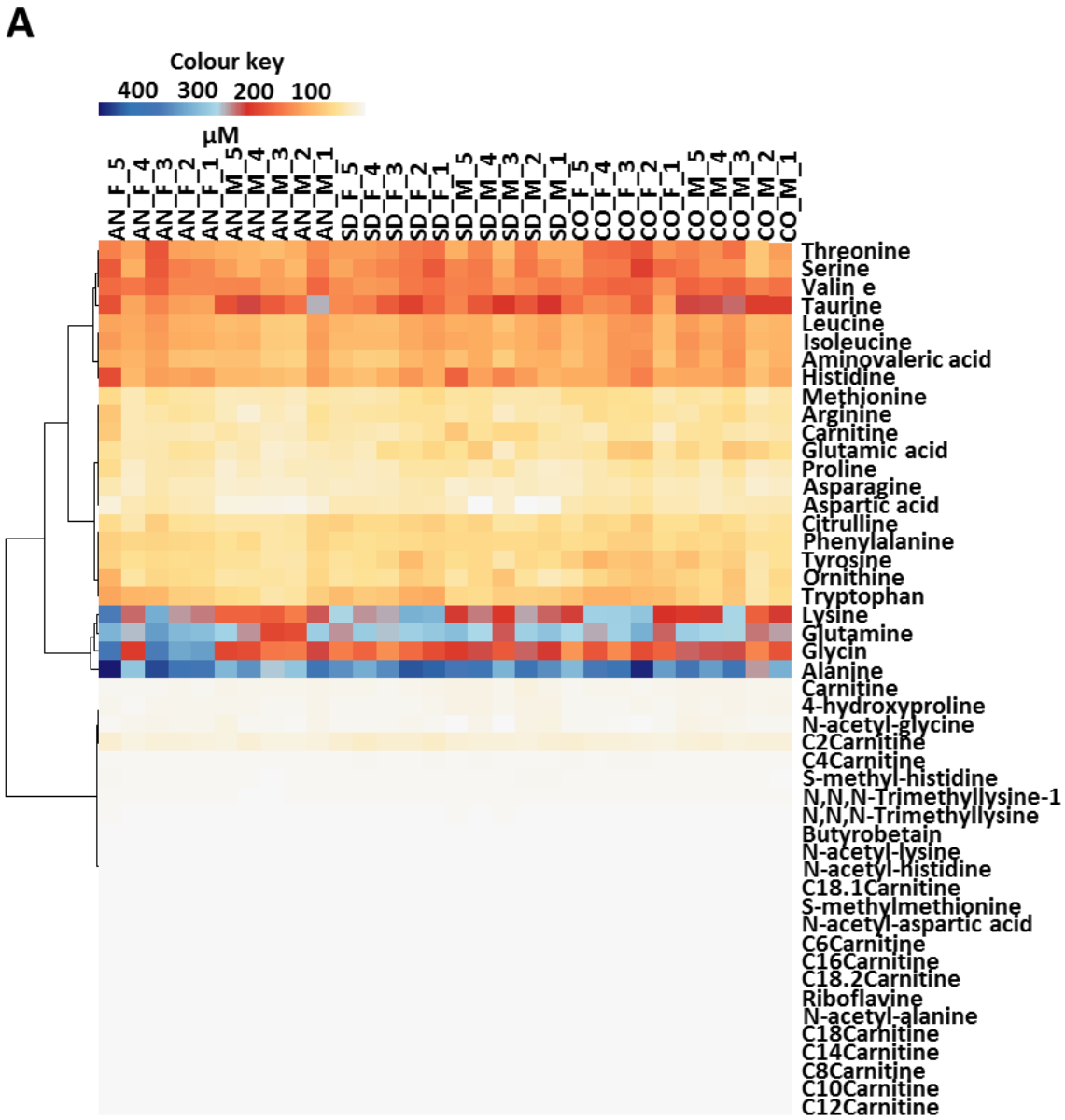


Figure 13: No difference was observed in the concentration of metabolites in the ceacum of wild-type C57Bl/6 mice fed chow, chow plus sourdough bread and chow plus analog bread.
A. Heatmap of absolute metabolites concentration in the mouse ceacum. **B.** Scaled heatmap of relative concentration of metabolites in each animal. AN - analog bread and chow group. SD – sourdough bread and chow group, CO – chow group. M – male. F – female. **C.** PCA analysis of metabolites shows no significant differences in metabolites concentration between different diet groups.

5.2.4. Metabolic profile of the plasma from the wild-type mice fed sourdough bread

In parallel to ceacal content metabolites, metabolites in plasma of mice were measure using LC-MS/MS analysis to estimate the impact of sourdough consumption at the systemic level. The metabolites concentration were depicted using heatmaps and analysed with PCA as described earlier. The concentrations of all analytes are given in the Appendix. The metabolites were analyzed for the wild-type 129/Sv group (Fig. 14) and C57Bl/6 group (Fig. 15). Analogous to caecal content profile no significant variations were observed in the plasma metabolites concentration between three different mice groups. This is in line with the earlier observation that sourdough bread feeding does not have no significant effect on the weight, the morphology of the intestinal lining and the metabolites in the caecum.



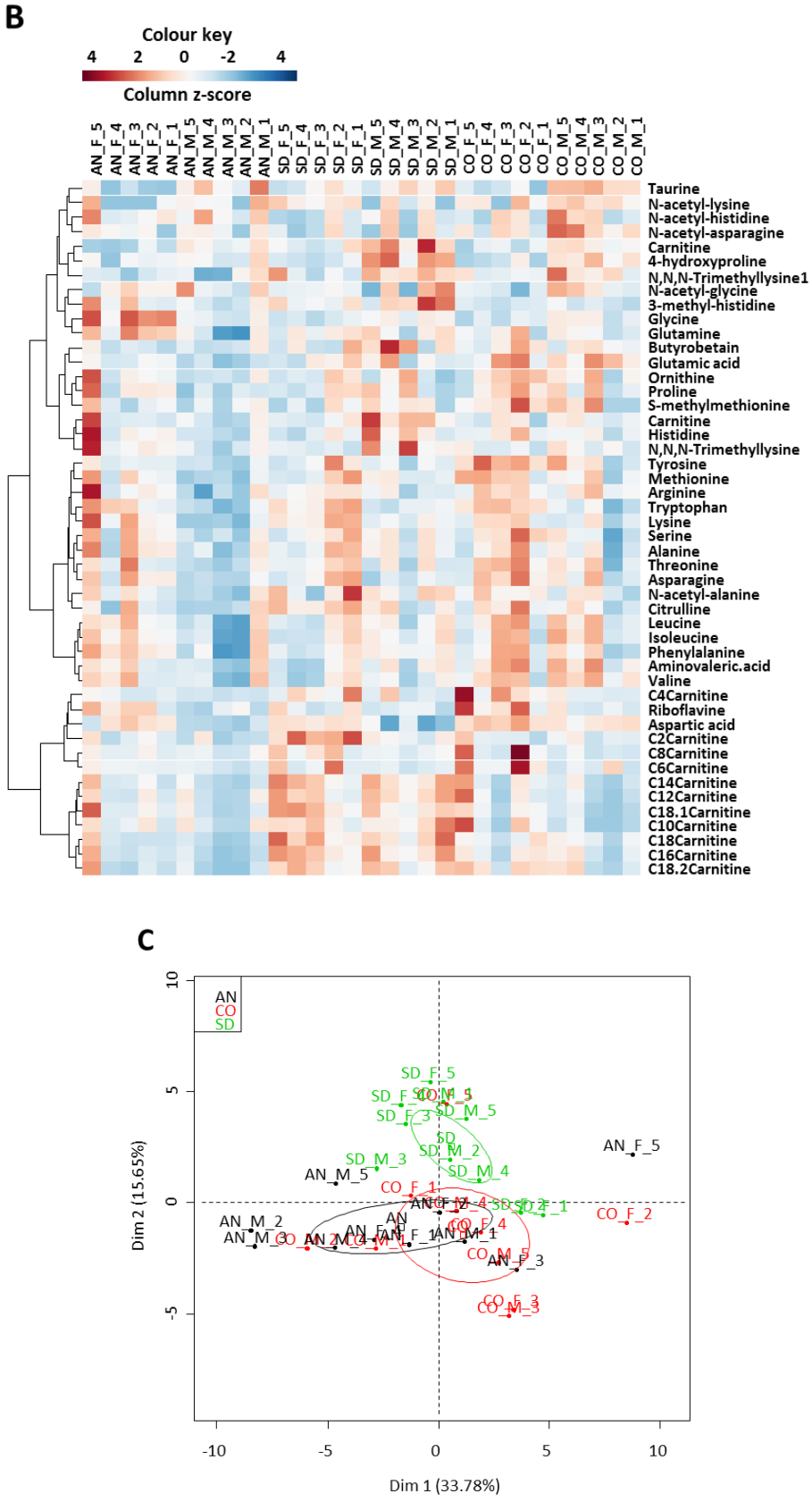
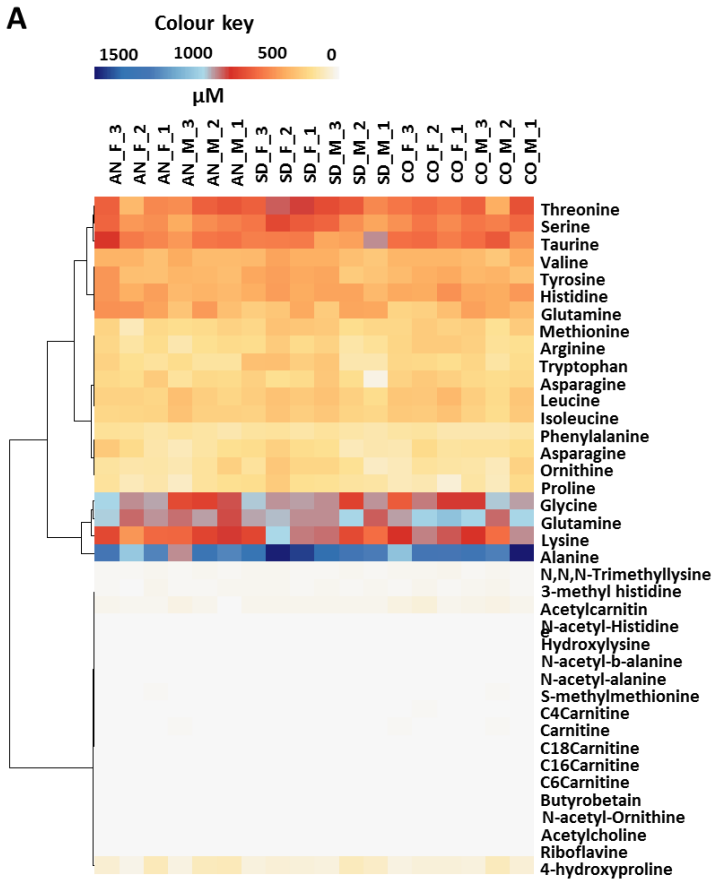


Figure 14: No difference was observed in the concentration of metabolites in plasma of wild-type 129/Sv mice fed chow, chow plus sourdough bread and chow plus analog bread.

A. Heatmap of absolute metabolites concentration in plasma of 129/Sv mice. **B.** Scaled heatmap of relative concentration of metabolites in each animal. AN - analog bread and chow group. SD – sourdough bread and chow group, CO – chow group. M – male. F – female. **C.** PCA analysis with confidence eclipse of 95% of metabolites shows no significant differences in metabolites concentration between different diet groups.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Proline
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Lysine
Alanine
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N-acetyl-Histidine
Hydroxylysine
N-acetyl-b-alanine
N-acetyl-alanine
S-methylmethionine
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Carnitine
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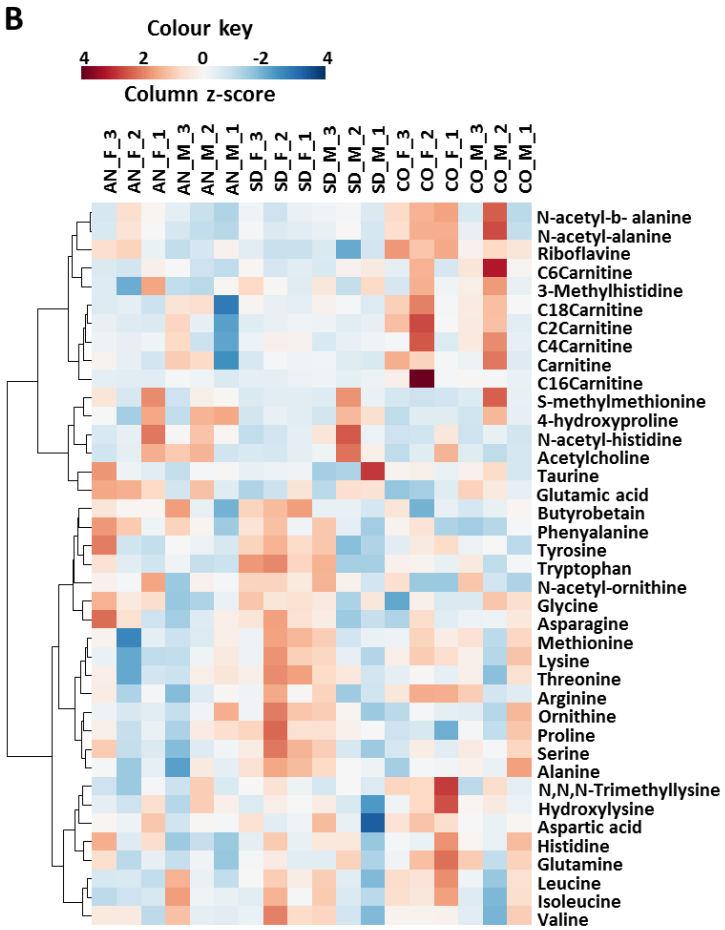
N-acetyl-Ornithine
Acetylcholine
Riboflavine
4-hydroxyproline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C18Carnitine
C2Carnitine
C4Carnitine
Carnitine
C16Carnitine
S-methylmethionine
4-hydroxyproline
N-acetyl-histidine
Acetylcholine
Taurine
Glutamic acid
Butyrobetain
Phenyalanine
Tyrosine
Tryptophan
N-acetyl-ornithine
Glycine
Asparagine
Methionine
Lysine
Threonine
Arginine
Ornithine
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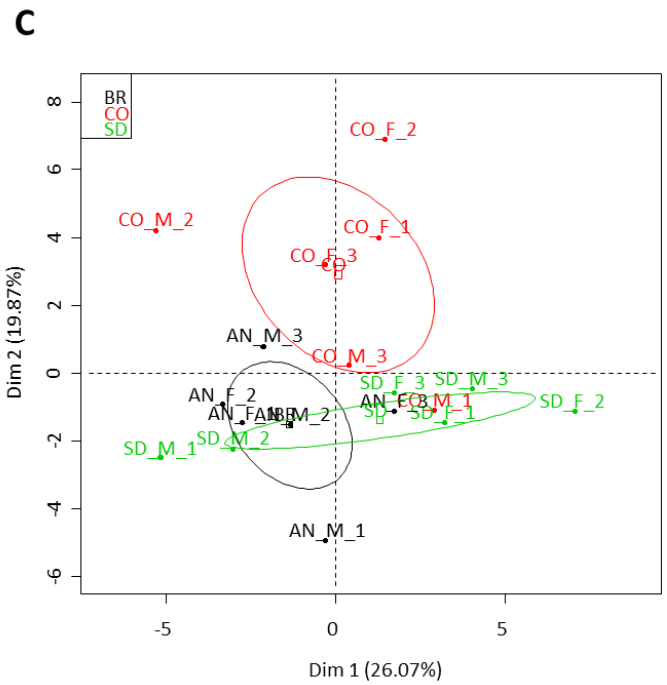


Figure 15: No difference was observed in the concentration of metabolites in plasma of wild-type C57Bl/6 mice fed chow, chow plus sourdough bread and chow plus analog bread.
A. Heatmap of absolute metabolites concentration in plasma of 57Bl/6 mice. **B.** Scaled heatmap of relative concentration of metabolites in each animal. AN - analog bread and chow group. SD – sourdough bread and chow group, CO – chow group. M – male. F – female. **C.** PCA analysis with confidence eclipse of 95% of metabolites shows no significant differences in metabolites concentration between different diet groups.

There is a separation trend observed in the PCA plots between control/chow group and analog as well as sourdough bread groups of mice. This could be explained by consumption of basic bread components, such as wheat and rye flour, but not due to fermentation as there is no clear separation between analog and sourdough groups.

Neither acetylcholine nor canavanine were detected in the ceacum or plasma of mice fed sourdough bread.

5.2.5. 2D-SDS gel electrophoresis of proteins isolated from the gut of wild-type mice

The effect of sourdough bread feeding on the expression of proteins in the intestinal mucosa of wild-type mice was investigated employing proteomics. The proteins were isolated from the ileum epithelial cells of C57Bl/6 mice and proximal colon tissue of 129/Sv mice and subjected to 2D-SDS gel electrophoresis. The difference in the protein spots was analyzed using following criteria: Mann-Whitney test $p<0.05$, fold change compared to chow control group $0.8>FC>1.2$ and spot frequency at least 2 out of 4 gels analysed. Selected spots were picked, digested and subjected to MALDI-TOF sequence identification. No proteins were consistently identified by MALDI-TOF as significantly regulated in the sourdough bread group as compared to analog bread and/or chow group.

5.3. Sourdough and sourdough bread extracts do not inhibit secretion of pro-inflammatory compounds *in vitro*

5.3.1. Effect of sourdough extracts *in vitro* on the secretion of pro-inflammatory chemokine IP-10 by IECs

In order to identify bioactive molecules in sourdough in the context of inflammatory response TNF-activated intestinal epithelial cell-line Mode-K was stimulated with water extracts of raw sourdough, baked sourdough bread and baked analog bread. TNF activation of Mode-K cells stimulates production and secretion of pro-inflammatory chemokine IP-10 into cell medium. Therefore we measured the conectration of IP-10 in the cell media with and without sourdough extracts.

The extracts were re-suspended to 25% in MilliQ water, filter-sterilized and added to the cell culture to a final concentration of 0.1%. The 0.1% concentration was the highest achieveable without causing cytotoxicity. The extracts of sourdough, analog as well as sourdough bread do not have significant effect on the IP-10 production and/or secretion by TNF-activated Mode-K cells. Sourdough extracts also did not significantly induce non-activated Mode-K (Fig. 16).

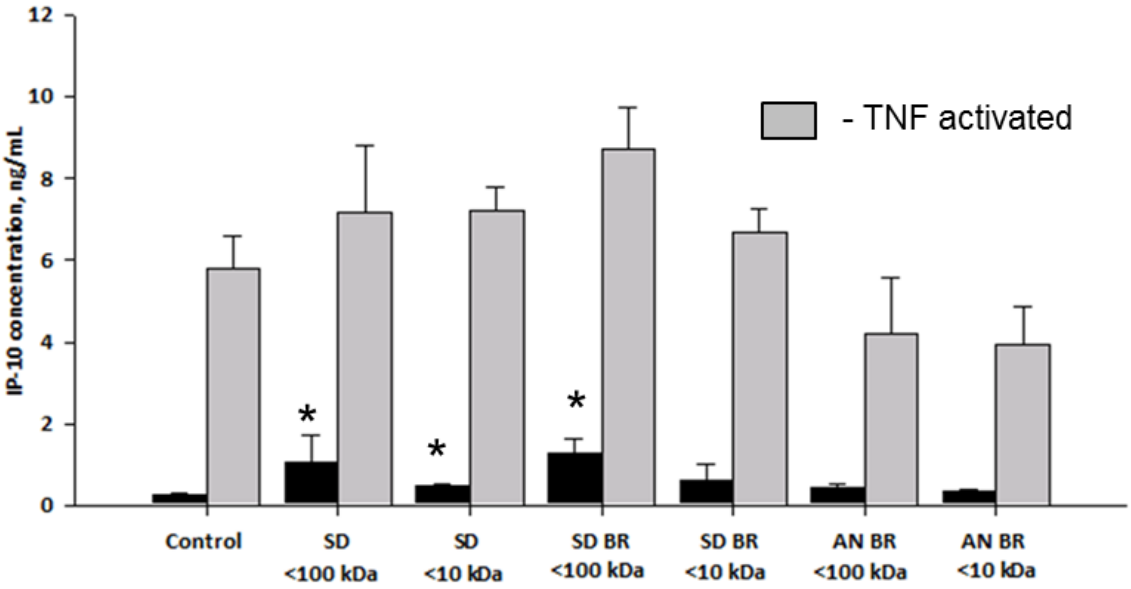


Figure 16: Impact of sourdough, sourdough bread and analog bread extracts on the secretion of pro-inflammatory chemokine IP-10 by intestinal epithelial cells activated with TNF.
The figure shows the mean concentration of IP-10 in the culture media of Mode-k cells as measured by ELISA in triplicates. The cells where incubated for 24 hours with 0.1% of water extracts (SD – raw sourdough, SD BR – sourdough bread, AN BR – analog bread; <100 kDa –fraction of water soluble extract between 100 kDa and 10 kDa, <10 kDa – fraction of water soluble extract under the size of 10 kDa). Sourdough extracts demonstrated no significant effect on the secretion of IP-10 by unstimulated Mode-K cells (■) and had no inhibitory effect on IP-10 secretion when cells where activated by 10 ng/mL TNF (□). *p<0.05 when compared to untreated control (■).

5.3.2. Effect of sourdough extracts on the secretion of pro-inflammatory cytokine TNF by murine macrophages

Extracts of raw sourdough, baked sourdough bread and baked analog bread were further tested for effects on the secretion of pro-inflammatory cytokine TNF RAW 264 macrophage cell line. LPS-stimulation of macrophages leads to production of TNF. Therefore we measured the concentration of IP-10 in the cell media with and without sourdough extracts. Extracts exhibited significantly induced the secretion of TNF by unstimulated RAW 264.7 cells and did not inhibit TNF secretion by LPS-activated RAW 264.7 (Fig. 17).

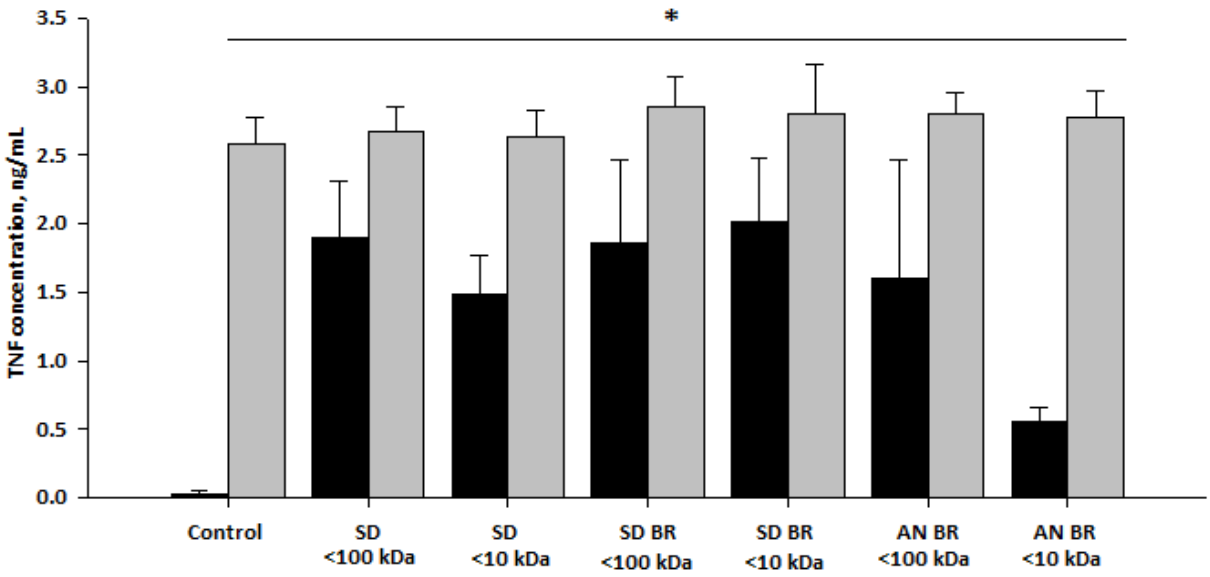


Figure 17: Sourdough extracts have significant stimulatory effect on the secretion of pro-inflammatory cytokine TNF by the macrophages.
The figure shows the mean concentration of TNF in the culture media of RAW 264.7 cells as measured by ELISA in triplicates. The cells where incubated for 24 hours with 0.1% of water extracts (SD – raw sourdough, SD BR – sourdough bread, AN BR – analog bread; <100 kDa – fraction between 100 kDa and 10 kDa, <10 kDa – fraction under 10 kDa). Extracts had no inhibitory effect on TNF secretion by RAW 264.8 cells activated with 1 µg/mL LPS (▭) and furthermore stimulated the secretion of TNF by non-activated cells (■). * $p<0.05$.

Water extraction was performed from non-sterile sourdough and bread, therefore the stimulatory effect on macrophages could be due to the contamination with external endotoxins as well as antigens already present in the bread. To test this hypothesis, endotoxin content in all extracts was measured by LAL Chromogenic Endpoint Assay. The analysis showed presence of endotoxin in all extracts (up to ~500 EU/mg). In order to differentiate between the effects of endotoxins from other bioactive sourdough components the extracts were cleaned-up by Detoxi-Gel™ Endotoxin Removing columns with polymixin B

bound gel. The concentration of endotoxins was again measured after the clean-up and all extracts had under 0.5 EU/mg of endotoxin (Table 1).

Table 1: Water soluble extracts contain high amounts of endotoxin that can be effectively removed by Detoxi-Gel™ Endotoxin Removing Columns.
The table shows the concentration of endotoxin in the water soluble extracts as determined by LAL Chromogenic Endpoint Assay. The last column shows the concentration of endotoxin after the extracts have been clean-up using Detoxi-Gel™ Endotoxin Removing Columns.

Water extract	LPS conc. before clean-up	LPS conc. after clean-up
Sourdough bread, 10 kDa	5.6 ± 0.5 EU/mg	0.5 ± 0.0 EU/mg
Sourdough bread, 100 kDa	498.3 ± 12.7 EU/mg	0.5 ± 0.0 EU/mg
Sourdough, 10 kDa	7.3 ± 0.0 EU/mg	0.5 ± 0.0 EU/mg
Sourdough, 100 kDa	30.5 ± 6.9 EU/mg	0.5 ± 0.0 EU/mg
Analog bread, 10 kDa	2.0 ± 0.3 EU/mg	0.5 ± 0.0 EU/mg
Analog bread, 100 kDa	63.9 ± 30.9 EU/mg	0.5 ± 0.0 EU/mg

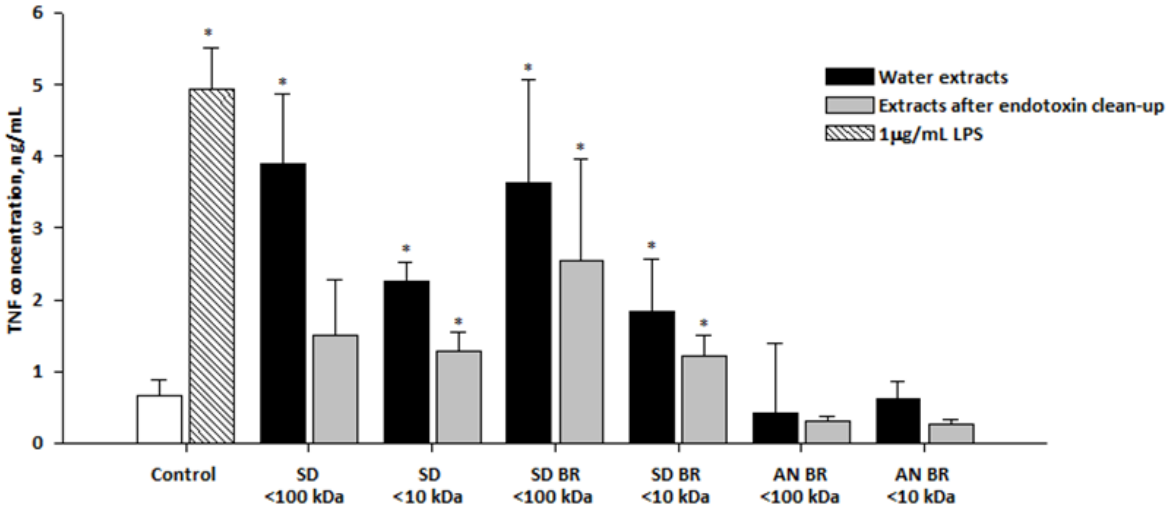


Figure 18: Sourdough extracts maintain mild stimulatory effect on the secretion of pro-inflammatory cytokine TNF by macrophages even after removal of endotoxins.
The figure shows the concentration of TNF in the culture media of RAW 264.7 cells as measured by ELISA. The cells where incubated for 24 hours with 0.1% of water extracts and 0.1% of water extracts after endotoxin removal. SD – raw sourdough, SD BR – sourdough bread, AN BR – analog bread; <100 kDa – fraction between 100 kDa and 10 kDa, <10 kDa – fraction under 10 kDa. Endotoxin-free extracts stimulated the secretion of TNF by RAW 264.7 cells to a lesser degree compared to untreated extracts however the effect remained significant for the sourdough and sourdough bread extracts. *p<0.05 when compared to untreated control (□).

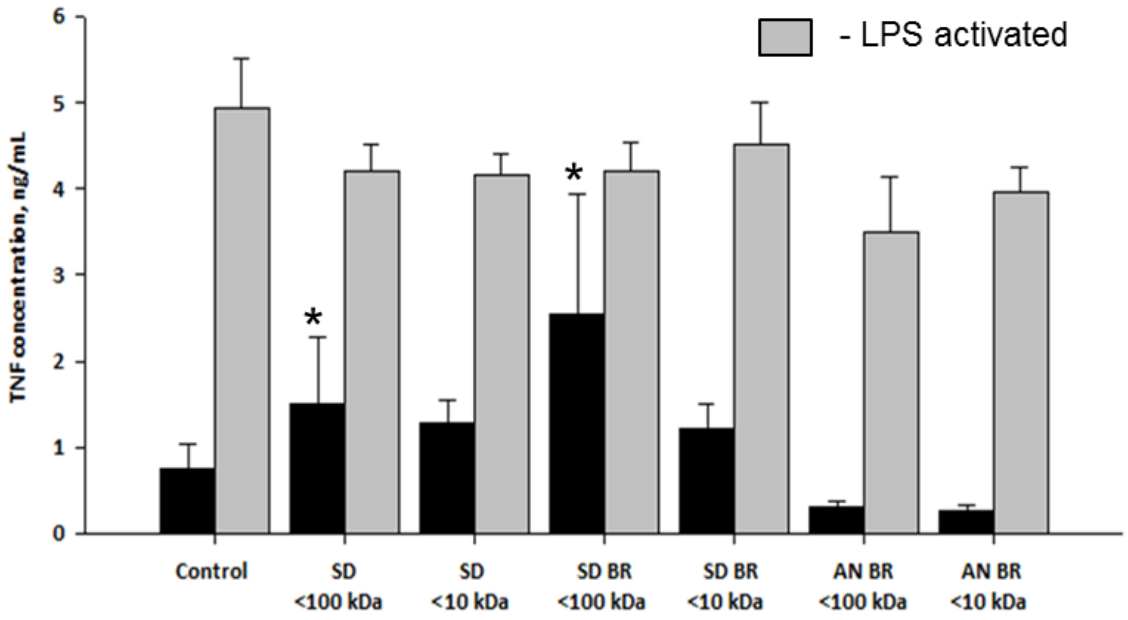


Figure 19: Endotoxin-free extracts do not inhibit the secretion of pro-inflammatory cytokine TNF by LPS-activated macrophages.
The figure shows the mean concentration of TNF in the culture media of RAW 264.7 cells as measured by ELISA in triplicates. The cells where incubated for 24 hours with 0.1% of endotoxin-free extracts (SD – raw sourdough, SD BR – sourdough bread, AN BR – analog bread; <100 kDa – fraction between 100 kDa and 10 kDa, <10 kDa – fraction under 10 kDa) and 1 µg/mL LPS. Extracts and had no inhibitory effect on TNF secretion by RAW 264.8 cells activated with 1 µg/mL LPS (▭) and mildly stimulated the secretion of TNF in control non-activated cells (■).*p<0.05 when compared to untreated control (■).

After the clean-up of endotoxins the water extracts were tested again for the effect on the secretion of TNF by the macrophages. The stimulation of TNF secretion was reduced as compared to the extracts before clean-up but remained significant when compared to control unstimulated macrophages (Fig. 18). Interestingly only raw sourdough and sourdough bread extracts, but not analog bread extracts, stimulated TNF secretion. Furthermore endotoxin-free extracts did not decreas the secretion of TNF by LPS-activated RAW 264.7 macrophages (Fig. 19).

5.4. Extracts of sourdough and sourdough bread contain physiologically active neurotransmitter acetylcholine

5.4.1. Sourdough-derived acetylcholine triggers muscle contraction *in vitro*

Metabolimic analysis of raw sourdough and sourdough bread indicated presence of significant amount of acetylcholine as compared to unfermented analog bread (see Table 2).

Table 2: Sourdough and sourdough bread contain high amounts of acetylcholine of with 38,6 mg/kg in dry bread.
Concentration was determined using LC-MS/MS by comparing the peak height to the height of solutions with standard concentrations of acetylcholine.

Water extracts	Ach conc. in water extracts	Ach conc in bread or sourdough, dry mass
Sourdough, 10 kDa	1819 ±717 µM	26.5 ±10.4 mg/kg
Sourdough bread, 10 kDa	2644 ± 273 µM	38.6 ± 4.03 mg/kg
Analog bread, 10 kDa	44.5 ±1.9 µM	0.64 ± 0.03 mg/kg

Acetylcholine (Ach) is a neurotransmitter that is responsible for the activation of motility in the gastro-intestinal tract by stimulating either muscarinic (mAchR) or nicotinic Ach receptors (nAchR) on the muscle cells. To determine if the sourdough-derived acetylcholine mimicks this activity, isolated corpus muscle of guinea pig was stimulated with extracts of sourdough, sourdough bread and analog bread and the contraction stimulation was measured. Both sourdough and sourdough extracts but not analog bread extract induced muscle contractions similar to acetylcholine at equivalent concentration (Fig. 20). Atropine, mAchR-specific antagonist, was used to determine whether extracts stimulate contraction by activating muscarinic or nicotinic AchR. Pre-treatment of muscle strips with atropine completely abrogated stimulation by Ach as well as by sourdough and sourdough bread extracts indicating that sourdough-derived acetylcholine is acting via mAchR.

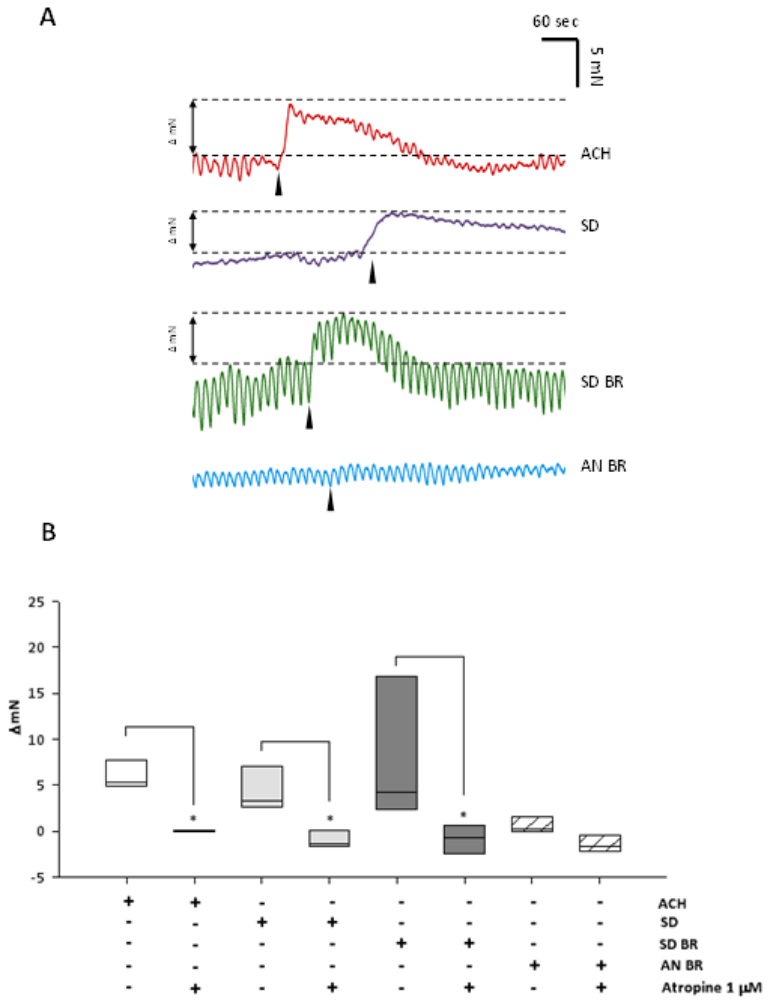


Figure 20: Sourdough extracts effectively stimulate stomach muscle motility by acting directly on mAchR. Figure A shows the muscle tone change induced by either acetylcholine (2.5 µM) or extracts at 0.02%. Increase in tone was immediately observed upon addition of the stimulants (arrow sign) except for extract of analog bread. Figure B shows the median (n>4) change of muscle tone upon differential treatments. The pro-kinetic effect of Ach and extracts is completely abolished by mAchR specific antagonist atropine. This indicates that acetylcholine in extracts directly acts on mAchR. (*p<0.05).

Furthermore to clarify whether sourdough-derived Ach acts through the activation of neurons that consequently stimulate muscle cells muscle preparations were pre-treated with tetrodotoxin (TTX). TTX blocks action potential generated by neurons that abrogates downstream signalling. TTX pre-treatment had no significant effect on muscle contraction induced by acetylcholine and extracts suggesting that both directly activate mAchR on muscle cells (Fig. 21).

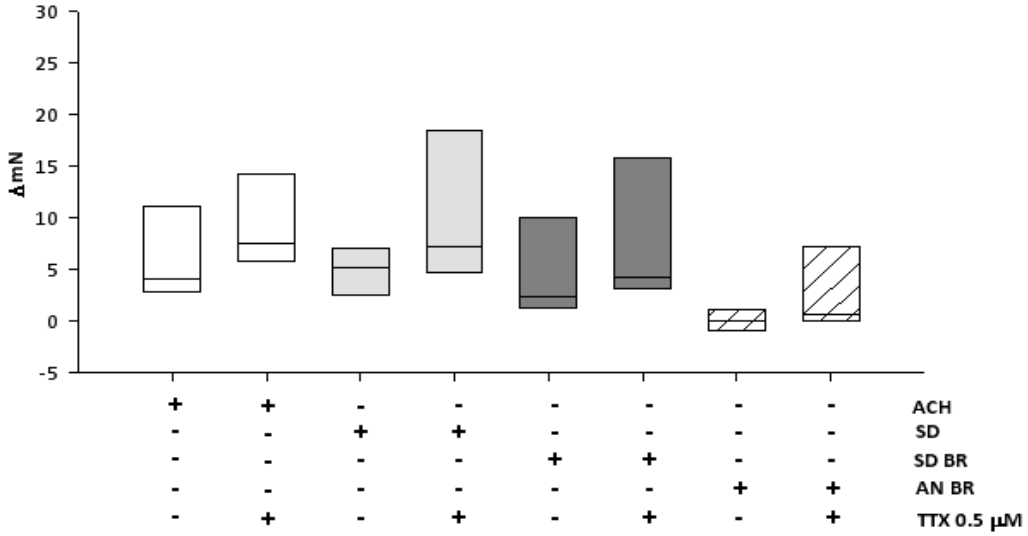


Figure 21: TTX has no significant effect on the stimulation of muscle contraction by sourdough-derived Ach.
The figure shows the muscle tone change stimulated by either acetylcholine (2.5 µM) or 0.02% extracts, and the effect of TTX pre-treatment. TTX did not exhibit significant effect on the muscle contraction induced by sourdough Ach suggesting stimulation is induced by direct action on muscle mAchR without neuronal mediation.

Acetylcholine is known to act through both the activation of mAchR and nAchR. To elucidate the role of nAchR the effect of sourdough Ach, muscle strips were pretreated with hexamethonium (HXM), a specific inhibitor of nAchR (Fig. 22). HXM did not exhibit significant effect on the muscle contraction induced by sourdough Ach corroborating the earlier observation that nAchR are not involved in the observed muscle stimulation.

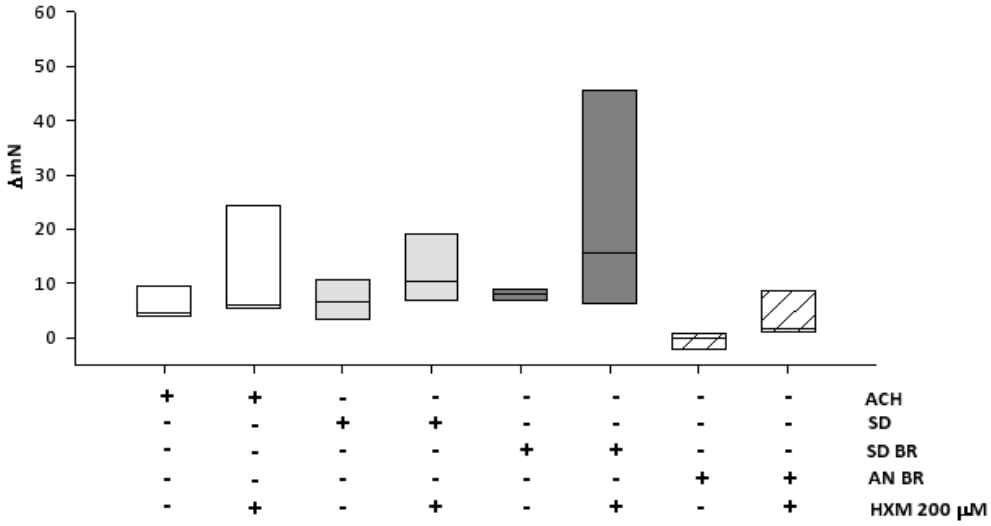


Figure 22: HXM pre-treatment increases the muscle contraction stimulated by sourdough Ach although the effect is statistically insignificant.
The figure shows the muscle tone change stimulated by either acetylcholine (2.5 µM) or extracts at 0.025% and the effect of HXM pretreatment. HXM did not exhibit significant effect on the muscle contraction induced by sourdough Ach indicating that nAchR play insignificant role in the observed muscle stimulation.

5.4.2. Sourdough-derived acetylcholine stimulates secretion by the intestinal mucosa from the luminal side

Acetylcholine, released by the enteric neurons to the serosal side of the intestinal wall, stimulates secretion of chloride ions by the mucosa subsequently driving the passive transport of water into the lumen. This effect is transient due to rapid degradation of Ach by acetylcholine esterase.

The effect of sourdough-derived Ach on the intestinal secretory function was tested in guinea pig colon. Extracts of sourdough, sourdough bread, analog bread as well as (pure) Ach were applied on either luminal (mucosal) or serosal side of intestinal mucosa/submucosa preparations from guinea pig distal colon. The experiment was performed in Ussing chamber and the change in short-circuit current (I_{sc}) was measured. In this system, passive flow of ions across a tissue or epithelial cell layer is eliminated by balancing electrical, osmotic, hydrostatic and chemical gradients across the preparation, such that only active ion transport is measured. In the Ussing chamber, electrodes are placed close to each side of the tissue to allow detection of the spontaneous potential difference (PD) across the epithelium, generated as a consequence of active ion transport (Hirota and McKay, 2006).

Surprisingly an increase in I_{sc} could be observed when the preparations were stimulated from both mucosal and serosal side by Ach as well as Ach-containing extracts (analog bread extract had no effect) (Fig. 23).

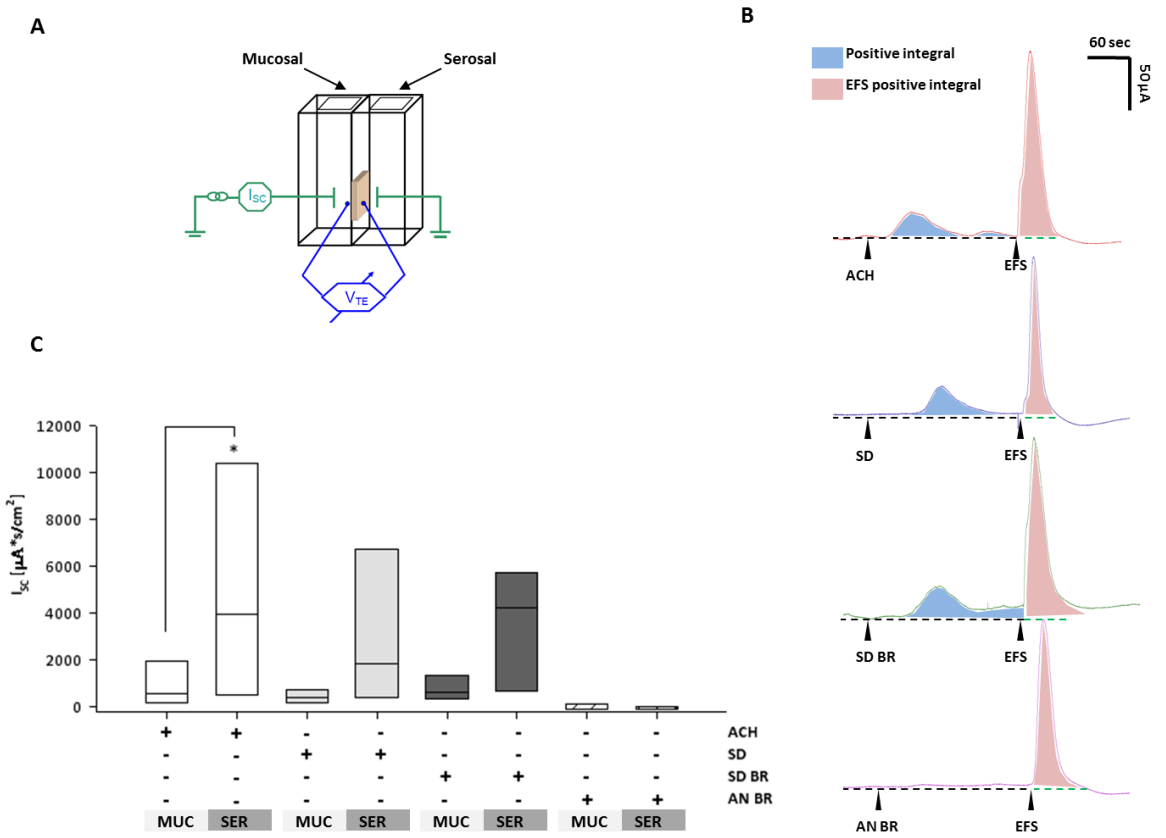


Figure 23: Sourdough and sourdough bread extracts stimulate chloride ions secretion when applied from either serosal or mucosal side of intestinal mucosa.
The figure A (Kerstin Müller, 2008) shows Ussing chamber set-up with mucosa piece separating the two chambers. The blue set of electrodes measure transepithelial voltage (VTE) and the green set – the short circuit current (I_{SC}). The secretion is measured estimated by the change in I_{SC} necessary to maintain V_{TE} at 0 mV. Figure B gives representative I_{SC} traces stimulated by acetylcholine (ACH), sourdough (SD), sourdough bread (SD BR) or analog bread (AN BR) extracts. Area under the curve is calculated using an integral (µA*s/cm^2) where blue shows response to extracts and red shows response to EFS. Figure C shows median (n>4) value of change in I_{SC} upon treatment on either mucosal side (MUC) or serosal side (SER) of guinea pig colonic mucosa. Acetylcholine and sourdough as well as sourdough bread extracts clearly stimulate secretion when applied to either side of mucosa while analog brad extract has no effect. Suggesting that acetylcholine in sourdough and sourdough bread is responsible for the stimulation. (*p<0.05).

The tissue was pre-treated with atropine and the effect of extracts on secretion measured again (Fig. 24). Atropine completely abolished the response corroborating the role of mAchR in the pro-secretory effect of sourdough extracts.

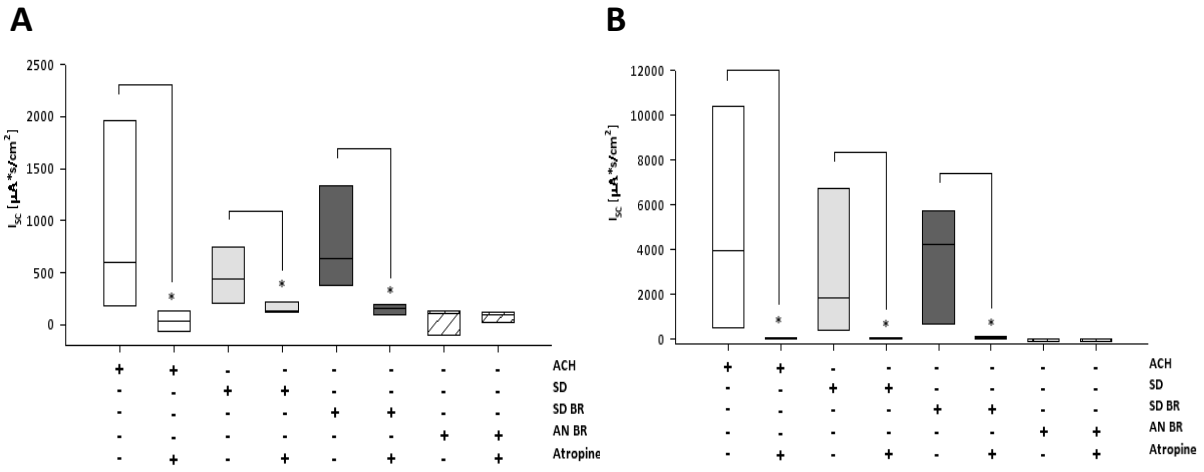


Figure 24: Atropine completely abrogates chloride ions secretion stimulated with sourdough and sourdough bread extracts.
Figure shows mean (n>4) value of change in I_{SC} over time upon Ach (10 µM) and extract (0.1%) application on either mucosal side (A) or serosal side (B) of guinea pig colonic mucosa with and without atropine pretreatment (1 µM). Atropine completely abrogated secretion stimulation suggesting the role of mAchR in the secretory effect of sourdough extracts. (*p<0.05).

Earlier research provides evidence of the expression of AchR in intestinal epithelial cells on the basolateral side but not on the apical side of the cell layer (Hirota and McKay, 2006). Therefore it is highly probable that Ach applied from apical side crosses the cell layer and stimulates the receptors on the basolateral side. This hypothesis was verified by the fact that when Ach is applied apically (in other words on the mucosal or luminal side) the secretory effect was significantly inhibited when atropine was applied to the serosal but not to the mucosal side (Fig. 25). Atropine applied on the serosal side blocked all mAchR on basolateral side abrogating Ach activity. However, when atropine is applied on the mucosal side this results into smaller amounts reaching basolateral side and thus only partially inhibiting Ach activity. The observation that atropine can cross epithelial layer and block basolateral mAchR was confirmed by the fact that when Ach was applied serosally and atropine mucosally secretion was inhibited (data not shown).

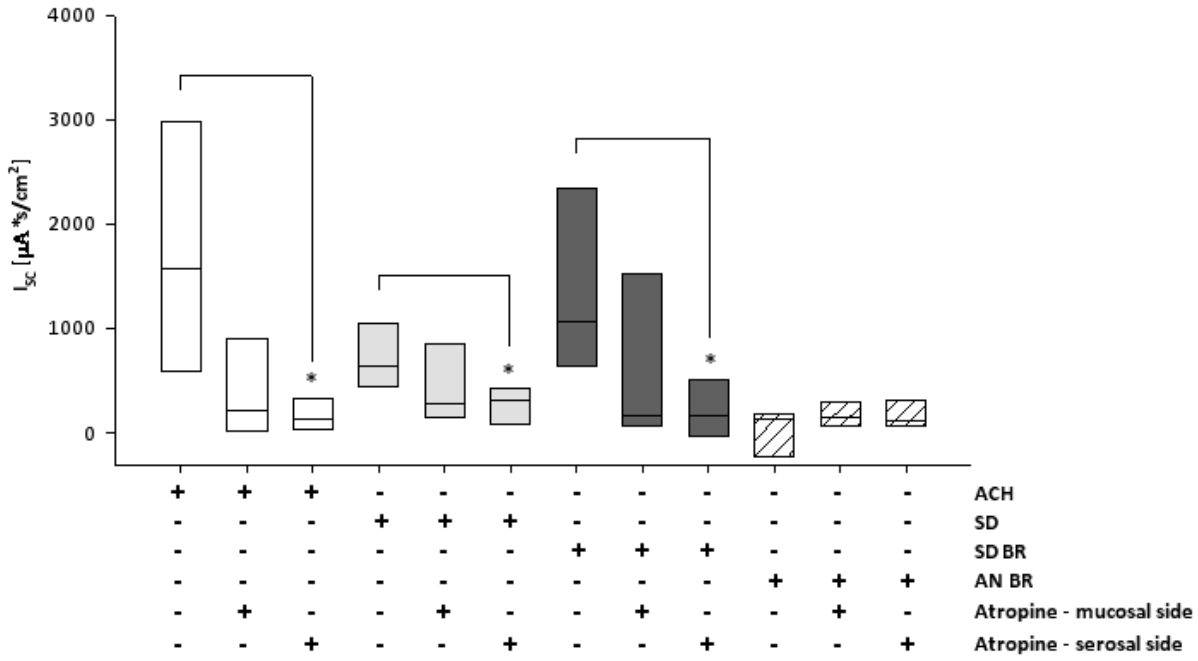


Figure 25: Atropine completely abrogates secretion stimulation by Ach and extracts when applied serosally but not mucosally.
Figure shows the mean (n>4) value of change in I_{SC} over time subsequent to Ach and extract treatment on the mucosal side with and without atropine pre-treatment (1 µM). Atropine was added on either serosal or mucosal side. Atropine completely abrogated secretion when applied on serosal but not mucosal side. (*p<0.05).

Trans-epithelial resistance (TER) was evaluated as a quality control to monitor alteration to tissue permeability during the experiment. TER was calculated using Ohm's law from I_{SC} and V_{TE} values obtained by switching intermittently to an open current mode. No significant differences were observed in TER throughout the experiments and across treatments, indicating that Ach as well extracts do not alter tissue integrity (Fig. 26).

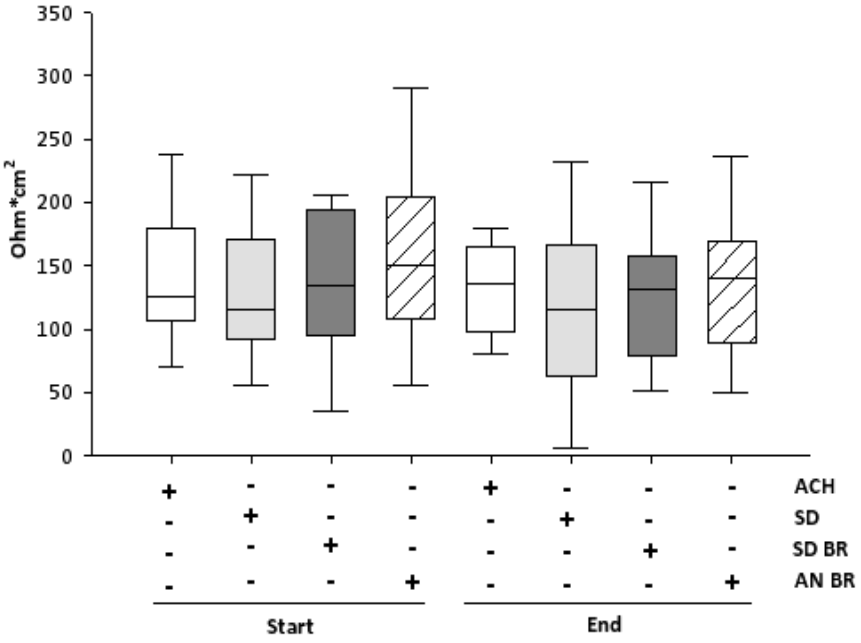
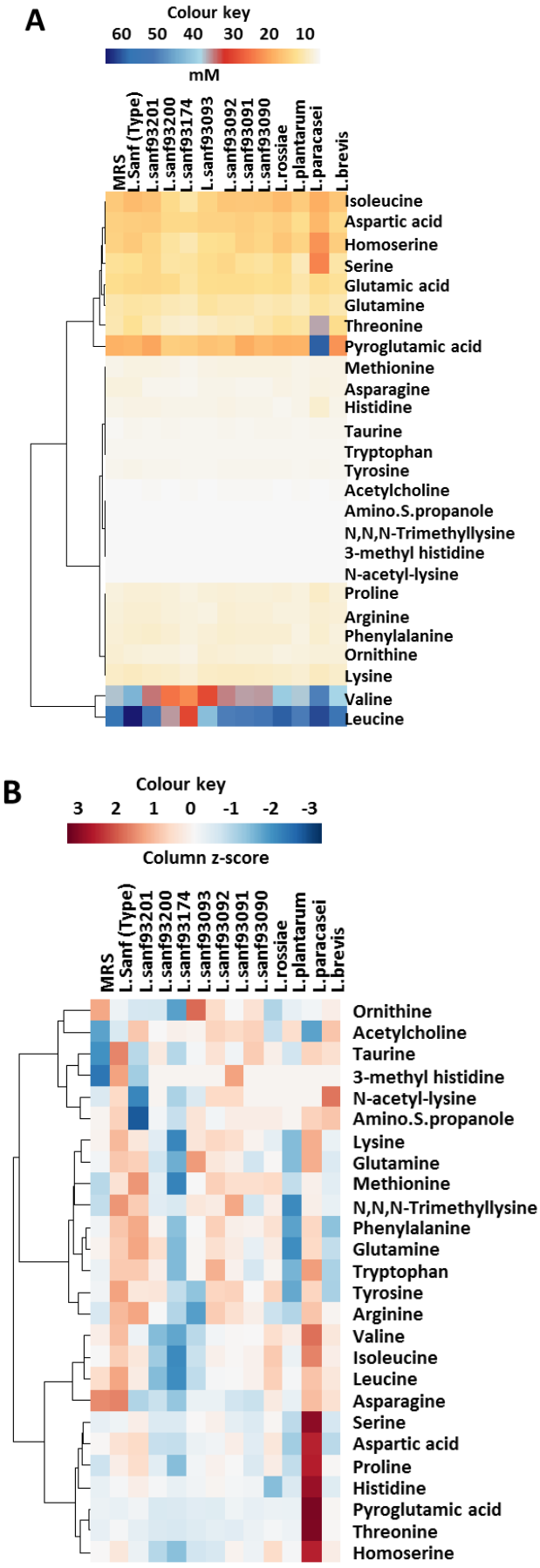


Figure 26: Trans-epithelial resistance of mucosa before and after extracts' treatment.
Figure shows mean (n>10) value of change in transepithelial resistance (TER= V_{TE}/I_{SC}*1000/2) over time upon Ach and extract treatment. No significant differences were observed in TER throughout the experiments and across treatments, indicating that Ach as well as extracts do not alter tissue integrity.

5.5. Functional analysis of sourdough lactic acid bacteria

5.5.1. LC-MS/MS analysis revealed presence of acetylcholine in growth media of sourdough LAB

Seven strains of *L. sanfranciscensis* (23090-23201) and one strain of *L. rossiae* (E1) were isolated from Hofpfisterei sourdough. To identify if sourdough LAB produce acetylcholine detected in sourdough and also to identify other potentially interesting metabolites we analyzed the growth media (MRS) after 24 hour inoculation with sourdough LAB using. In addition we analyzed the growth media of *L. plantarum FUA 3038* and *L. brevis 3113* isolated from other sourdoughs, and *L. paracasei* from VSL#3. For the measured concentrations of all analytes see Appendix.


A
Colour key
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mM
MRS
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L.sanf93200
L.sanf93174
L.sanf93093
L.sanf93092
L.sanf93091
L.sanf93090
L.rossiae
L.plantarum
L.paracasei
L.brevis
Isoleucine
Aspartic acid
Homoserine
Serine
Glutamic acid
Glutamine
Threonine
Pyroglutamic acid
Methionine
Asparagine
Histidine
Taurine
Tryptophan
Tyrosine
Acetylcholine
Amino.S.propanole
N,N,N-Trimethyllysine
3-methyl histidine
N-acetyl-lysine
Proline
Arginine
Phenylalanine
Ornithine
Lysine
Valine
Leucine
B
Colour key
3 2 1 0 -1 -2 -3
Column z-score
MRS
L.Sanf (Type)
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L.brevis
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Amino.S.propanole
Lysine
Glutamine
Methionine
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Phenylalanine
Glutamine
Tryptophan
Tyrosine
Arginine
Valine
Isoleucine
Leucine
Asparagine
Serine
Aspartic acid
Proline
Histidine
Pyroglutamic acid
Threonine
Homoserine

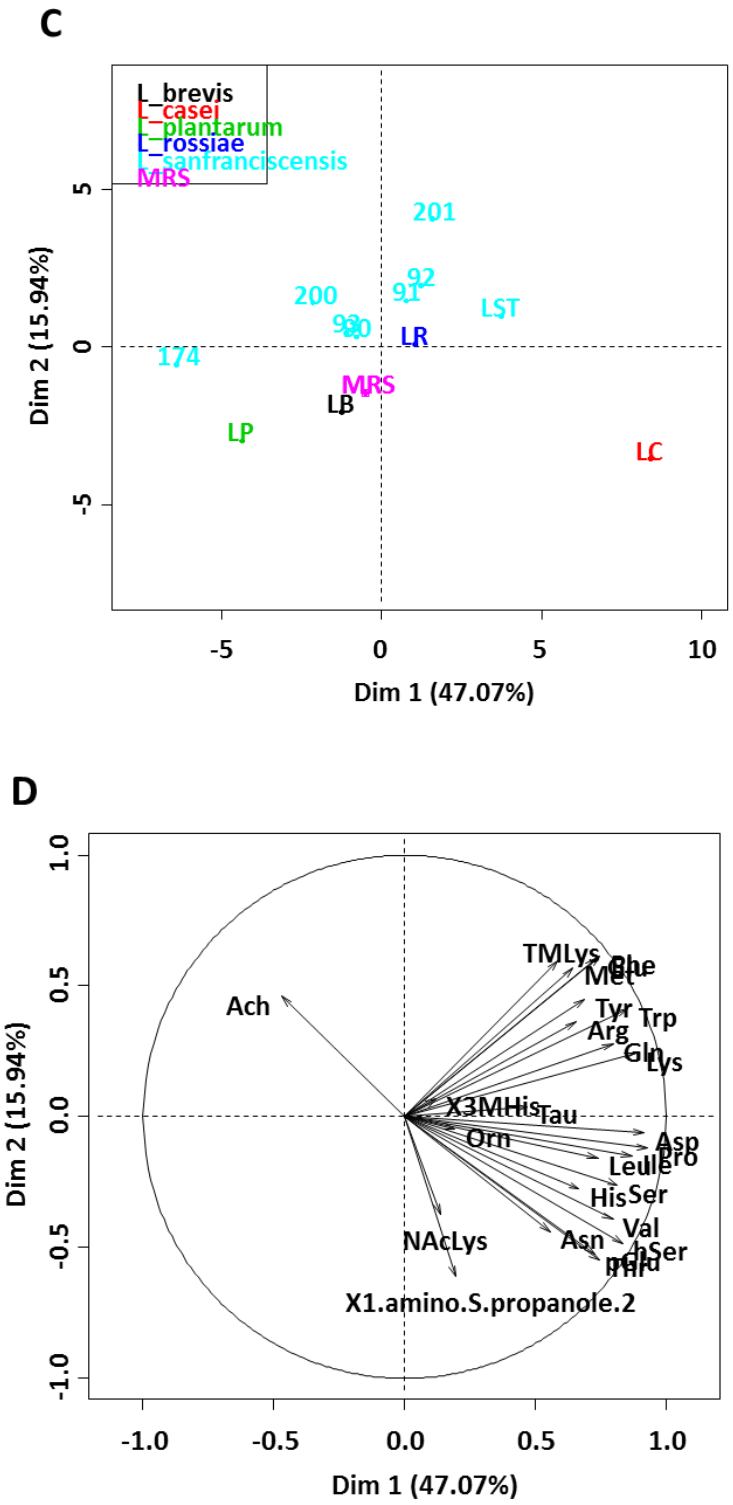

Figure 27: Metabolic analysis of sourdough lactic acid bacteria growth medium identified high amounts of acetylcholine.

A. Heatmap of absolute metabolites values in MRS broth after 24 hour LAB inoculation (mean of three experiments). MRS – control growth media without bacteria, L.sanf23090-23201 and *L. rossiae* – strains isolated from Hofpfisterei sourdough, L.sanf(Type) – *L. sanfranciscensis* type strain 24051, L. plantarum and L.brevis - *L. plantarum* FUA 3038 and L. brevis 3113 isolated from other sourdoughs, and *L. paracasei* from VSL#3. **B.** Scaled heatmap of relative concentration of metabolites. **C.** PCA plot of metabolites indicates sharp distinction between all sourdough bacteria and *L. paracasei* (LC). **D.** Acetylcholine plays the strongest role in the separation observed in PCA plot since *L. paracasei* does not produce any detectable level of the neurotransmitter**.**

PCA analysis shows significant difference in the metabolites profiles of all sourdough isolated bacteria compared to *L. paracasei*. In major parts, the separation is due to the impact of acetylcholine present in sourdough bacteria growth media but not in *L. paracasei* media (Fig. 27). This observation allows for assumption that the acetylchiline found in the sourdough and sourdough bread is produced by LAB during fermentation process.

L. brevis 3113 strain produced the highest concentration of acetylcholine in 24 hours; however this strain has also the highest growth. When the concentration is adjusted to the bacterial number in the growth media (MRS both), the most acetylcholine per bacterial cell is produced by *L. sanfranciscensis* strains 23090 and 23093 isolated from the sourdough used for this study (Fig. 28).

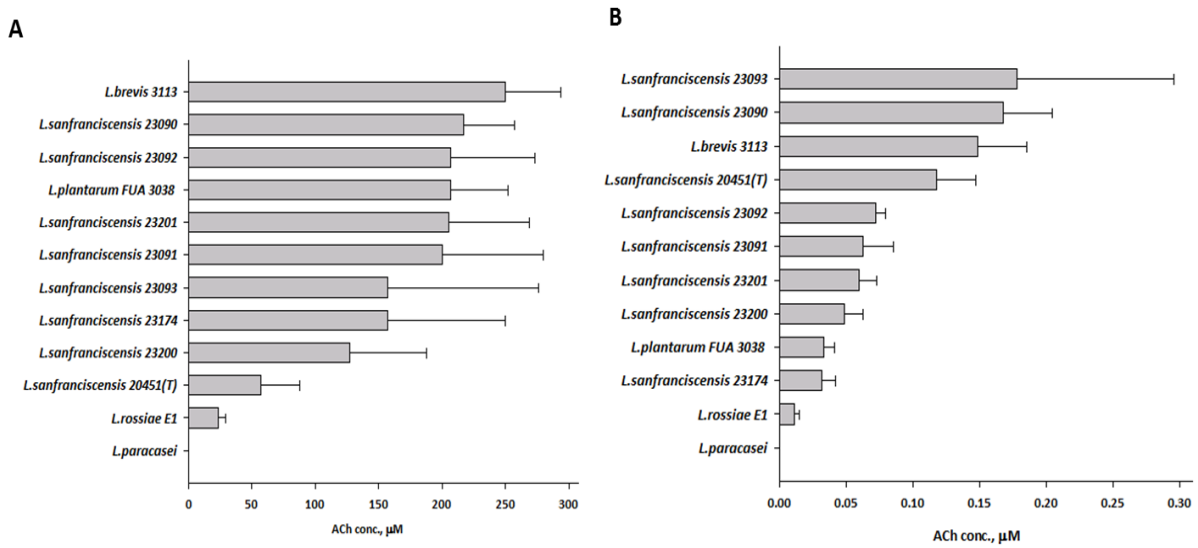

Figure 28: Acetylcholine concentration in MRS media upon 24 hours of incubation with LAB.
A. Acetylcholine concentration in MRS broth upon 24 hour inoculation with $0.25*10^7$ of bacteria/mL. **B.** Acetylcholine concentration adjusted to 10^6/mL bacteria count in MRS. Concentration was determined using LC-MS/MS by comparing the peak height to the height of solutions with known concentration of acetylcholine.

5.5.2. Sourdough LAB inhibit secretion of IP-10 by TNF-activated IECs

In order to detect the presence of bioactive compounds from sourdough LAB we incubated Mode-K cells with bactreail conditioned media as well as fixed bacteria. Eight sourdough LAB strains and *L. paracasei* were tested for their effects on the secretion of pro-inflammatory chemokine IP-10 by unstimulated and TNF-activated Mode-K cells. The conditioned media was prepared from the bacterial growth media.

No conditioned media alone affected the secretion of IP-10 by unstimulated Mode-K cells. Incubation of TNF-activated epithelial cells with the conditioned media from seven sourdough strains (*L. sanf.* 23201, 23090, 23092, 23093, 23091, 23174, 23200 and *L.rossiae*) and *L.sanf.* type strain significantly decreased IP-10 secretion, while conditioned media from *L. paracasei* completely abbrogated IP-10 secretion (Fig. 29). Only sourdough strains *L.rossiae, L.sanf.* 23174 and *L.sanf.* 23200 decreased IP-10 concentration by >50%. Interestingly, preparation of fixed bacteria of the same strains also inhibited IP-10 secretion (Fig. 30). However only strains *L. sanf.* 23090, 23092, 23093, 23091, 23200 and *L.sanf.* type strain significantly decreased IP-10 secretion. Fixed *L. paracasei* inhibited IP-10 secretion similar to *L. sanf.* 23090 and 23092. This suggests that there are both secreted and cell surface factors produced by sourdough LAB that affect IP-10 production in epithelial cells.

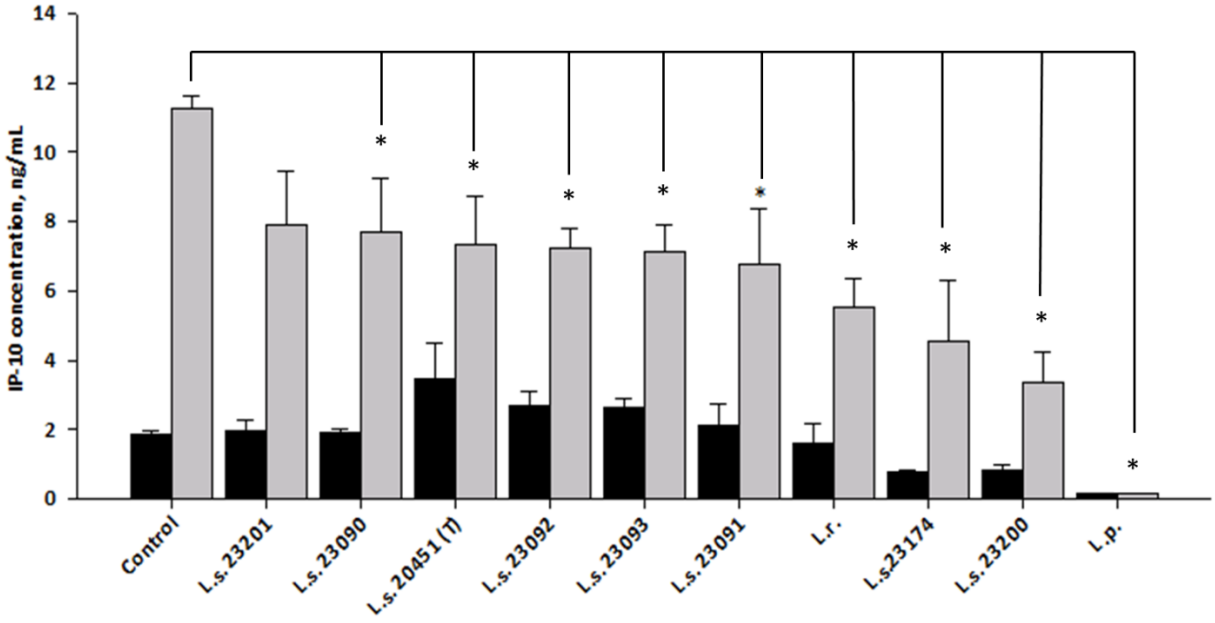

Figure 29: Concentrated conditioned media of sourdough LAB significantly inhibits the secretion of IP-10 by TNF-activated IEC.

The figure shows the concentration of IP-10 in the culture media of Mode-k cells as measured by ELISA. The cells where incubated for 24 hours with concentrated conditioned media (cCM) (■) and cCM plus 10 ng/mL TNF. *L. rossiae, L. sanfranciscensis* strains 23174 and 23200 inhibited IP-10 secretion by more than 50% following *L. paracasei* that completely abrogated IP-10 secretion. *p<0.05.

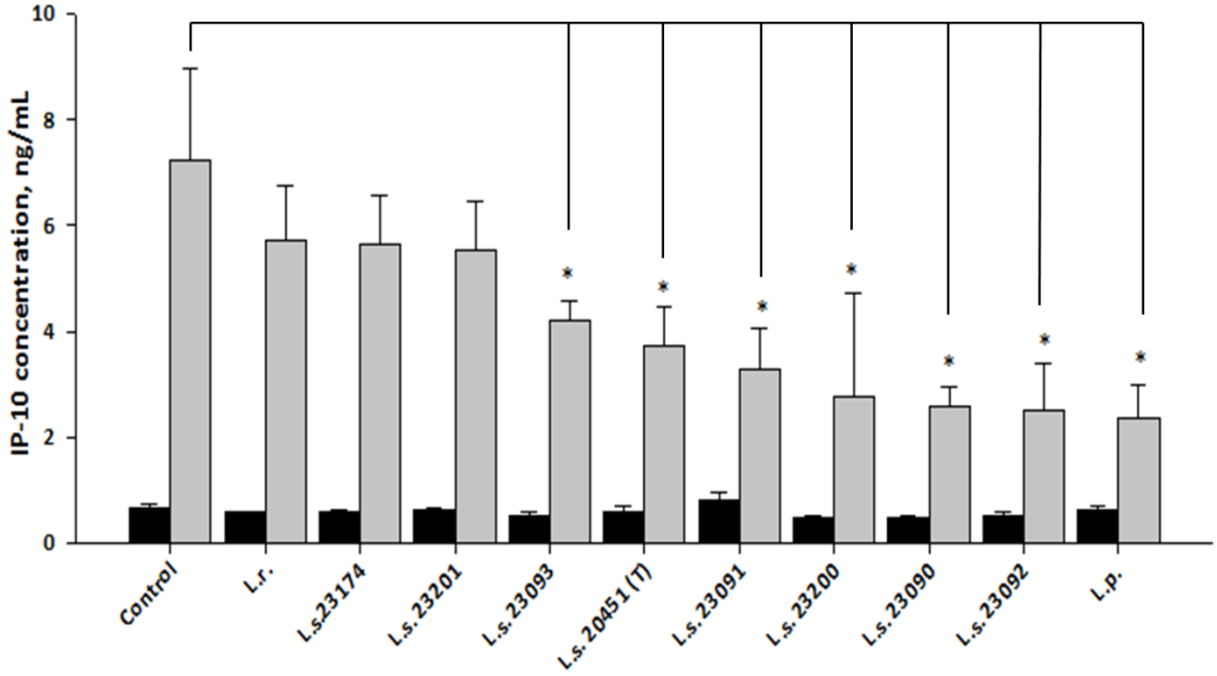

Figure 30: Formaldehyde-fixed sourdough LAB significantly inhibit the secretion of IP-10 by TNF-activated IECs.

The figure shows the concentration of IP-10 in the culture media of Mode-k cells as measured by ELISA. The cells where incubated for 24 hours with 20 MOI of fixed LAB (■) and 20 MOI of fixed LAB plus 10 ng/mL TNF (□). Fixed *L. paracasei* (L.p.) has, similar to fixed *L. sanfranciscensis* strains 23090 and 23092, the highest inhibitory activity on the IP-10 secretion by TNF-activated IECs (□) as compared to TNF-activated control. *p<0.05.

5.5.3. Sourdough LAB do not express cytokine-degrading enzyme Lactocepin PrtP

L. paracasei, actively degradeds pro-inflammatory chemokine IP-10 as do the sourdough LAB. The active player in IP-10 degradation by *L. paracasei* was earlier found to be lactocepin PrtP, a serine protease. In order to investigate if sourdough LAB inhibition of IP-10 secretion is also due to the presence of lactocepin, we analyzed sourdough LAB DNA. Total bacterial DNA was isolated from eight sourdough strains: *L. sanfranciscensis* DSMZ 23090, DSMZ 23091, DSMZ 23092, DSMZ 23093, DSMZ 23174, DSMZ 23200, DSMZ 23201 and *L. rossiae* (E1) as well as *L. paracasei* which served as positive control. DNA was amplified using Lactocepin PrtP specific primers and visualized on agarose gel. No lactocepin PrtP gene could be detected in sourdough LAB (Fig. 31). It remains to be investigated what other cell surface and secreted factors are reponsible for the decrease in IP-10 observed during incubation of TNF-stimulated epithelial cells with sourdough LAB.

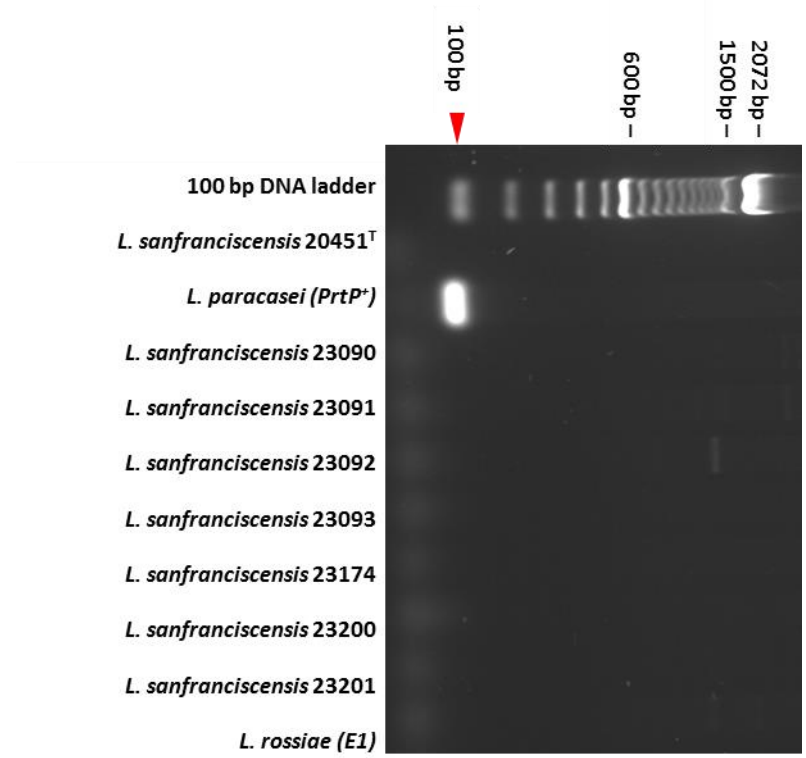

Figure 31: Sourdough lactic acid bacteria do not express Lactocepin PrtP gene.
The figure shows the agarose gel with PCR amplification products of bacterial DNA using Lactocepin PrtP primers. *L. paracasei* strain is a positive control for Lactocepin PrtP gene and is the only strain with PrtP gene amplification band, while sourdough *L. sanfranciscensis* strains (*L. sanfranciscensis* 23090-23201), *L. rossiae* (E1) and *L. sanfranciscensis* type strain 20451 do not exhibit detectable Lactocepin PrtP gene.

6. DISCUSSION

We hypothesized that sourdough bread, as a common LAB fermented food, may contain bioactive compounds capable of modulating intestinal inflammation. Long-term sourdough bread feeding did not mitigate intestinal inflammation in two IBD-related models of colitis and ileitis. On the contrary sourdough bread aggravates tissue pathology in colitic IL-$10^{-/-}$ mice. Sourdough bread feeding, however, did not alter the intestinal metabolic profile of healthy mice. Interestingly, the metabolic analysis identified biologically active acetylcholine in sourdough produced by LAB, capable of stimulating stomach motility and mucosa secretion *ex vivo*. Sourdough LAB also showed capacity to inhibit production of pro-inflammatory IP-10 by intestinal epithelial cells activated with TNF, suggesting that fermented sourdough harbours biologically active compounds.

Diet and the intestinal microbiota are widely implicated in the development of inflammatory disorders of gut. IBD-related models are frequently used to demonstrate the specific effects of dietary interventions on the disease pathogenesis. Experimental models of colitis have been used to demonstrate beneficial effect of dietary interventions such as probiotics and prebiotic GBF (Hoermannsperger et al., 2009; Kanauchi et al., 2008; Kanauchi et al., 2003). On the other hand high-fat and gluten have detrimental effects in the colitic mouse models (Gruber et al., 2013; von Schillde et al., 2012; Wagner et al., 2013). Contrary to our initial hypothesis that sourdough bread might mitigate intestinal inflammation, IL-$10^{-/-}$ mice fed sourdough bread demonstrated increased histopathology scores in the ceacum. No significant change was observed in mice fed unfermented analog bread, implicating the role of fermentation metabolites and/or bacterial factors in the above observation. Subsequent analysis of metabolic profile in the ceacum of IL-$10^{-/-}$ mice however did not reveal significant variations between the mice fed different diets and thus did not reflect the change in histopathology. Sourdough fermentation increases bioavailability of micronutrients such as iron (Chaoui et al., 2006), a factor that may play role in disease aggravation observed in IL-$10^{-/-}$ mice.

Recently the role of dietary iron in modulating intestinal inflammation was demonstrated in $TNF^{\Delta ARE}$ mice (Werner et al., 2011). These mice exhibited high sensitivity to luminal iron availability as demonstrated by complete amelioration of disease in animals kept on iron-free diet. The effect of iron might be stemming from its influence on gut microbiota, as the reactivity of major effector T-cells was not altered in mice on iron-free diet in contrary to compositional changes of microbiota. The microbiota composition in sourdough fed IL-$10^{-/-}$

mice was not evaluated in this study and therefore could not be interpreted with respect to the observed pro-inflammatory effect.

The pro-inflammatory effect of sourdough bread in IL-10$^{-/-}$ mice was not confirmed in TNF$^{\Delta ARE}$ mice. Ileum histopathology of the latter animals was not affected in the presence of sourdough bread. No effects in TNF$^{\Delta ARE}$ mice might not be surprising as this model has a very strong TNF-induced immunopathology and shows higher resistance to anti-inflammatory impact of probiotics as compared to IL-10$^{-/-}$ mice (Hoermannsperger et al., 2009).

Sourdough fermentation produces a range of bioactive compounds that may have impact on host and/or microbiota function in the absence of extreme IBD-modeled inflammation. EPS produced by sourdough LAB during cereal fermentation have bifidogenic properties that may lead to enhanced SCFA production with concomitant health effects (Korakli et al., 2002; Pepe et al., 2013). Sourdough fermentation by LAB also produces bioactive peptides through proteolysis of native peptides that exhibit antioxidant action on stressed fibroblasts *in vitro* (Coda et al., 2012). Clinical studies with subjects, exhibiting high risk for glucose intolerance, the ingestion of sourdough bread, in particular rye-based, improved glycaemia by lowering glucose and glucagon-like peptide-1 (GLP-1) response (Najjar, Parsons et al. 2009, Bondia-Pons, Nordlund et al. 2011, Mofidi, Ferraro et al. 2012). Furthermore, changes in plasma metabolites were associated with tryptophan, suggested to regulate the pro-inflammatory responses of macrophages (Bondia-Pons et al., 2011; Bosco et al., 2000; Mofidi et al., 2012; Najjar et al., 2009). Our findings however did not support any changes in wild-type mice upon pro-longed feeding with sourdough bread, as exemplified by metabolomic analysis of local intestinal metabolites in ceacum and systemic metabolites in plasma. Furthermore no significant changes were detected in the protein expression profiles of intestinal epithelium in mice fed sourdough bread compared to mice fed conventional chow diet. The analysis however did not include the fatty acid content in the mice colon, the data that might be relevant in connection with EPS presence in sourdough.

In contrast to our findings, dietary interventions with pro- and prebiotics display a significant influence on the metabolic profile of plasma and urine, although not so much on microbial composition(Martin et al., 2008a; Martin et al., 2008b). The particular pathways involved were carbohydrate metabolism as well as lipid and lipoprotein metabolism Further evidence for the role of diet on microbial composition and host metabolism was demonstrated in iron-deficient rats harbouring increased numbers of *Lactobacilli* and

Enterobacteriaceae as well as decreased numbers of *Roseburia* spp./*E. rectal* species. The latter bacteria are a known group of butyrate producers. In accordance lower levels of ceceal SCFA (butyrate and propionate) were measured in animals fed with a iron-deficient vs. iron-sufficient diet (Dostal et al., 2012).

In concert with the insignificant effect of sourdough bread feeding on ceacal and plasma metabolite profiles, metabolic analysis of water extracts of sourdough and analog bread also revealed few differences. The significantly different concentrations of metabolites observed in raw sourdough are likely diluted in sourdough bread due to the addition of unfermented flour before baking. Nevertheless, a notable exception in the fermented sourdough bread is the high amount of the neurotransmitter acetylcholine (Ach) reaching approximately 0,2 µmol/g dry mass .

Presence of Ach in plants, fungi and bacteria has been established long time ago (Stephenson and Rowatt, 1947). However its function in bacteria and fungi remains unclear. In plants it has been associated with the regulation of differentiation, phytochrome-mediated processes, plus transport of ions and water (Smallman and Maneckjee, 1981; Wessler et al., 1999). A study investigating Ach production by different organisms confirmed it is ubiquitous in bacteria, archae, fungi and eucarya alike with highest level detected so far in the top portion of bamboo shoot (2,9 µmol/g). This concentration is about 80 times that found in rat brain. Ach abundance across species earns it a characteristic of a proto-molecule active as a local mediator and modulator of physiological functions since the early beginning of life (Kawashima et al., 2007). To clarify the origin of the Ach in the sourdough, we analysed the growth media of sourdough-isolated bacteria that mainly belong to *L. sanfranciscensis* species. Sourdough LAB secreted significant amounts of Ach into the growth medium, suggesting that bacteria are the main source of Ach in sourdough. This property may be inherent to LAB specializing in fermentation of plant material as no Ach was produced by *L. paracasei* of intestinal origin.

In humans Ach is a well-known neurotransmitter but also has been detected in a number of non-neuronal tissues such as immune cells, endothelial, gastrointestinal, respiratory and urogenital epithelial cells participating in basic cell functions like proliferation, differentiation, cell-to cell signalling, immune function, secretion and absorption earning itself a reputation of “universal cytotransmitter” (Fujii and Kawashima, 2001; Horiuchi et al., 2003; Kirkpatrick et al., 2001; Wessler et al., 2001). In the context of GI tract Ach is known to be one the principal signaling molecules of the ENS regulating muscle tone, secretion and immune

reaction (Wessler and Kirkpatrick, 2008). There are two categories of Ach receptors (AchRs): five subtypes metabotropic G-protein-coupled muscarinic receptors (mAchr), and ionotropic nicotinic receptors (nAch). Both receptor categories are expressed on neuronal as well as on non-neuronal cells (Barrett and Keely, 2000; Hirota and McKay, 2006). Stimulation of mAchR induces motility and luminal chloride secretion in the GI tract, while stimulation of cholinergic nAchR has been shown to have anti-inflammatory effect in macrophages by inhibiting LPS-stimulated TNF secretion as well as smooth muscle relaxing effect (Borovikova et al., 2000; Tracey, 2002). Therefore deregulated ENS typically results in altered bowel movement and intestinal hydration what may lead to increased burden of microbiota that in turn may induce inflammatory processes. From reverse perspective inflamed tissues often display perturbations in the enteric cholinergic system and reduced ion transport responses to cholino-mimetics (Hirota and McKay, 2006).

We investigated the activity of sourdough-produced Ach in the context of neuronal GI functions. Both stomach muscle motility and mucosal chloride secretion were activated by sourdough extracts but not analog bread extracts *ex vivo* in guinea pig isolated tissue. Sourdough-derived Ach acts via mAchR as demonstrated by complete inhibition of its effect with atropine (mAchR-specific antagonist) pre-treatment. Stomach motility was stimulated despite application of TTX, neuronal action potential inhibitor, suggesting direct effect of sourdough Ach on muscle cells. The stimulatory effects of sourdough Ach were transient and lasted for under 2 to 5 minutes. This is likely due to the rapid Ach degradation by acetylcholinesterase (AChE) abundant at neuromuscular and neuroeffector junctions (Wessler et al., 2003). These observations suggest that luminal application of Ach at local sites may modulate ENS-mediated process such as motility and fluid secretion. Fluid secretion into the intestine is hypothesized to provide the ideal environment for enzymatic digestion and to facilitate the passage of stool through the intestinal tract. Furthermore a number of studies suggest that acute and locally targeted water secretion serves as a protective measure against epithelial damage at points of particular mechanical stress (Barrett and Keely, 2000; Sidhu and Cooke, 1995). Motility is another crucial function of the GI tract. Agonists and antagonists of serotonin (5-hydroxytryptamine) receptors are a common treatment option for modulating motility and consequently bowel movements of IBS patients (Camilleri and Andresen, 2009). It may be possible to use dietary interventions to modulate GI motility as rats fed a synbiotic composed of chicory inulin, *L. rhamnosus* and

B. lactis exhibited significantly increased intestinal motility response *in vivo* (Lesniewska et al., 2006).

Observations from sourdough bread feeding to mouse models were complemented with *in vitro* findings using sourdough bread extracts and sourdough LAB. While sourdough extracts induced inflammatory response, sourdough LAB mitigated the inflammatory response in cell culture. There was observed a small but significant increase in TNF production by murine macrophages incubated with fermented extracts. This pro-inflammatory response is in agreement with the observed effects of *in vivo* sourdough feeding of IL-$10^{-/-}$ mice. Moreover acetylcholine in the extracts did not inhibit secretion of pro-inflammatory TNF by LPS-stimulated macrophages as was observed earlier in human macrophage cell line (Borovikova et al., 2000).

Contrary to pro-inflammatoy effects of sourdough, the sourdough-isolated bacteria exhibited anti-inflammatory properties. LAB isolated from Hofpfisterei sourdough was identified as *L. sanfranciscensis* and *L.rossaie* – strains typically found in rye and wheat flour based doughs. Fixed (in other words dead) *L. sanfranciscensis* bacteria as well as secreted bacterial metabolites (conditioned media) were able to inhibit secretion of pro-inflammatory chemokine IP-10 by intestinal epithelial cells. The inhibition of IP-10 was previously demonstrated by *L. paracasei* from VSL#3 mix that expresses lactocepin prtP, a serine protease that specifically degrades IP-10 *in vitro* and *in vivo* (Hoermannsperger et al., 2009; von Schillde et al., 2012). However this scenario is unlikely for the species *L. sanfranciscensis* since no lactocepin prtP gene was detected in the sourdough strains. At this stage it cannot be concluded if the effect of sourdough LAB on IP-10 secretion by IEC is a pre- or post-translational effect, or what cell-surface structures are involved. However, this observation deserves further investigation as IP-10 is a crucial payer in the development of IBD. IP-10 secretion is triggered by pathogens in the gut and leads to enhanced recruitment of immune cells (i.e. effector Th1 cells and monocytes) into the gut mucosa, which neutralizes the pathogen. When the pathogen stimuli is gone the IP-10 secretion shuts-off, however in the case of IBD it is continuously produced even without any pathogenic stimuli resulting in constant immune cell recruitment and eventually loss of epithelial cell integrity and tissue damage (Hyun et al., 2005). Therefore, interventions that decrease IP-10 concentration may mitigate IBD pathogenesis, as demonstrated in murine colitis model fed *L. casei* with active IP-10 degrading lactocepin (von Schillde et al., 2012).

In summary, this work provided a first look on the effect of sourdough bread on GI functions in the healthy and inflamed setting. Sourdough bread feeding augmented inflammation in the murine model of colitis but had no effect in the murine model of ileitis. Furthermore sourdough bread feeding did not alter metabolic profiles of healthy mice. Sourdough isolated LAB and their metabolites on the other hand may have modulatory properties on gut function and inflammation. Acetylcholine produced by sourdough LAB stimulates gut functions such as intestinal fluid secretion and stomach motility. Additionally unidentified sourdough LAB cell-surface and secreted factors can impede IP-10 secretion by IECs. Altered intestinal fluid secretion and motility are common symptoms in IBS, while IP-10 has been implicated in re-enforcing intestinal inflammation in IBD patients. Above observations should warrant further investigation into the functionality of sourdough LAB and their metabolites in the context of GI tract health.

7. OUTLOOK

The present finding that Ach produced by sourdough LAB actively stimulates stomach motility as well as chloride ion secretion by intestinal mucosa, provides a completely new possibility for the targeted dual stimulation through selection and specific administration of acetylcholine-producing LAB. These bacteria could be orally administered in protected form (by encapsulation) as pharmaceuticals or "functional foods" and hence locally deliver acetylcholine to the effected tissue without systemic side-effects. This supplementation might be a promising addition to the traditional therapies for chronic IBS and other disorders associated with an impaired intestinal motility and secretion. Considering enzymatic and physiochemical fragility of acetylcholine it would be recommended to encapsulate acetylcholine-producing bacteria, thus bypassing harsh gut environment and enabling bacteria to exert local acetylcholine-mediated stimulation of intestinal wall.

8. APPENDIX

Parameters of compounds for LC-MS/MS analysis.

Compound ID	Mass transition Q1 → Q3 (Da)				DP [V]	CE [V]	CXP [V]
1-amino-S-propanole	*m/z*	75.88	→	76	26	5	2
1-amino-S-propanole 2	*m/z*	75.88	→	58	26	13	8
3-methyl-histidine	*m/z*	170.03	→	96.2	51	29	6
3-methyl-histidine 2	*m/z*	170.03	→	109	51	23	6
4-aminobenzoic acid	*m/z*	137.89	→	94.2	71	19	6
4-hydroxyproline	*m/z*	131.93	→	85.9	36	21	4
5-aminolevulinic acid	*m/z*	131.93	→	86	36	19	6
5-aminovaleric acid	*m/z*	117.91	→	55	31	23	8
Acetylcarnitin	*m/z*	203.85	→	84.9	31	27	4
Alanine	*m/z*	90.2	→	44.1	41	19	2
Alanine 2	*m/z*	90.11	→	90	26	5	6
Arachidonylcarnitin	*m/z*	448.38	→	85	61	49	4
Arachinylcarnitin	*m/z*	456.43	→	85	71	49	4
Arginine	*m/z*	175.09	→	70.1	36	33	4
Asparagin	*m/z*	132.93	→	73.9	1	19	6
Aspartic acid 1	*m/z*	134.07	→	74	46	19	6
Aspartic acid 2	*m/z*	134.07	→	87.9	46	15	6
Behenylcarnitin	*m/z*	484.39	→	85.1	71	53	4
Butyrobetain	*m/z*	145.98	→	146	21	11	4
Butyrylcarnitin	*m/z*	232.16	→	85	41	27	6
C12brDC	*m/z*	374.33	→	85	76	41	4
C16brDC	*m/z*	430.31	→	85	81	45	4
C3DC	*m/z*	248.14	→	84.9	26	35	6
C4brDC	*m/z*	262.19	→	85.2	56	35	6
C4DC	*m/z*	262.21	→	85.1	61	35	4
C5DC	*m/z*	276.2	→	85	35	33	6
C6brDC	*m/z*	290.26	→	85.1	56	33	0
Canavanine	*m/z*	177.01	→	76.1	46	23	2
Carnitin	*m/z*	162.01	→	102.9	56	25	6
Cimetidine 1	*m/z*	253.04	→	159.1	46	21	8
Cimetidine 2	*m/z*	253.04	→	95	46	39	6
Citrulline 1	*m/z*	176.12	→	70	31	33	4
Citrulline 2	*m/z*	176.12	→	113	31	23	8
Cystein	*m/z*	122	→	58.9	86	35	4
cysteic acid	*m/z*	169.92	→	123.9	56	19	6
Decanoylcarnitin	*m/z*	316.29	→	85	41	35	4
Erucaylcarnitin	*m/z*	482.36	→	85	71	49	4
Erucylcarnitin	*m/z*	482.36	→	60.2	71	55	4
Glutamin	*m/z*	147.03	→	84	46	23	6
Glutamic acid	*m/z*	148.08	→	84	31	23	6
Glycin	*m/z*	76.08	→	76	31	5	6
Hexanoylcarnitin	*m/z*	260.07	→	85	31	29	4

Histidine	*m/z*	156.15	→	110	41	21	8
Histidinol	*m/z*	141.97	→	81	41	29	2
Homoserine	*m/z*	119.93	→	74	41	17	2
Hydroxyarginine	*m/z*	191.4	→	70	51	35	10
Hydroxy-lysine	*m/z*	163.07	→	127.9	46	17	6
Isoleucin	*m/z*	132.06	→	86	41	15	6
Lauroylcarnitin	*m/z*	344.29	→	85	46	37	4
Leucin	*m/z*	132.06	→	86	41	15	6
Lignocerylcarnitin	*m/z*	512.41	→	85	71	53	4
Linolenoylcarnitin	*m/z*	422.39	→	85	61	47	4
Linolylcarnitin	*m/z*	424.33	→	85	61	43	4
Lysin	*m/z*	147.03	→	84	46	23	6
Methionine	*m/z*	150.07	→	104	31	15	8
Myristoylcarnitin	*m/z*	372.36	→	85	56	41	4
N,N,N-trimethyllysine	*m/z*	189.03	→	84.1	51	31	4
N,N-dimethyl-glycine	*m/z*	104.28	→	104	16	7	6
N-acetyl-alanine	*m/z*	131.93	→	72	36	19	2
N-acetyl-asparagine	*m/z*	174.93	→	88	41	23	6
N-acetyl-aspartic acid	*m/z*	175.96	→	134	46	15	6
N-acetyl-b-alanine 1	*m/z*	131.93	→	72	36	19	2
N-acetyl-b-alanine 2	*m/z*	131.93	→	90	36	17	6
N-acetyl-glycine	*m/z*	117.93	→	76	26	13	2
N-acetyl-histidine	*m/z*	198	→	110	46	29	6
N-acetyl-lysine	*m/z*	188.99	→	84.1	46	33	4
N-acetyl-ornithine 1	*m/z*	175.01	→	114.9	41	19	6
N-acetyl-ornithine 2	*m/z*	175.01	→	70.1	41	35	4
N-acetyl-phenylalanine	*m/z*	207.99	→	120.1	16	27	6
NAD	*m/z*	664.09	→	135.09	71	67	6
O-acetyl-homoserine	*m/z*	162.06	→	102	36	15	6
Octanoylcarnitin	*m/z*	288	→	85	41	33	4
Oleoylcarnitin	*m/z*	426.41	→	85	76	45	4
Ornithine	*m/z*	132.98	→	70	31	25	12
Palmitoylcarnitin	*m/z*	400.36	→	85	61	43	4
Phenylalanine	*m/z*	166.04	→	120	51	19	10
Proline	*m/z*	116.08	→	70	21	21	4
Propionylcarnitin	*m/z*	217.88	→	85	26	27	4
Pyroglutamic acid	*m/z*	130.07	→	84	36	19	6
Riboflavine	*m/z*	377.02	→	243.1	66	33	12
Serine	*m/z*	106.07	→	60	26	17	4
S-lactoyl-glutathione	*m/z*	380.02	→	142.1	46	21	8
S-methyl-methionine	*m/z*	164.02	→	102	31	15	6
Stearoylcarnitin	*m/z*	428.44	→	369.3	56	27	4
Taurine	*m/z*	125.99	→	44.1	46	29	2
Threonine 1	*m/z*	120.08	→	73.9	36	17	6
Threonine 2	*m/z*	120.08	→	55.9	36	25	4
Thyroxine	*m/z*	777.66	→	731.6	71	35	20
Tryptophan	*m/z*	205.1	→	146	41	25	12

Tyrosine 1	*m/z*	182.1	→	136	26	19	10
Tyrosine 2	*m/z*	182.1	→	91	26	41	6
Valine	*m/z*	118.07	→	72.1	21	15	6
y-aminobuttersaeure	*m/z*	103.91	→	69	31	23	4

Metabolites (mM) in ceacum of 57/Black-6 wildtype mice:

mM	CO_M_1	CO_M_2	CO_M_3	CO_F_1	CO_F_2	CO_F_3	SD_M_1	SD_M_2	SD_M_3	SD_F_1	SD_F_2	SD_F_3	AN_M_1	AN_M_2	AN_M_3	AN_F_1	AN_F_2	AN_F_3
Class	CO	CO	CO	CO	CO	CO	SD	SD	SD	SD	SD	SD	AN	AN	AN	AN	AN	AN
Ala 2	12,26	10,27	6,42	7,45	3,96	7,24	2,67	3,01	3,06	8,62	8,16	7,54	10,96	8,86	5,66	1,90	9,85	5,23
Arg	15,31	12,29	9,88	9,65	10,00	10,55	10,89	9,43	9,83	12,37	9,60	10,85	10,68	10,34	9,97	9,64	12,82	12,16
Asn	1,21	0,45	0,18	NA	0,22	0,55	0,14	0,08	0,16	0,17	NA	0,13	0,36	0,11	NA	0,12	0,74	1,15
Asp 2	6,02	4,45	3,63	2,96	4,68	3,93	4,89	3,75	5,04	2,87	2,85	3,02	2,04	2,38	3,62	5,15	4,43	3,43
Cys	1,19	1,25	1,32	1,36	2,19	2,62	1,71	1,21	2,10	1,83	1,43	1,59	1,68	1,58	1,34	2,73	2,03	1,81
Gln	2,10	1,11	0,45	0,99	2,38	1,60	5,14	1,67	2,52	1,55	0,42	0,91	0,62	1,48	1,80	6,97	3,03	2,32
Glu 1	10,36	9,01	8,20	8,40	5,44	6,59	7,25	13,13	8,72	9,24	11,78	7,35	7,48	9,65	7,29	6,74	8,74	7,80
Gly	4,56	3,75	1,81	2,84	2,14	2,83	1,96	1,56	1,89	2,62	2,66	2,10	2,52	2,74	2,31	2,04	3,95	1,94
His 1	3,38	2,69	2,65	2,67	2,79	2,67	3,05	2,70	2,67	2,96	2,24	2,72	3,00	2,44	2,40	3,18	3,50	3,07
Ile	7,57	6,46	4,47	4,47	4,27	5,16	4,43	3,94	4,42	5,33	4,70	4,80	5,27	5,48	4,56	4,06	6,77	4,60
Leu	8,68	7,95	5,83	5,63	5,76	6,54	5,81	5,66	5,93	6,81	6,20	6,54	6,63	6,92	6,21	5,61	8,35	6,11
Lys	18,98	10,77	5,50	6,44	5,23	8,61	5,98	4,65	6,17	9,11	6,21	6,59	7,94	8,42	5,65	4,28	12,30	7,00
Met 1	3,54	2,40	1,58	1,70	1,39	1,84	1,45	1,31	1,53	2,13	1,53	1,72	2,11	2,02	1,45	1,27	2,69	1,69
Phe	4,55	3,24	1,75	1,90	1,49	2,46	1,58	1,29	1,71	2,41	1,76	2,04	2,36	2,38	1,67	1,37	3,69	1,94
Pro	3,50	2,77	1,72	1,93	1,70	2,06	1,63	1,37	1,59	1,84	1,95	1,61	1,80	1,60	1,47	1,82	2,52	1,78
Ser 1	6,38	5,35	3,80	3,64	3,56	4,30	3,47	3,12	3,73	4,21	4,05	4,20	3,62	3,61	3,55	3,28	4,97	3,73
Thr 1	3,74	2,98	2,08	2,14	1,93	2,12	1,97	1,76	2,10	2,52	2,30	2,37	2,16	2,28	2,00	1,71	3,13	2,18
Trp 1	0,66	0,51	0,28	0,27	0,23	0,38	0,27	0,16	0,26	0,43	0,29	0,35	0,41	0,28	0,24	0,32	0,61	0,34
Tyr 1	5,03	3,43	1,75	1,68	1,67	2,62	1,63	1,46	1,76	2,75	1,80	2,23	2,83	2,69	1,80	1,54	4,25	2,04
Val 1	5,92	5,23	3,39	3,10	2,83	3,70	2,59	2,46	2,99	3,69	3,74	3,85	4,05	4,11	3,25	2,56	5,40	3,50
4-hydroxyproline	1,68	1,85	1,81	1,52	1,72	1,70	1,36	1,31	1,48	1,81	1,70	1,76	1,48	1,76	1,70	1,33	1,84	1,83
5-aminovaleric acid	2,66	2,28	2,84	2,35	1,86	2,27	1,58	1,02	1,72	1,56	2,51	3,10	1,88	3,98	3,15	4,02	2,74	5,58
N,N,N-trimethyllysine	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,05	NA	0,04	0,04	0,04	0,05	0,05	NA	0,05	0,04
pyroglutamic acid	13,97	14,55	15,57	13,13	15,43	14,47	4,61	4,13	4,43	17,07	16,83	14,85	13,12	14,52	15,32	2,61	15,73	15,86
riboflavine	0,02	0,02	0,02	0,02	0,01	0,02	0,01	0,01	0,01	0,01	0,02	0,01	0,01	0,02	0,01	0,02	0,02	0,01
taurine	0,51	0,96	0,31	1,37	0,41	0,61	0,59	NA	0,80	0,88	1,22	1,14	1,98	1,19	0,31	0,94	4,59	1,63
carnitine	0,03	0,03	0,02	0,03	NA	NA	NA	0,02	NA	NA	0,02	NA	NA	NA	NA	NA	0,03	NA
citrulline	NA	1,54	NA	NA	NA	NA	NA	0,83	NA	2,01	2,21	NA	2,04	NA	NA	NA	NA	NA
cysteic acid	NA	NA	NA	NA	0,02	NA	NA	0,02	NA	0,02	NA	NA	0,02	NA	NA	NA	0,02	NA
N-acetyl-glycine	NA	NA	0,03	NA	NA	NA	NA	0,04	0,02	NA	0,04	0,04	NA	0,02	NA	0,02	NA	NA
N-acetyl-lysine	NA	NA	NA	0,02	0,01	NA	0,01	0,01	NA	NA	0,02	0,02	NA	NA	NA	0,01	0,01	NA
ornithine	NA	0,66	0,77	0,67	NA	NA	0,23	0,66	NA	NA	0,38	NA	NA	0,52	0,29	NA	0,62	0,37

Metabolites (mM) in ceacum of 12/Sv wildtype mice:

Name mM	CO_M_1	CO_M_2	CO_M_3	CO_M_4	CO_M_5	CO_F_1	CO_F_2	CO_F_3	CO_F_4	CO_F_5	SD_M_1	SD_M_2	SD_M_3	SD_M_4	SD_M_5	SD_F_1	SD_F_2	SD_F_3	SD_F_4	SD_F_5	AN_M_1	AN_M_2	AN_M_3	AN_M_4	AN_M_5	AN_F_1	AN_F_2	AN_F_3	AN_F_4	AN_F_5
Class	CO	CO	CO	CO	CO	CO	CO	CO	CO	CO	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
Ala 2	4,89	3,73	4,65	2,87	3,63	4,31	6,62	5,58	5,54	5,23	3,74	5,33	5,00	4,55	3,74	4,06	4,35	5,12	4,00	5,42	3,19	4,30	3,58	4,30	3,53	4,49	4,99	5,39	5,41	4,48
Arg	3,25	2,47	2,95	2,73	1,77	2,26	4,29	4,02	3,13	3,25	2,27	3,40	3,51	3,32	1,84	2,17	3,07	3,16	2,79	3,03	2,16	2,60	1,90	2,10	1,62	2,65	3,51	3,44	3,59	3,07
Asn	1,72	NA	0,99	0,16	NA	1,21	3,90	3,45	1,02	1,36	0,52	0,48	0,90	0,89	0,26	0,61	1,66	2,22	0,51	0,17	0,42	0,11	0,27	0,50	0,22	1,57	1,95	2,34	3,13	1,78
Asp 2	3,71	1,43	3,63	0,74	1,28	3,56	6,92	4,74	5,11	4,04	2,48	5,65	4,09	3,05	2,37	2,22	3,48	4,71	2,45	4,05	2,48	2,50	2,40	3,37	2,47	2,42	2,98	5,00	5,08	2,00
Gln	2,04	0,93	1,58	0,63	0,97	1,41	4,08	2,45	2,36	2,20	1,22	2,22	1,64	1,64	0,95	1,51	1,39	2,12	1,13	1,58	0,83	1,20	0,78	1,36	1,04	1,79	2,45	2,69	3,08	1,45
Glu 1	0,08	NA	0,05	NA	NA	0,04	0,14	0,08	0,09	0,08	0,01	0,07	0,04	0,07	0,01	0,03	NA	0,05	0,02	0,02	NA	0,02	0,01	0,04	NA	0,02	0,06	0,07	0,12	NA
Gly	NA	2,79	5,49	0,92	4,21	5,67	NA	NA	NA	NA	3,01	NA	NA	4,46	2,56	2,61	5,01	NA	4,06	NA	1,68	4,24	1,88	3,85	1,81	4,64	NA	NA	NA	4,97
His 1	1,38	0,34	0,82	0,22	0,83	0,60	2,39	1,92	1,64	1,11	0,95	1,69	1,36	1,33	0,62	1,07	1,05	2,10	0,42	0,95	0,39	0,64	0,68	1,21	0,46	1,25	2,00	1,68	2,05	1,20
Ile	4,03	2,00	3,62	1,33	2,21	3,17	5,53	4,30	4,31	3,87	2,58	3,33	3,98	3,86	2,48	3,07	3,57	4,47	2,57	3,28	1,97	2,62	2,17	2,91	2,20	3,25	3,86	4,97	4,69	3,14
Leu	6,53	2,92	5,85	1,97	3,03	4,56	NA	8,72	8,03	6,99	3,43	7,96	7,08	6,66	3,43	4,37	5,65	9,08	3,87	6,49	2,62	3,99	2,96	4,53	3,20	5,20	7,31	NA	NA	4,84
Lys	9,38	6,49	9,65	4,29	4,95	7,42	20,64	12,89	12,79	10,83	5,52	10,41	11,09	9,41	5,12	8,14	7,73	10,26	6,76	10,12	4,93	6,59	4,21	6,55	5,71	7,97	11,49	11,17	11,83	7,48
Met 1	2,94	1,60	2,82	1,29	1,45	2,15	5,03	3,70	3,13	3,16	1,77	3,01	2,87	2,90	1,70	2,23	2,54	3,02	2,08	2,83	1,42	1,92	1,43	2,29	1,70	2,77	3,17	3,48	3,65	2,41
Phe	3,19	1,81	2,87	1,19	1,70	2,63	5,61	4,03	3,45	3,63	1,72	3,41	3,38	3,47	1,97	2,25	2,62	3,38	2,15	3,06	1,59	2,05	1,66	2,41	1,87	2,86	3,35	3,93	4,16	2,57
Pro	1,95	0,95	1,63	0,63	0,87	1,48	3,14	2,36	2,62	2,01	1,07	2,60	2,22	1,42	1,02	1,32	1,62	2,37	0,98	2,25	0,81	1,16	0,91	1,51	0,79	1,55	2,26	2,43	2,94	1,65
Ser 1	5,34	2,12	4,11	1,27	2,65	3,67	NA	NA	7,27	6,30	2,70	NA	4,81	3,85	2,32	3,22	4,12	7,25	2,57	5,46	1,61	2,90	2,05	3,25	2,10	4,08	7,22	NA	NA	4,22
Thr 1	NA	1,20	3,11	0,80	1,40	2,38	NA	NA	NA	NA	1,60	NA	3,16	0,00	1,52	2,10	3,32	NA	1,47	NA	1,04	2,30	1,46	2,59	1,41	NA	NA	NA	NA	2,90
Trp 1	0,55	0,29	0,47	0,20	0,27	0,41	1,03	0,72	0,63	0,63	0,31	0,75	0,47	0,70	0,34	0,43	0,50	0,62	0,36	0,55	0,24	0,37	0,29	0,47	0,29	0,47	0,66	0,76	0,73	0,44
Tyr 1	3,51	1,91	3,47	1,41	1,69	2,74	7,51	4,97	4,21	4,03	2,06	4,14	4,08	4,49	2,13	2,75	3,29	4,14	2,55	3,47	1,61	2,36	1,97	2,62	2,02	3,39	4,55	4,78	4,89	3,08
Val 1	NA	2,69	5,63	1,37	2,81	4,90	NA	NA	NA	NA	3,35	NA	NA	NA	2,85	3,76	6,25	NA	3,09	NA	1,87	3,61	2,43	4,03	2,38	4,92	NA	NA	NA	5,11
citrulline	1,12	1,38	1,70	0,56	1,39	1,76	4,66	1,28	3,92	2,22	0,96	1,64	1,52	2,10	0,98	2,59	0,87	1,74	0,93	2,53	1,04	1,61	1,05	2,13	1,72	2,57	1,87	1,92	1,39	1,02
N-acetyl-glycine	NA	NA	NA	NA	NA	NA	0,01	0,01	NA	NA	0,01	NA	0,01	NA	0,01	NA	0,01	NA	0,02	NA	NA	NA	NA	0,02	NA	NA	0,02	0,01	NA	NA
N-acetyl-histidine	NA	NA	NA	0,01	NA	0,01	0,01	NA	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	NA	0,01	NA	NA	0,01	NA	NA	0,01	0,01	NA	0,01	0,01	0,01	NA
ornithine	1,47	1,44	0,72	0,28	1,71	1,33	0,78	0,83	1,57	0,68	1,76	0,68	0,91	0,55	1,34	1,76	1,51	1,00	1,57	1,68	0,88	1,49	0,95	0,68	1,53	1,44	1,18	0,14	0,81	1,58
riboflavine	0,02	0,02	0,02	0,02	0,01	0,02	0,03	0,02	0,02	0,02	NA	0,02	0,02	0,02	0,01	0,02	0,02	0,02	0,02	0,02	0,01	0,02	0,01	0,02	0,01	NA	0,02	0,02	0,02	0,01
taurine	2,41	2,13	1,60	1,18	2,72	NA	3,33	5,47	1,98	3,29	1,62	NA	2,41	2,56	NA	2,31	1,04	NA	2,55	2,18	0,79	2,86	NA	2,15	0,70	1,45	2,26	1,63	3,76	1,74

Metabolites (mM) in ceacum of IL-10 knockout mice:

Name mM	SD Female 1	SD Female 2	SD Female 3	SD Female 4	SD Female 5	SD Male 1	SD Male 2	SD Male 3	SD Male 4	SD Male 5	CO Female 1	CO Female 2	CO Female 3	CO Female 4	CO Female 5	CO Male 1	CO Male 2	CO Male 3	CO Male 4	CO Male 5	AN Female 1	AN Female 2	AN Female 3	AN Female 4	AN Female 5	AN Male 1	AN Male 2	AN Male 3	AN Male 4	AN Male 5
Class	CO	CO	CO	CO	CO	CO	CO	CO	CO	CO	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
5-aminovaleric aci	32,29	50,49	15,93	27,71	23,33	20,08	21,14	11,28	36,75	24,68	5,15	2,48	9,29	21,03	29,04	30,02	57,41	18,44	6,23	6,58	17,93	40,85	6,53	29,76	19,81	9,58	42,50	29,38	16,44	2,48
Ala	64,71	151,36	34,60	82,95	66,67	40,97	38,25	24,05	91,76	63,05	17,67	9,11	36,84	52,92	99,56	55,59	80,20	48,94	20,68	19,90	40,21	72,96	17,54	53,96	31,51	20,83	72,99	52,00	35,08	NA
Arg	22,14	7,74	14,45	17,19	14,91	12,35	11,84	4,56	21,34	16,96	2,59	0,72	3,16	23,82	34,39	42,16	36,39	17,74	5,80	3,56	16,93	17,57	1,76	22,06	11,63	4,34	16,52	11,28	4,26	0,64
Asn	7,04	8,10	1,74	4,75	3,66	3,33	3,20	2,35	7,24	4,09	NA	NA	1,51	3,02	2,52	6,41	17,02	0,57	0,92	0,88	3,04	7,35	2,81	4,56	5,18	1,21	5,70	5,79	3,27	NA
Asp 1	12,09	28,15	5,88	14,25	10,83	5,82	4,60	3,41	15,23	11,41	3,87	1,17	4,54	10,77	17,54	9,30	25,69	12,27	5,88	3,58	7,43	16,13	5,78	11,87	11,55	7,48	26,78	19,03	11,54	1,15
Asp 2	9,67	20,74	6,22	14,84	9,49	5,60	5,19	4,23	13,55	9,32	4,45	1,31	4,71	10,15	12,54	2,37	10,44	5,00	2,05	0,88	3,08	8,50	0,69	NA	5,42	2,00	6,80	4,49	2,39	NA
citrulline 2	2,41	39,04	4,56	9,59	5,72	1,80	0,89	4,18	8,26	5,20	1,15	0,35	0,62	5,03	6,34	0,61	0,93	3,85	3,93	2,04	1,01	3,70	1,04	2,04	1,17	0,80	4,06	2,64	1,85	1,18
Gln	11,31	14,60	4,75	12,29	7,96	5,91	6,37	2,94	14,39	8,99	4,82	0,72	3,45	9,96	18,27	10,66	21,42	7,94	3,01	3,14	5,21	15,01	3,09	9,69	6,54	3,52	15,56	13,11	7,36	0,58
Glu 1	35,12	53,51	18,71	32,01	28,29	15,27	21,95	13,06	39,66	30,06	13,23	4,94	17,52	28,26	46,85	19,49	51,08	34,41	42,00	22,21	20,83	44,23	9,39	25,01	21,53	26,37	44,52	35,26	25,76	2,10
His 1	2,49	1,57	1,12	1,44	0,97	0,80	1,34	0,78	2,02	1,27	0,28	NA	0,06	1,01	1,51	2,09	4,05	0,31	0,00	NA	1,65	1,39	0,16	1,23	1,49	0,10	1,31	1,22	0,29	NA
homoserine	6,09	11,66	3,20	7,74	5,22	4,73	3,09	1,69	8,46	5,35	2,36	0,15	2,57	6,18	10,02	7,82	11,72	4,92	1,69	1,37	4,66	9,79	2,68	5,35	5,59	2,00	9,99	6,24	3,89	0,16
Ile	19,29	27,67	10,46	21,33	15,67	11,22	10,70	7,31	27,56	13,86	2,61	0,88	7,05	14,09	22,26	20,93	32,48	12,38	3,89	4,19	12,35	24,43	6,86	21,08	14,87	5,79	25,29	21,94	10,46	2,41
Leu	54,37	293,31	23,37	69,60	41,51	31,12	34,59	17,88	95,60	53,04	5,46	2,14	10,21	24,40	52,87	77,05	113,28	33,47	7,34	8,09	30,19	120,55	9,29	49,97	28,40	9,05	76,01	42,33	19,17	3,47
Lys	52,52	92,55	27,53	52,89	28,61	23,63	27,88	19,99	58,37	32,37	9,02	2,05	9,83	53,15	73,35	71,40	71,18	42,98	18,20	11,38	39,37	46,74	11,17	41,57	33,77	14,83	49,30	40,10	17,39	4,95
Met 1	45,59	188,49	16,15	63,75	39,44	18,87	19,45	8,89	79,43	39,69	3,38	0,94	7,81	45,59	90,09	29,67	82,10	41,75	15,97	10,02	32,54	73,28	7,56	38,23	23,31	7,64	60,87	41,82	17,73	1,41
ornithine	0,08	1,31	1,00	1,02	0,55	0,04	0,10	0,37	0,63	0,49	NA	NA	NA	0,57	1,71	NA	NA	NA	NA	NA	NA	0,42	NA	NA	NA	NA	0,93	0,12	NA	0,05
Phe	13,29	22,63	6,96	14,39	11,90	8,79	10,16	6,63	18,99	14,21	2,16	0,67	4,08	11,47	16,56	14,69	19,93	11,54	3,67	3,39	9,76	18,63	4,00	12,87	9,57	3,81	17,45	14,74	7,97	1,13
Pro	4,61	8,48	2,84	12,37	8,97	3,08	4,57	3,37	19,55	7,19	2,33	0,50	1,57	4,03	6,32	4,26	8,71	3,50	1,19	1,19	2,74	6,62	1,73	4,90	4,73	2,28	8,79	6,17	3,34	0,66
Ser 1	17,35	26,88	8,36	17,56	13,06	12,52	8,17	6,60	21,71	14,74	6,65	2,87	6,96	13,52	18,90	18,04	30,09	12,14	4,59	6,20	8,99	20,23	7,49	15,83	13,69	6,13	25,03	16,25	11,12	3,38
taurine	50,76	6,06	16,34	20,58	15,65	13,32	18,69	9,61	39,40	27,20	105,24	7,36	9,80	25,86	120,74	9,96	5,64	9,51	1,98	5,12	7,73	24,76	1,86	6,41	6,81	11,83	24,78	12,79	11,66	2,62
Thr 2	3,22	5,71	1,65	3,13	2,72	2,41	1,43	1,16	4,75	2,69	1,17	0,11	1,28	2,66	5,08	3,92	5,60	2,11	0,80	0,91	2,33	4,32	1,32	2,67	2,63	0,88	4,90	3,39	2,12	2,53
Trp 1	3,87	1,11	0,73	2,98	0,77	1,64	0,87	0,71	1,55	2,23	0,33	0,01	0,88	0,78	1,85	1,85	6,06	1,20	0,48	0,44	0,96	2,96	0,92	2,85	2,66	0,65	3,24	1,30	1,75	0,18
Trp 2	3,76	1,74	0,59	1,30	0,75	1,77	2,09	0,74	1,66	1,19	0,37	NA	0,45	1,97	1,74	3,59	2,80	2,25	0,22	0,52	1,06	3,11	0,94	3,05	1,45	1,14	1,31	1,69	1,76	0,16
Tyr 1	36,74	84,51	13,75	32,89	23,90	13,94	20,12	7,57	38,96	27,46	2,63	0,57	5,15	23,62	29,93	6,05	55,20	18,14	5,80	3,74	17,27	25,61	5,65	20,79	20,12	4,88	28,66	17,23	10,88	1,05
Tyr 2	22,12	44,26	8,49	20,26	16,07	10,76	13,08	6,07	27,55	19,92	2,53	0,62	5,16	16,09	23,18	5,73	35,60	14,94	4,97	3,95	13,78	22,61	5,39	17,98	14,85	5,06	22,06	16,96	9,93	1,16
Val 1	30,88	50,66	16,16	36,02	24,76	21,32	24,71	12,65	43,09	29,49	5,77	1,97	10,17	22,92	35,51	36,64	54,89	24,76	8,29	6,98	19,19	45,37	9,96	36,51	20,97	8,49	44,09	31,67	16,05	2,27
N,N,N-trimethylly	0,01	0,01	0,01	0,03	0,01	0,01	0,01	0,01	0,03	0,01	0,01	0,00	0,00	0,01	0,04	0,02	0,03	0,01	0,00	0,00	0,01	0,02	0,00	0,02	0,02	0,00	0,03	0,02	0,01	0,01

Metabolites (mM) in plasma of 57/Black-6 wildtype mice:

microM	CO_M_1	CO_M_2	CO_M_3	CO_F_1	CO_F_2	CO_F_3	SD_M_1	SD_M_2	SD_M_3	SD_F_1	SD_F_2	SD_F_3	AN_M_1	AN_M_2	AN_M_3	AN_F_1	AN_F_2	AN_F_3
Class	CO	CO	CO	CO	CO	CO	SD	SD	SD	SD	SD	SD	AN	AN	AN	AN	AN	AN
Ala 2	1981,06	1497,30	1603,93	1534,90	1549,50	1211,65	1510,21	1568,02	1766,68	1885,66	1961,98	1707,20	1472,61	1656,13	1000,63	1479,34	1177,41	1548,93
Arg	218,22	205,91	289,03	311,71	314,00	263,66	203,33	174,40	284,27	244,31	315,44	235,10	244,66	217,51	158,52	238,43	183,09	255,85
Asn	230,48	198,04	196,49	185,86	244,44	147,86	161,45	134,54	224,12	238,84	292,65	234,18	222,72	182,68	137,41	167,39	240,81	323,23
Asp 2	253,89	232,61	251,34	290,06	320,17	284,66	45,11	231,70	329,26	240,48	221,96	287,50	245,34	252,27	200,26	315,91	236,70	254,23
Cys	50,31	0,00	19,17	46,34	36,63	35,80	NA	19,43	16,24	40,23	70,61	21,44	38,09	29,28	27,77	NA	9,32	17,39
Gln	1112,25	958,98	1120,12	1213,86	1135,17	1026,38	938,11	1112,94	1003,80	1003,45	1064,01	1032,88	916,55	1026,04	963,08	1012,01	952,82	1090,35
Glu 1	379,26	442,13	486,98	366,34	291,04	273,67	457,76	465,57	314,89	452,32	333,72	303,78	365,66	507,71	343,23	477,13	529,80	541,02
Gly	1029,08	1081,04	891,21	893,20	980,46	723,35	1009,10	814,61	1001,77	1025,75	1009,77	1083,03	925,84	818,60	786,63	1036,41	1002,44	1114,34
His 1	512,07	444,07	459,54	535,59	444,39	452,13	388,00	475,01	474,69	442,78	502,08	437,95	390,25	414,42	390,90	487,58	432,79	520,12
Ile	318,89	237,43	280,58	352,13	312,91	318,85	261,83	278,18	332,38	295,14	343,90	283,36	287,18	291,56	353,08	275,89	269,80	262,44
Leu	334,40	241,97	300,02	388,80	329,98	339,05	232,25	289,77	349,96	321,33	350,49	308,67	265,45	296,52	368,21	266,14	281,19	280,47
Lys	1005,21	664,21	867,03	934,41	988,79	876,27	658,74	786,32	961,43	979,90	1104,06	801,02	894,05	809,57	688,50	680,97	526,03	795,55
Met 1	312,27	216,07	299,34	285,37	314,99	258,36	256,35	230,41	319,48	337,77	351,85	256,92	283,25	241,60	225,42	251,69	136,18	276,82
Phe	179,28	161,39	157,73	160,36	188,63	181,91	157,58	174,07	197,20	177,16	198,68	188,60	157,36	181,37	194,02	176,89	196,23	207,61
Pro	243,94	128,33	179,80	74,96	147,14	136,67	182,66	192,43	216,58	217,36	310,17	230,69	220,62	200,25	113,44	167,50	132,59	196,65
Ser 1	678,92	596,61	631,82	562,83	629,92	536,19	468,30	542,85	692,72	726,50	795,49	628,97	591,38	553,80	443,32	549,04	515,26	695,10
Thr 1	758,90	440,73	706,85	628,36	680,83	627,10	572,11	733,72	777,79	902,87	940,65	708,11	742,95	709,79	555,74	570,43	389,10	711,47
Trp 1	239,73	180,29	270,04	226,24	255,35	256,82	161,29	161,78	333,94	300,40	367,97	355,98	191,16	186,36	237,19	195,10	219,68	282,04
Tyr 1	352,39	410,62	396,82	451,46	430,76	384,68	343,29	315,97	469,67	451,74	488,44	459,74	375,02	397,65	409,24	356,53	365,92	523,76
Val 1	437,48	327,67	382,17	406,03	404,07	405,09	332,02	370,05	431,94	427,67	476,75	389,47	382,47	384,18	443,46	358,78	412,60	414,65
acetylcholine 2	0,04	0,00	0,06	0,24	0,06	NA	0,13	0,32	0,09	0,07	0,04	0,05	0,03	0,24	0,21	0,25	0,07	0,03
3-methyl-histidine	14,08	24,83	16,95	14,84	23,34	11,95	20,00	10,44	18,01	12,93	15,82	19,90	15,97	9,79	10,24	24,25	4,88	12,78
4-hydroxyproline	69,77	126,56	54,31	64,16	64,16	46,87	102,56	125,08	55,69	65,19	70,95	56,15	133,74	127,25	47,29	133,55	34,64	80,69
carnitine	0,61	0,39	0,89	1,61	1,31	1,43	0,74	0,84	0,99	0,87	1,26	1,31	0,53	0,74	1,25	0,69	1,03	0,92
cimetidine 2	0,00	0,02	0,01	0,02	0,01	0,01	0,01	NA	0,01	0,01	0,01	NA	0,01	0,01	NA	0,01	NA	0,01
hydroxy-lysine	2,80	3,16	2,98	4,47	3,41	2,86	1,58	2,58	3,12	2,94	3,10	2,61	3,06	3,53	2,26	3,32	2,45	2,71
N,N,N-trimethyllysine	14,64	17,72	12,74	23,76	18,01	18,18	14,22	15,80	13,78	15,20	17,41	15,68	14,11	18,57	13,34	15,54	11,51	13,37
N-acetyl-alanine	1,42	4,09	1,93	3,22	3,21	2,64	1,61	2,15	1,91	1,82	1,71	2,08	1,31	1,40	1,65	2,14	2,55	1,68
N-acetyl-aspartic acid	0,00	3,64	0,00	NA	0,65	NA	NA	1,40	3,32	1,18	1,18	1,29	0,02	1,94	1,58	NA	NA	1,24
N-acetyl-b-alanine	1,45	4,59	1,87	3,85	3,67	3,09	1,89	2,37	2,26	2,18	1,67	2,26	1,33	1,63	2,11	2,49	3,00	1,81
N-acetyl-histidine	1,86	2,41	1,77	3,32	1,79	1,75	2,48	5,71	3,36	2,30	1,83	1,57	2,84	4,13	2,81	5,29	2,37	2,02
N-acetyl-ornithine 2	0,02	0,04	0,12	0,00	0,01	0,10	0,04	0,08	0,13	0,09	0,11	0,11	0,07	0,08	0,00	0,14	0,07	0,08
O-acetyl-homoserine	0,00	0,00	0,03	0,03	NA	0,01	0,22	NA	NA	NA	NA	NA	NA	0,01	NA	NA	NA	NA
ornithine	284,05	134,17	177,92	179,02	197,73	138,11	112,20	209,51	263,45	269,89	329,77	195,42	290,98	194,96	147,35	175,27	164,58	191,02
riboflavine	0,25	0,28	0,22	0,35	0,31	0,37	0,13	NA	0,15	0,12	0,12	0,16	0,22	0,14	0,11	0,18	0,29	0,27
S-methyl-methionine	2,76	9,09	2,14	1,63	1,51	1,80	2,81	7,67	2,19	2,42	2,43	2,05	3,57	3,96	1,49	7,90	1,90	4,56
taurine	548,94	730,72	659,21	606,47	669,60	655,61	998,40	482,22	463,84	618,86	608,47	600,48	646,42	631,24	515,38	580,10	614,06	864,56
Acetylcarnitin	23,92	46,35	35,42	30,64	66,89	46,77	25,81	24,26	27,45	28,29	26,24	23,38	NA	25,56	42,12	22,14	23,10	26,02
Butyrobetain	0,06	0,05	0,04	0,05	NA	0,07	0,04	0,05	0,05	0,10	0,09	0,08	NA	0,05	0,10	0,06	0,06	0,07
C3DC	NA	NA	0,26	NA	1,43	NA	NA	NA	0,05	NA	NA	0,09	NA	0,18	0,29	NA	0,14	0,10
Carnitin	4,36	10,25	5,06	5,32	7,52	8,75	4,17	4,40	5,25	5,14	5,43	4,04	NA	7,25	7,76	3,77	4,80	5,85
Erucylcarnitin	0,09	NA	NA	NA	NA	NA	NA	0,00	NA	0,02	0,13	NA	NA	0,06	NA	NA	0,12	0,05
Hexanoylcarnitin	0,82	3,07	1,08	0,22	1,74	0,47	1,00	0,86	0,30	0,41	0,24	0,79	NA	0,13	0,75	0,89	0,18	0,33
Lauroylcarnitin	0,03	0,09	0,07	NA	0,15	0,14	0,28	0,05	0,05	0,05	NA	0,02	NA	NA	0,05	0,05	NA	NA
Myristoylcarnitin	0,00	NA	NA	NA	0,77	0,16	0,24	NA	NA	0,04	NA	NA	NA	NA	NA	NA	NA	NA
N,N,N-trimethyllysin	0,04	0,00	0,14	0,04	NA	NA	0,18	0,22	0,30	0,07	0,13	0,22	NA	NA	NA	0,00	0,11	0,07
Palmitoylcarnitin	0,19	0,32	0,37	0,31	2,61	0,47	0,21	0,15	0,24	0,22	0,13	0,14	NA	0,24	0,30	0,10	0,11	0,14
Propionylcarnitin	3,09	7,02	4,39	2,72	8,07	3,63	3,41	3,15	2,60	3,98	4,07	3,28	NA	2,16	5,08	3,40	3,10	3,32
Stearoylcarnitn	0,56	0,79	0,62	0,55	0,95	0,74	0,45	0,55	0,59	0,48	0,50	0,58	NA	0,69	0,66	0,39	0,48	0,45

Metabolites (mM) in plasma of 129/Sv mice:

µM	CO_M	CO_M	CO_M	CO_M	CO_M	CO_F_	CO_F_	CO_F_	CO_F_	CO_F_	SD_M	SD_M	SD_M	SD_M	SD_M	SD_F_	SD_F_	SD_F_	SD_F_	SD_F_	AN_M	AN_M	AN_M	AN_M	AN_M	AN_F_	AN_F_	AN_F_	AN_F_	AN_F_
Class	CO	CO	CO	CO	CO	CO	CO	CO	CO	CO	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
Ala 2	313,49	244,97	387,18	331,84	375,22	332,12	468,05	399,13	367,25	302,10	346,27	393,52	309,56	387,46	369,67	431,18	438,75	347,56	316,97	343,80	387,85	289,36	259,33	343,23	304,68	377,30	384,71	447,68	288,01	475,62
Arg	43,60	40,13	58,15	49,80	37,89	42,91	53,76	51,12	56,69	39,84	36,91	37,69	27,17	46,84	41,82	49,28	49,11	45,58	43,37	39,64	51,55	25,83	32,35	18,02	31,29	42,97	46,96	42,65	34,56	80,88
Asn	25,62	22,05	37,15	26,42	31,79	29,83	41,67	33,53	36,31	25,39	25,96	27,93	23,30	26,21	20,67	38,45	36,96	29,24	26,92	24,27	28,35	21,68	21,87	23,35	22,03	30,70	30,42	38,56	23,62	35,38
Asp 2	36,59	34,74	32,41	26,50	34,20	34,66	47,93	39,20	41,88	35,74	5,50	NA	22,50	NA	26,92	33,66	35,82	31,76	34,46	35,20	18,08	7,93	11,07	12,34	12,75	32,16	34,86	40,97	34,40	16,34
cysteic acid	NA	NA	0,22	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0,24	NA	NA	0,17	NA	NA	NA	0,19	0,19	NA	NA	0,19	0,19	0,21	0,26	NA	NA
Gln	247,05	233,06	263,13	268,71	277,43	227,27	313,49	273,60	249,41	264,29	290,36	275,24	223,65	264,53	297,17	285,95	276,44	256,42	280,03	242,33	277,57	187,01	192,65	242,77	271,37	297,07	302,37	330,94	254,54	308,02
Glu 1	52,81	68,34	78,40	48,32	61,37	41,22	81,56	76,50	46,18	39,62	38,37	48,83	26,76	75,51	50,87	64,81	50,60	54,14	30,92	36,12	33,32	24,76	20,87	36,77	28,46	46,08	47,58	37,69	36,26	49,55
Gly	181,04	139,74	219,74	221,40	227,06	168,78	186,50	134,21	179,24	124,49	215,27	226,80	175,31	220,40	202,49	186,83	161,59	130,88	165,99	146,40	218,27	147,34	151,13	186,17	192,23	329,37	321,91	364,08	202,15	370,00
His 1	108,15	88,11	119,07	110,99	111,05	106,41	144,53	123,62	107,15	106,70	99,03	119,88	140,76	115,50	167,80	107,15	125,33	104,70	87,01	95,55	117,21	84,04	89,59	96,36	96,16	117,59	113,56	118,59	96,07	186,36
Ile	100,29	95,87	124,64	112,18	126,93	91,10	123,92	125,98	103,64	94,38	116,83	105,05	96,21	106,00	109,97	118,51	109,59	94,04	94,34	93,35	119,57	75,85	76,92	99,91	94,80	104,94	106,88	119,99	107,38	121,21
Leu	98,08	91,94	122,93	106,73	120,45	92,47	127,73	123,39	107,30	96,55	113,36	102,88	84,89	107,57	104,67	116,56	109,51	89,96	92,74	97,89	115,61	73,45	75,62	95,94	89,46	101,05	103,41	122,81	109,13	113,36
Lys	214,42	170,19	266,20	214,24	202,65	210,62	288,81	276,05	273,41	215,61	227,24	247,45	204,87	234,22	214,65	305,50	312,00	249,78	243,18	262,54	223,79	146,66	170,94	165,98	165,68	235,93	244,65	314,83	228,81	363,91
Met 1	42,29	30,03	53,15	42,69	44,84	36,22	51,84	52,48	57,30	56,87	40,38	38,00	38,30	46,68	45,27	58,78	51,70	39,91	36,22	37,43	41,38	28,01	33,58	32,52	30,18	41,02	42,89	51,70	35,18	58,94
Phe	51,30	48,91	65,44	58,30	64,41	54,57	70,07	69,77	58,66	55,27	62,05	58,18	57,87	59,69	61,20	63,56	59,20	54,06	52,64	56,69	66,20	43,34	42,47	58,78	50,85	60,14	61,90	66,65	62,99	70,13
Pro	32,25	26,06	48,21	29,63	44,08	38,81	51,20	43,43	37,09	26,37	23,81	28,01	47,68	35,08	45,82	42,96	43,33	30,70	26,51	29,76	28,35	30,86	22,93	30,72	20,20	38,13	39,51	38,69	25,30	57,46
Ser 1	109,76	80,31	128,65	129,84	151,58	161,00	197,73	152,01	147,73	113,85	129,08	151,77	108,43	148,30	138,12	175,65	151,06	139,64	129,69	112,52	143,16	95,20	115,94	101,62	118,28	136,69	143,25	174,99	105,48	174,70
Thr 1	118,70	81,22	154,59	130,33	142,29	128,32	176,00	160,93	155,47	110,94	110,44	145,23	94,23	138,85	103,93	155,43	147,88	126,85	119,54	120,47	134,36	102,38	91,04	98,47	103,76	113,00	116,44	179,82	116,94	143,26
Trp 1	55,58	39,71	78,22	60,03	72,59	89,80	92,94	101,63	100,55	85,03	73,89	70,36	70,66	55,77	60,84	109,09	103,54	81,67	77,07	91,66	73,94	45,78	39,52	54,79	58,07	69,65	73,64	94,77	96,98	112,50
Tyr 1	51,08	40,21	70,87	65,65	86,40	71,14	86,48	82,29	98,16	71,97	54,00	60,08	40,68	60,68	53,92	68,16	91,98	61,40	53,45	48,02	59,50	39,32	42,06	49,64	48,57	56,10	55,69	57,87	52,65	67,91
Val 1	154,38	142,04	169,74	158,56	165,52	127,45	164,28	166,12	158,22	141,87	149,60	134,83	123,43	146,23	141,57	157,62	150,37	124,59	121,00	133,76	163,98	114,81	117,20	139,78	138,03	136,45	136,75	168,34	152,33	160,31
3-methyl-his	1,81	2,01	2,30	2,01	2,62	2,27	2,20	1,91	2,10	2,31	4,25	4,80	3,23	3,33	2,14	3,14	2,42	2,62	2,48	3,34	2,90	2,06	1,84	2,19	2,59	2,26	2,39	3,62	2,49	4,08
4-hydroxypr	7,84	6,65	9,21	7,39	7,69	4,12	7,15	5,01	4,58	4,17	9,55	10,02	6,35	11,13	10,10	6,34	5,10	4,61	4,98	5,48	8,31	5,37	5,23	6,63	6,81	6,37	6,13	6,51	3,60	6,81
5-aminovale	100,34	82,09	129,57	104,87	122,05	84,36	130,60	124,49	103,50	89,02	105,81	100,98	73,89	106,84	88,12	106,41	97,82	74,15	72,39	82,52	113,42	67,91	71,24	86,84	88,80	85,43	88,68	113,89	99,32	107,22
acetylcholin	NA	0,04	0,03	0,03	NA	0,02	0,03	0,02	NA	NA	0,03	NA	NA	0,02	0,04	NA	NA	NA	NA	0,02	0,02	NA	0,02	0,03	0,02	NA	0,02	NA	0,03	0,05
carnitine	40,56	34,29	39,23	49,29	46,51	25,88	47,22	50,00	32,35	37,56	44,35	59,81	60,00	49,85	79,00	49,35	41,67	33,12	35,49	38,02	45,56	28,46	26,25	34,17	41,88	33,15	37,04	36,73	34,81	78,00
cimetidine 2	NA	NA	0,01	NA	0,01	NA	NA	0,01	NA	0,01	NA	NA	NA	0,00	0,01	0,01	NA	NA	NA	NA	NA	NA	NA	NA	0,00	NA	0,01	NA	NA	NA
citrulline	47,97	40,89	54,89	63,40	53,69	52,74	77,91	58,34	63,03	46,74	58,14	46,23	42,91	65,46	59,37	71,69	66,17	58,14	57,94	68,91	65,20	45,03	41,91	41,77	41,20	46,77	50,54	71,14	37,91	57,40
hydroxy-lysi	NA	NA	0,53	0,53	0,70	0,69	0,62	NA	0,55	NA	0,51	NA	NA	0,69	NA	0,58	0,77	NA	NA	0,47	0,62	NA	0,48	NA	NA	0,61	NA	NA	NA	0,91
N,N,N-trime	1,23	1,52	1,28	1,52	1,73	1,15	1,45	1,30	1,29	1,60	1,56	1,50	2,46	1,43	2,16	1,17	1,37	1,27	1,36	1,40	1,55	1,28	1,00	1,20	1,44	1,53	1,63	1,51	1,39	2,60
N-acetyl-ala	0,10	0,13	0,20	0,20	0,12	0,19	0,18	0,18	0,23	0,16	0,22	0,23	0,12	0,18	0,20	0,33	0,19	0,08	0,15	0,24	0,20	0,07	0,09	0,11	0,11	0,13	0,16	0,21	0,14	0,22
N-acetyl-asp	0,14	0,11	0,15	0,22	0,24	0,06	0,12	0,15	0,05	0,13	0,07	0,14	NA	0,16	NA	0,06	0,15	0,04	NA	0,08	0,12	NA	0,09	0,11	0,11	NA	0,10	0,09	0,10	0,12
N-acetyl-asp	1,72	0,73	0,79	1,21	0,45	NA	1,55	NA	1,04	NA	NA	NA	NA	0,67	NA	1,39	0,76	0,19	0,75	NA	1,43	NA	NA	NA	1,03	1,70	0,81	NA	NA	0,95
N-acetyl-gly	4,09	4,74	7,13	9,10	10,15	4,21	NA	3,71	4,57	4,93	11,81	9,73	NA	8,87	NA	4,93	5,87	5,05	4,86	4,45	8,79	2,75	4,36	5,09	11,53	6,16	6,10	8,59	6,29	2,54
N-acetyl-hist	0,39	0,29	0,66	0,65	0,90	0,30	0,59	0,67	0,25	0,40	0,35	0,62	0,23	0,69	0,50	0,37	0,61	0,30	0,24	0,27	0,70	0,36	0,50	0,80	0,47	0,58	0,50	0,43	0,39	0,87
N-acetyl-lysi	0,24	0,34	0,31	0,39	0,35	0,22	0,42	NA	0,29	0,28	NA	0,27	0,13	0,35	NA	0,33	0,30	0,23	0,27	0,38	0,44	NA	0,23	0,24	0,26	0,22	NA	NA	NA	0,43

Metabolites (mM) in plasma of 129/Sv mice (continuation):

µM	CO_M_1	CO_M_2	CO_M_3	CO_M_4	CO_M_5	CO_F_1	CO_F_2	CO_F_3	CO_F_4	CO_F_5	SD_M_1	SD_M_2	SD_M_3	SD_M_4	SD_M_5	SD_F_1	SD_F_2	SD_F_3	SD_F_4	SD_F_5	AN_M_1	AN_M_2	AN_M_3	AN_M_4	AN_M_5	AN_F_1	AN_F_2	AN_F_3	AN_F_4	AN_F_5
Class	CO	CO	CO	CO	CO	CO	CO	CO	CO	CO	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
N-acetyl-orn	0,14	NA	0,15	NA	0,11	NA	0,09	0,10	NA	NA	NA	NA	NA	NA	NA	NA	NA	0,09	0,07	NA	0,13	NA	0,09	NA	0,12	NA	0,09	NA	0,08	NA
ornithine	60,87	37,57	82,77	59,09	65,38	77,27	82,58	74,77	63,07	41,97	33,44	46,74	82,99	60,23	41,55	66,44	75,95	47,46	50,42	44,66	63,67	41,06	45,64	44,17	41,82	55,15	58,33	62,35	44,36	100,34
riboflavine	0,12	0,12	0,11	0,12	0,14	0,12	0,18	0,14	0,13	0,18	0,11	0,13	0,13	0,12	0,11	0,14	0,12	0,11	0,12	0,15	0,11	0,13	0,12	0,10	0,11	0,12	0,15	0,15	0,14	0,16
S-methyl-me	NA	NA	0,15	0,09	0,12	0,09	0,18	0,09	0,08	0,07	NA	0,08	0,09	0,06	0,10	0,06	0,10	NA	0,05	NA	0,07	0,06	NA	0,06	NA	0,06	0,05	NA	0,04	0,12
taurine	199,28	200,64	228,69	220,85	218,66	111,03	172,47	141,19	132,48	155,33	212,03	178,79	207,07	178,11	141,15	166,96	196,48	174,31	144,15	135,16	251,70	155,21	172,91	217,98	182,62	113,70	117,46	138,63	112,07	182,42
Acetylcarniti	14,95	15,23	13,41	17,68	16,12	12,50	14,84	15,02	15,39	20,25	17,68	13,76	15,68	16,64	17,73	25,56	21,62	21,07	24,70	18,66	13,22	12,91	11,65	18,00	12,82	14,65	16,62	14,99	12,79	18,77
Carnitin	7,13	6,66	7,51	8,52	8,33	6,68	4,84	6,61	6,33	4,32	8,67	13,14	7,40	11,02	9,54	8,64	5,92	5,22	5,95	6,96	8,39	6,66	6,14	7,23	7,90	4,95	6,64	4,43	4,09	4,31
Arachidonyl	0,56	0,52	0,41	0,96	0,83	1,31	1,54	0,78	1,15	1,62	1,01	0,86	0,70	0,89	1,12	1,24	1,18	1,36	1,43	1,74	0,63	0,30	0,37	0,44	0,55	0,84	0,55	0,72	0,65	1,60
Arachinylcar	1,27	0,39	0,80	1,22	2,22	1,13	0,56	0,39	0,50	0,43	2,99	3,27	0,99	2,64	3,48	0,92	0,92	1,52	0,84	1,71	0,91	0,51	0,42	0,88	1,14	0,48	0,83	0,28	0,20	0,64
Behenylcarn	0,10	0,03	0,07	0,05	0,13	0,06	0,04	0,04	0,03	0,02	0,23	0,21	0,07	0,21	0,23	0,06	0,06	0,06	0,05	0,08	0,06	0,03	0,01	0,03	0,07	0,01	0,04	0,01	0,01	0,05
Butyrobetair	0,34	0,36	0,44	0,42	0,29	0,42	0,53	0,39	0,30	0,34	0,44	0,25	0,54	0,69	0,44	0,52	0,26	0,27	0,41	0,28	0,41	0,29	0,27	0,26	0,33	0,24	0,29	0,31	0,26	0,39
Decanoylcarn	0,01	0,00	0,00	0,01	0,01	0,01	0,02	0,00	0,01	0,02	0,02	0,01	0,01	0,01	0,01	0,01	0,01	0,02	0,01	0,01	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01
Erucaylcarnit	0,00	0,00	0,00	0,01	0,01	0,01	0,00	0,00	0,00	0,00	0,13	0,04	0,02	0,02	0,02	0,01	0,00	0,01	0,01	0,01	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00
Hexanoylcar	0,04	0,20	0,06	0,11	0,04	0,16	0,43	0,10	0,11	0,32	0,09	0,07	0,04	0,10	0,08	0,08	0,33	0,11	0,06	0,23	0,07	0,03	0,05	0,08	0,10	0,09	0,10	0,10	0,11	0,15
Lauroylcarnit	0,01	0,01	0,01	0,02	0,01	0,01	0,02	0,01	0,02	0,03	0,03	0,02	0,02	0,02	0,03	0,01	0,02	0,02	0,02	0,03	0,02	0,01	0,01	0,01	0,02	0,01	0,02	0,01	0,01	0,02
Linolenoylca	0,13	0,12	0,10	0,38	0,26	0,37	0,34	0,08	0,24	0,32	0,32	0,18	0,15	0,25	0,32	0,19	0,20	0,32	0,23	0,29	0,10	0,03	0,12	0,12	0,09	0,14	0,15	0,15	0,14	0,38
Linolylcarniti	0,09	0,05	0,06	0,16	0,14	0,15	0,16	0,07	0,14	0,18	0,17	0,12	0,09	0,16	0,17	0,12	0,11	0,16	0,19	0,15	0,08	0,04	0,04	0,05	0,10	0,07	0,09	0,05	0,06	0,20
Myristoylcar	0,03	0,02	0,03	0,06	0,04	0,05	0,06	0,02	0,04	0,09	0,09	0,06	0,05	0,07	0,09	0,05	0,05	0,08	0,09	0,10	0,03	0,02	0,02	0,03	0,05	0,03	0,05	0,04	0,04	0,08
N,N,N-trime	2,09	2,33	2,21	2,26	2,59	2,12	2,15	2,10	2,07	2,28	2,32	2,43	2,29	2,25	2,44	2,02	2,16	1,95	2,10	2,50	2,31	2,19	1,82	1,80	2,12	2,11	2,04	1,89	2,06	2,07
Octanoylcarn	0,01	0,01	0,01	0,01	0,01	0,01	0,04	0,01	0,01	0,03	0,01	0,01	0,01	0,01	0,01	0,01	0,02	0,02	0,01	0,02	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01
Oleoylcarniti	0,22	0,11	0,16	0,43	0,32	0,51	0,56	0,20	0,48	0,75	0,79	0,64	0,46	0,56	0,84	0,50	0,63	0,87	1,02	0,99	0,38	0,20	0,17	0,29	0,59	0,40	0,56	0,38	0,39	1,19
Palmitoylcar	0,13	0,06	0,10	0,19	0,16	0,18	0,17	0,07	0,13	0,18	0,27	0,19	0,15	0,16	0,26	0,15	0,17	0,22	0,25	0,27	0,08	0,05	0,04	0,08	0,12	0,09	0,11	0,09	0,10	0,25
Propionylcar	3,12	3,09	3,21	3,21	3,59	3,45	3,77	4,41	3,27	5,36	3,01	3,15	2,91	4,01	3,15	4,58	3,34	3,50	3,89	3,19	2,94	2,97	2,88	3,03	2,98	3,21	3,03	3,00	3,33	3,45
Stearoylcarn	0,03	0,01	0,03	0,03	0,04	0,04	0,03	0,02	0,03	0,03	0,07	0,05	0,03	0,04	0,05	0,04	0,03	0,06	0,05	0,07	0,02	0,01	0,01	0,02	0,02	0,02	0,02	0,02	0,02	0,05
C12brDC	0,01	0,02	0,03	0,07	0,06	0,02	0,08	0,02	0,05	0,09	0,11	0,09	0,07	0,07	0,13	0,03	0,06	0,08	0,11	0,11	0,06	0,03	0,02	0,01	0,03	0,05	0,04	0,05	0,05	0,09
C16brDC	NA	0,55	0,33	0,29	NA	0,33	0,44	0,29	0,49	NA	0,38	0,49	0,39	0,43	0,38	0,44	0,29	0,37	0,27	0,32	0,49	0,33	0,37	0,44	0,38	NA	0,27	0,49	0,54	0,33
C3DC	6,06	3,98	5,88	5,20	4,90	1,96	4,42	3,37	2,51	7,28	6,16	4,31	0,82	3,29	4,80	4,45	4,36	4,61	5,60	4,77	3,03	3,37	3,43	6,50	6,40	2,57	6,01	2,54	2,93	4,26
C4DC	0,02	0,01	0,02	0,04	0,01	0,03	0,01	0,02	0,01	0,01	0,03	0,04	0,02	0,01	0,01	0,04	0,02	0,02	NA	0,03	0,02	0,04	0,01	0,01	0,02	0,01	0,02	0,02	0,01	0,03
C5DC	NA	NA	0,01	0,01	0,01	0,02	0,10	0,06	0,06	0,38	NA	0,04	0,05	0,06	NA	0,10	0,12	0,08	0,14	0,31	0,09	0,02	0,02	0,04	0,15	0,11	NA	0,14	0,00	0,30

Metabolites (mM) in MRS broth after 24 hour incubation with sourdough lactobacilli:

Name	Class	Arg	Asn	Asp 1	Gln	Glu 1	His 1	Ile	Leu	Lys	Met 1	Phe	Pro	Ser 1	Thr 1	Trp 1	Tyr 2	Val 1	homo serine	1-amino-S-propanole 2	3-methyl-histidine 2	acetylcholine 2	N,N,N-trimethyllysine	N-acetyl-b-alanine 2	N-acetyl-lysine	N-acetyl-ornithine 2	ornithine	pyroglutamic acid	taurine
LB 1	L_brevis	2,66	1,95	8,89	5,47	6,82	1,08	15,62	84,74	3,22	1,56	2,18	2,45	7,42	16,98	0,65	0,70	57,49	15,47	0,23	0,03	0,42	0,02	0,12	0,07	0,19	2,74	21,68	1,14
LB 2	L_brevis	2,27	1,33	8,82	4,57	7,00	1,02	11,18	49,82	3,29	1,47	1,92	2,33	8,27	4,13	0,58	0,65	35,43	9,32	0,22	0,03	0,34	0,01	0,10	0,06	NA	2,32	22,12	0,74
LB 3	L_brevis	2,23	1,34	7,73	5,35	6,86	1,27	7,67	33,86	3,51	1,23	1,93	2,34	2,03	2,57	0,60	0,62	20,79	6,38	0,18	0,03	0,36	0,02	NA	0,06	NA	1,72	10,47	0,71
LC 1	L_casei	2,16	1,32	21,85	8,52	8,17	6,26	23,75	92,85	5,19	1,58	3,39	5,43	40,86	96,54	0,99	0,77	89,46	38,64	0,22	0,03	NA	0,02	0,12	0,05	0,23	2,14	152,05	1,00
LC 2	L_casei	3,28	2,27	9,94	4,76	7,82	1,25	12,85	61,06	3,09	1,54	2,63	2,57	9,85	5,40	0,62	1,24	39,02	10,40	0,22	0,03	NA	0,01	NA	0,06	NA	2,34	15,67	0,92
LC 3	L_casei	2,72	1,73	8,50	5,74	8,75	1,33	8,10	35,59	3,82	1,48	2,75	2,53	8,96	3,25	0,69	1,01	23,97	5,05	0,18	0,03	NA	0,02	0,10	0,05	NA	1,94	16,11	0,76
LP 1	L_plantar	2,05	1,35	9,88	4,77	7,04	1,20	15,96	79,44	2,83	1,62	2,19	2,25	5,25	12,01	0,59	0,59	57,62	14,92	0,21	0,03	0,35	0,01	0,12	0,06	0,19	2,69	13,57	0,77
LP 2	L_plantar	2,35	1,51	8,75	5,02	6,72	1,14	10,78	56,45	3,05	1,40	2,10	2,26	6,14	4,24	0,65	0,64	38,04	9,40	0,20	0,03	0,34	0,01	NA	0,06	NA	2,20	20,99	0,91
LP 3	L_plantar	1,46	0,78	5,63	3,39	3,53	0,90	4,83	18,14	2,39	0,92	1,19	1,47	2,53	1,94	0,49	0,38	14,25	4,05	0,17	0,03	0,28	0,02	0,11	0,04	0,15	1,35	7,54	0,38
LR 1	L_rossiae	2,15	1,52	10,41	4,89	6,39	0,57	14,69	76,69	2,75	1,51	2,45	1,98	7,24	11,21	0,59	1,02	53,01	13,93	0,22	0,03	0,15	0,01	0,14	0,06	0,17	2,03	13,92	1,02
LR 2	L_rossiae	2,17	1,49	12,46	6,12	8,97	0,55	15,85	71,17	4,05	2,05	3,39	2,93	10,13	6,65	0,79	1,08	43,83	14,50	0,20	0,03	0,15	0,02	0,12	0,05	0,18	1,69	20,50	0,68
LR 3	L_rossiae	1,88	1,35	8,04	5,46	8,36	0,55	8,60	35,94	3,10	1,39	2,69	2,47	7,63	3,02	0,66	0,93	21,86	6,23	0,17	0,03	0,14	0,03	0,10	0,05	NA	1,75	8,90	0,74
90 1	L_sanfran	2,69	1,18	10,93	4,75	7,12	1,16	15,57	77,38	3,57	1,67	2,67	1,98	6,51	9,99	0,67	0,92	51,39	11,38	0,21	0,03	0,30	0,01	0,13	0,06	0,20	2,82	18,89	1,27
90 2	L_sanfran	2,33	1,18	8,31	4,58	7,02	1,22	10,35	50,54	3,26	1,74	2,74	2,10	6,37	4,73	0,63	0,80	31,33	10,03	0,20	0,03	0,33	0,01	NA	0,05	0,18	2,09	15,29	0,65
90 3	L_sanfran	2,27	0,63	8,41	5,63	8,06	1,45	8,11	32,69	3,67	1,45	2,65	2,36	6,11	2,56	0,63	0,87	20,82	5,71	0,18	0,03	0,43	0,02	NA	0,05	0,16	2,16	6,02	0,81
91 1	L_sanfran	2,29	1,02	9,24	4,92	7,32	1,20	12,58	63,92	3,31	1,57	2,45	2,04	5,83	6,23	0,64	0,92	44,90	10,45	0,21	0,03	0,26	0,02	0,12	0,06	0,17	2,50	16,51	0,86
91 2	L_sanfran	2,79	1,11	11,34	5,75	8,61	1,35	12,40	58,82	4,13	1,87	3,03	2,70	10,08	4,57	0,75	1,14	39,10	12,50	0,20	0,04	0,40	0,02	NA	0,06	0,17	2,01	18,30	0,77
91 3	L_sanfran	2,60	0,98	9,70	6,12	7,78	1,38	8,17	32,05	3,71	1,43	2,63	2,31	4,93	2,12	0,67	0,99	20,65	6,92	0,18	0,03	0,34	0,03	0,09	0,05	0,19	2,01	10,61	0,66
92 1	L_sanfran	2,37	1,52	9,62	5,33	7,83	1,46	13,01	62,67	3,64	1,76	2,80	2,22	8,22	5,78	0,75	0,95	44,09	8,71	0,20	0,03	0,24	0,02	0,12	0,06	NA	2,56	8,77	1,06
92 2	L_sanfran	3,39	1,24	10,94	5,70	8,79	1,46	12,95	60,23	3,94	1,85	3,34	2,57	10,80	5,10	0,82	1,12	36,74	8,86	0,19	0,03	0,40	0,02	0,13	0,06	NA	2,43	13,33	0,82
92 3	L_sanfran	2,28	0,92	8,17	6,17	7,82	1,26	8,10	30,42	3,75	1,43	2,78	2,48	7,30	3,02	0,69	0,95	20,00	5,95	0,18	0,03	0,41	0,02	0,10	0,05	0,18	2,22	12,35	0,68
93 1	L_sanfran	0,04	1,94	9,89	8,62	7,59	1,29	9,61	49,06	3,84	1,24	2,65	2,38	8,55	5,50	0,70	0,08	31,14	9,12	0,22	0,03	0,17	0,02	0,11	0,05	0,65	5,26	11,48	0,92
93 2	L_sanfran	2,76	0,74	9,43	5,03	8,14	1,26	10,89	48,33	3,63	1,76	2,73	2,41	8,44	3,82	0,68	0,89	31,47	8,23	0,19	0,03	0,38	0,02	0,12	0,05	NA	1,59	14,76	0,71
93 3	L_sanfran	2,06	1,00	9,15	5,85	7,39	1,29	7,13	27,97	3,45	1,48	2,46	2,15	7,26	1,95	0,66	0,86	17,18	5,32	0,19	0,03	0,25	0,02	NA	0,05	NA	1,74	10,03	0,68
174 1	L_sanfran	2,22	1,29	9,79	4,55	6,99	1,37	6,61	34,81	2,21	1,04	2,34	1,85	6,00	4,03	0,62	0,86	23,22	7,16	0,20	0,03	0,16	0,02	NA	0,05	NA	1,68	9,66	0,73
174 2	L_sanfran	1,53	0,45	7,86	3,35	4,73	0,95	4,91	23,84	1,99	0,66	1,46	1,44	6,43	2,22	0,48	0,49	18,49	3,90	0,16	0,03	0,28	0,01	0,09	0,04	NA	1,41	12,66	0,54
174 3	L_sanfran	2,13	0,52	8,28	4,92	7,21	1,28	6,14	22,63	3,00	1,28	2,19	1,90	6,56	2,02	0,65	0,78	15,48	3,98	0,17	0,03	0,38	0,02	NA	0,05	NA	1,50	9,69	0,64
200 1	L_sanfran	2,32	1,35	9,06	4,80	7,89	1,32	6,78	30,34	3,31	1,13	2,65	2,00	5,63	3,55	0,65	0,95	20,66	6,62	0,21	0,03	0,17	0,02	NA	0,06	0,20	2,03	9,40	0,89
200 2	L_sanfran	2,53	0,98	10,08	5,51	9,04	1,43	10,91	49,67	3,77	1,88	3,02	2,48	9,05	3,88	0,82	0,99	28,46	9,34	0,19	0,03	0,30	0,01	0,10	0,05	0,19	1,99	13,66	0,72
200 3	L_sanfran	2,17	0,64	6,84	4,62	7,54	1,20	6,78	23,74	2,71	1,24	2,33	2,10	4,18	2,72	0,61	0,90	15,86	3,57	0,17	0,03	0,28	0,02	0,09	0,05	NA	1,88	7,17	0,88
201 1	L_sanfran	2,66	1,07	10,88	4,99	8,19	1,20	15,11	76,16	3,30	1,67	2,90	2,42	9,72	7,58	0,65	1,03	47,46	11,28	0,21	0,04	0,28	0,01	NA	0,06	0,19	2,36	18,44	1,22
201 2	L_sanfran	2,97	0,79	12,55	6,36	8,89	1,73	12,05	55,23	3,64	2,01	3,41	2,78	9,78	4,90	0,79	0,99	32,07	10,31	0,19	0,03	0,43	0,02	0,09	0,05	0,17	2,07	14,30	0,69
201 3	L_sanfran	3,00	0,78	8,55	6,77	9,47	1,48	8,48	33,13	3,93	1,81	3,24	2,63	7,31	2,48	0,76	0,80	19,51	5,31	NA	NA	0,39	0,03	NA	NA	NA	1,50	14,62	NA
LST 1	L_sanfran	2,28	1,64	9,24	5,76	7,71	1,27	14,23	85,88	3,29	1,68	2,67	2,07	7,56	11,88	0,66	0,92	59,67	13,50	0,23	0,03	0,19	0,02	0,13	0,06	0,17	2,31	18,48	1,20
LST 2	L_sanfran	3,31	2,64	12,81	6,78	9,41	1,44	15,30	78,41	4,29	1,71	3,43	2,85	8,50	7,18	0,79	1,42	47,16	12,24	0,21	0,04	0,20	0,02	NA	0,06	0,18	2,30	14,38	1,02
LST 3	L_sanfran	2,76	1,86	9,26	5,81	8,14	1,26	9,22	37,48	4,32	1,42	3,00	2,45	6,48	2,65	0,75	1,04	23,82	6,71	0,18	0,03	0,16	0,03	0,09	0,05	0,19	1,75	8,36	0,87
MRS 1	MRS	1,69	1,41	8,70	4,36	5,92	1,13	10,51	62,63	2,33	0,99	2,00	1,49	7,09	5,62	0,68	0,46	41,26	11,50	0,20	NA	NA	0,01	NA	0,05	NA	3,14	14,47	NA
MRS 2	MRS	2,55	2,52	11,28	6,04	8,29	1,27	13,31	74,71	4,20	1,44	3,00	2,35	8,30	6,22	0,64	1,08	45,20	11,98	0,20	0,03	NA	0,02	NA	0,05	0,17	2,71	17,21	0,91
MRS 3	MRS	2,17	2,10	9,50	5,78	9,04	1,19	9,06	38,35	3,97	1,43	2,69	2,33	5,76	3,58	0,67	1,00	22,81	5,47	0,18	0,03	NA	0,02	NA	0,05	NA	2,09	11,51	0,50

Metabolites (mM) in water extract of sourdough bread, analog bread and raw sourdough:

mM	AN WF	AN WF	AN WF	BR WF 1	BR WF 2	BR WF 3	SD WF 1	SD WF 2	SD WF 3
Group	Analog	Analog	Analog	SD bread	SD bread	SD bread	Sourdough	Sourdough	Sourdough
Ala 2	7,34	6,31	6,82	7,75	4,98	6,38	16,34	19,48	11,34
Arg	2,71	2,58	2,54	2,96	2,86	2,80	3,65	3,82	3,16
Asn	9,36	9,30	8,98	4,28	1,32	4,01	4,05	5,58	3,63
Asp 1	6,50	6,25	6,26	7,02	7,53	6,44	6,66	7,94	5,66
Gln	1,51	0,86	1,38	0,25	NA	0,07	14,97	11,93	6,70
Gly	3,42	2,68	3,36	3,25	2,37	2,64	7,53	8,83	4,60
His 1	NA	1,27	2,12	0,43	NA	0,23	NA	NA	NA
Ile	1,99	1,48	1,71	1,66	0,43	1,34	7,29	7,04	4,49
Leu	4,97	4,48	4,54	5,81	4,24	5,03	13,01	14,02	10,80
Lys	3,47	2,56	3,18	1,73	0,16	1,27	15,99	14,84	10,39
Met 1	0,90	0,65	0,73	0,92	0,00	0,69	NA	5,01	2,40
Phe	1,89	1,84	1,76	2,63	1,81	2,27	5,81	6,80	4,61
Pro	1,06	0,93	1,01	1,70	1,54	1,54	4,00	3,94	2,68
Ser 1	1,86	1,76	2,06	1,97	2,22	1,77	9,90	10,67	5,50
Thr 1	1,27	0,97	1,24	0,99	0,70	0,75	5,29	5,10	3,46
Trp 1	1,11	1,02	1,04	1,13	1,09	0,93	1,99	2,47	1,53
Tyr 1	1,26	1,17	1,13	1,79	1,13	1,45	4,87	5,47	3,66
Val 1	2,95	2,53	2,62	3,38	2,29	2,77	9,40	9,79	7,08
3-methyl-histidine	0,05	0,05	0,05	0,05	0,06	0,05	0,02	0,03	0,02
4-hydroxyproline	0,05	0,04	0,05	0,04	0,05	0,04	NA	0,03	0,00
5-aminovaleric acid	1,47	1,26	1,34	1,69	1,19	1,44	7,74	8,37	4,73
acetylcholine 2	0,05	0,04	0,05	2,96	2,46	2,51	1,30	2,64	1,52
canavanine	0,16	0,15	0,19	1,25	1,16	1,13	0,79	1,18	0,83
carnitine	0,17	0,15	0,16	0,07	0,07	0,06	0,04	0,06	0,03
cimetidine 2	NA	NA	NA	0,00	0,00	0,00	NA	NA	0,00
cysteic acid	0,01	0,02	0,01	0,01	0,02	0,01	NA	0,01	0,01
homoserine	0,18	0,20	0,18	0,46	0,54	0,35	NA	0,55	NA
N,N,N-trimethyllysin	0,15	0,15	0,13	0,22	0,22	0,18	0,19	0,25	0,14
N-acetyl-alanine	0,01	0,01	0,01	0,01	0,02	0,01	0,00	0,01	0,00
N-acetyl-asparagine	NA	0,00	0,00	0,01	0,01	0,01	0,00	0,01	0,01
N-acetyl-aspartic acic	0,04	NA	0,04	0,26	0,17	0,16	NA	NA	NA
N-acetyl-b-alanine	NA	NA	NA	0,00	NA	0,00	NA	NA	NA
N-acetyl-glycine	0,04	0,05	0,04	0,40	0,52	0,31	0,12	0,27	0,11
ornithine	0,22	0,51	0,14	0,18	0,33	0,40	0,07	0,03	0,26
pyroglutamic acid	3,44	3,28	3,31	2,53	3,23	1,77	0,90	1,30	0,56
S-methyl-methionine	0,01	0,01	0,01	0,02	0,01	0,01	0,03	0,04	0,02
taurine	0,03	0,01	0,02	0,02	0,03	0,02	NA	0,01	NA

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Figure 1. General anatomy of gastrointestinal tract adopted from Encyclopedia Britannica (2003) and the intestinal mucosa section with its major cellular actors in the intestinal immune homeostasis.

Figure 2. Overview of gastro-intestinal tract disorders.

Figure 3. Dietary intervention effects with probiotics, prebiotics and other functional foods in IBS and IBD.

Figure 4: Weekly energy consumption (N=10/group) (A) and weight (B) at week 24 of age of IL-10-/- mice fed chow, chow plus sourdough bread and chow plus analog bread.

Figure 5: Sourdough bread feeding significantly increases the histopathological score in the ceacum but not in the colon of IL-10-/- mice.

Figure 6: Average energy consumption per TNFΔARE mice diet group (N=6/group) (A), weight of TNFΔARE mice at 12 weekd (B) and ileum histopathology (C).

Figure 7: Addition of sourdough bread to the standard chow diet of wild-type 129/Sv mice has no impact on the weight and he morphology of the intestinal mucosa.

Figure 8: Addition of sourdough bread to the standard chow diet of wild-type C57Bl/6 mice has no impact on the weight and physiology of the intestinal lining.

Figure 9: Significantly higher concentration of free amino acids was observed in the in water extracts of raw sourdough vs. sourdough bread and analog bread.

Figure 10: The levels of acetylcholine and canavanine metabolites are significantly higher in fermented sourdough bread and raw sourdough extracts when compared to unfermented analog bread.

Figure 11: No difference was observed in the concentration of metabolites in the ceacum of IL-10-/- mice fed chow, chow plus sourdough bread and chow plus analog bread.

Figure 12: No difference was observed in the concentration of metabolites in the ceacum of wild-type 129/Sv mice fed chow, chow plus sourdough bread and chow plus analog bread.

Figure 13: No difference was observed in the concentration of metabolites in the ceacum of wild-type C57Bl/6 mice fed chow, chow plus sourdough bread and chow plus analog bread.

Figure 14: No difference was observed in the concentration of metabolites in plasma of wild-type 129/Sv mice fed chow, chow plus sourdough bread and chow plus analog bread.

Figure 15: No difference was observed in the concentration of metabolites in plasma of wild-type C57Bl/6 mice fed chow, chow plus sourdough bread and chow plus analog bread.

Figure 16: Impact of sourdough, sourdough bread and analog bread extracts on the secretion of pro-inflammatory chemokine IP-10 by intestinal epithelial cells activated with TNF.

Figure 17: Sourdough extracts have significant stimulatory effect on the secretion of pro-inflammatory cytokine TNF by the macrophages.

Figure 18: Sourdough extracts maintain mild stimulatory effect on the secretion of pro-inflammatory cytokine TNF by macrophages even after removal of endotoxins.

Figure 19: Endotoxin-free extracts do not inhibit the secretion of pro-inflammatory cytokine TNF by LPS-activated macrophages.

Figure 20: Sourdough extracts effectively stimulate stomach muscle motility by acting directly on mAchR

Figure 21: TTX has no significant effect on the stimulation of muscle contraction by sourdough-derived Ach.

Figure 22: HXM pre-treatment increases the muscle contraction stimulated by sourdough Ach although the effect is statistically insignificant.

Figure 23: Sourdough and sourdough bread extracts stimulate chloride ions secretion when applied from either serosal or mucosal side of intestinal mucosa.

Figure 24: Atropine completely abrogates chloride ions secretion stimulated with sourdough and sourdough bread extracts.

Figure 25: Atropine completely abrogates secretion stimulation by Ach and extracts when applied serosally but not mucosally.

Figure 26: Trans-epithelial resistance of mucosa before and after extracts' treatment

Figure 27: Metabolic analysis of sourdough lactic acid bacteria growth medium identified high amounts of acetylcholine.

Figure 28: Acetylcholine concentration in MRS media upon 24 hours of incubation with LAB.

Figure 29: Concentrated conditioned media of sourdough LAB significantly inhibits the secretion of IP-10 by TNF-activated IEC.

Figure 30: Formaldehyde-fixed sourdough LAB significantly inhibit the secretion of IP-10 by TNF-activated IECs.

Figure 31: Sourdough lactic acid bacteria do not express Lactocepin PrtP gene.

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Table 1: Water soluble extracts contain high amounts of endotoxin that can be effectively removed by Detoxi-Gel™ Endotoxin Removing Columns.

Table 2: Sourdough and sourdough bread contain high amounts of acetylcholine of with 38.6 mg/kg in dry bread..

11. ABBREVIATIONS

Ach	acetylcholine
AchR	acetylcholine receptor
AChE	acetylcholinesterase
AN BR	analog bread
APC	antigen presenting cell
B.	bifidobacterium
CGRP	calcitonin gene-related protein
CD	Crohn´s disease
cfu	colony forming unit
CLA	conjugated linoleic acid
CM	conditioned media
CP	crypt patch
DNA	deoxyribonucleic acid
DC	dendritic cell
DSS	dextran sodium sulphate
E.coli	Escherichia coli
EFS	electrical field stimulation
ELISA	enzyme linked immune-sorbent assay
ENS	enteric nervous system
EPS	exopolysaccharide
ER	endoplasmic reticulum
FOS	fructooligosaccharide
GI	gastrointestinal
GALT	gut associated lymphoid tissue
GABA	gamma-aminobutyric acid
GBF	germinated barley foodstuff
GOS	galactooligosaccharide
GPR	G-protein coupled receptor
H&E	hematoxylin and eosin
HXM	hexamethonium
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IBD-C	constipation-predominant IBS
IBS-D	diarrhoea-predominant IBS
IEC	intestinal epithelial cell
IEL	intestinal epithelial lymphocyte
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IL-$10^{-/-}$	interleukin 10 knockout
ILC	innate lymphoid cell
ILF	isolated lymphoid follicle
IP-10	interferon inducible protein 10
L.	lactobacillus
L.s.	lactobacillus sanfranciscensis
LAB	lactic acid bacteria
LC MS MS	liquid chromatography mass spectrometry

LPS	lipopolysaccharide
LAL	limulus amebocyte lysate
M cell	microfold cell
mAchR	muscarinic acetylcholine receptor
MAPK	mitogen-activated protein kinase
MLN	mesenteric lymph node
moi	multiplicity of infection
nAchR	nicotinic acetylcholine receptor
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cell
NGF	nerve growth factor
NOD	nucleotide oligomerization domain receptor
PAMP	pathogen-associated molecular pattern
PCA	principal component analysis
PCR	polymerase chain reaction
PI-IBS	post-infectious IBS
PP	Peyer's patch
PPARγ	peroxisome proliferator-activated receptor
PRR	pattern recognition receptors
PUFA	polyunsaturated fatty acid
RNA	ribonucleic acid
SCFA	short chain fatty acid
SD	sourdough
SD BR	sourdough bread
SDS	sodium dodecyl sulphate
SIBO	Small intestine bacterial overgrowth
TCR T	cell receptor
TER	transepithelial resistance
TGF	transforming growth factor
Th	helper T cell
TLR	toll like receptor
TNF	tumour necrosis factor
Treg	regulatory T cell
TTX	tetrodotoxin
UC	ulcerative colitis
UPR	unfolded protein response
VIP	vasoactive intestinal peptide
Wt	wild-type

12. REFERENCES

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