Interactions between *Salmonella* Typhimurium and human gut bacteria

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I certify that the work contained in the thesis is entirely the result of my own work, except where reference is made to other authors. It has not been submitted in any form to the University of East Anglia or any other University.

Author's declaration

Dr. Carmen Pin at IFR developed the mathematical model described in chapter four and performed statistical data treatment. Dr. Gwenaelle Le Gall performed the metabolomic analysis. Mrs Kathryn Cross operated the electron microscope.

Publications

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Abstract

Salmonella enterica subsp. *enterica* serovar Typhimurium is an important enteropathogen that causes human morbidity and mortality worldwide. It is essential to study the interaction between *Salmonella* and the gut bacteria to elucidate the elements that influence the ability of the pathogen to overcome the colonisation barrier mediated by the gut microbiota and why *Salmonella* can persist in 'healthy' individuals in a carrier state after infection.

In this study the effect of faecal bacteria on the growth and survival of *S.* Typhimurium was investigated. Initially, experiments involved co-cultures of the pathogen and single strains of intestinal bacteria obtained from culture collections; results showed that when *E. coli* reached its maximum concentration density, the growth of *S.* Typhimurium was halted. *S.* Typhimurium was then inoculated with multi-strain gut bacteria from culture collections and also with faecal samples in batch cultures mimicking the conditions of the human colon. A significant reduction of *S.* Typhimurium concentration was observed in mixed cultures with faecal samples from different human donors; however, bacteria obtained from culture collection had no effect on *S.* Typhimurium. Close proximity with faecal bacteria was required as the pathogen was not affected when it was separated from the faecal bacteria by a 0.45 µm pore size membrane. *S.* Typhimurium was also affected in a continuous culture system.

Transcriptomic analysis indicated that some of the functions associated with the genes expressed by *S.* Typhimurium during *Salmonella* inactivation were related to stress responses. Molecular profiling of faecal bacteria measured by denaturing gradient gel electrophoresis did not show any change specifically associated to *S.* Typhimurium inactivation.

It was not possible to identify the bacterial strains responsible for the inactivation of *S.* Typhimurium; however, this effect caused by cell-cell contact with human faecal bacteria is reported for the first time in this study.

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Abbreviations

µl – Microlitre

AHL – N-acyl-L-homoserine lactone

ca – Circa

CA – Coefficient of association

CCF – Commensal colonisation factor

CD – Crohn's disease

cfu – Colony form unit

DGGE – Denaturing gradient gel electrophoresis

EHEC – Enterohemorrhagic *Escherichia coli*

Fig – Figure

g – Gram

GI – Gastrointestinal

GIT – Gastrointestinal tract

h – Hour

HSP – Heat shock proteins

Hz – Hertz

IBD – Inflammatory bowel disease

IgA – Immunoglobulin A

IgE – Immunoglobulin E

IL – Interleukins

kDA – Kilodalton

Kg – Kilogram

LPS – Lipopolysaccharides

m – Metre

M – Molar

MAMP – Microbial associated molecular pattern

mg – Milligram

min – Minute

ml – Millilitre

MV – Membrane vesicles

NHS – National Health Service

NLR – Nod-like receptors

nm – Nanometre

NMR – Nuclear magnetic Resonance

NTS – Non-typhoidal salmonellosis

^{o}C – Grade centigrade

ppm – Parts per million

QS – Quorum sensing

R – Retention time

rpm – Revolutions per minute

s – Second

SCFA – Short chain fatty acids

SCV – *Salmonella*-containing vacuoles

se – standard error

SEM – Scanning electron microscopy

SIgA – Secretory immunoglobulin A

SPI-1 – *Salmonella* pathogenicity island 1

SPI-2 – Salmonella pathogenicity island 2

spp – species

T3SS-1 – Type III secretion system 1

T3SS-2 – Type III secretion system 2

T4SS – Type IV secretion system

T6SS – Type VI secretion system

TCS – Two-component system

TEM – Transmission electron microscopy

TJ – Tight junctions

TLR – Toll-like receptors

UC – Ulcerative colitis

w/v – Weigh/volume

WHO – World Health Organisation

x *g* – gravity

1 Chapter 1 – Introduction

1.1 *Salmonella*

Salmonella is a Gram-negative, facultative rod-shaped bacterium belonging to the family *Enterobacteriaceae,* order *Enterobacteriales,* class *Gammaproteobacteria* and the division or phylum of the *Proteobacteria* [1]. Members of this genus are generally motile with peritrichous flagella (*Salmonella gallinarum* is one of the notable exceptions), aerogenic, non-lactose fermenting, oxidase-negative, urease-negative, citrate-utilising, acetylmethyl carbinol-negative and potassium cyanide-negative [2].

Salmonella comprises a large and closely related population of medically important pathogens. It has long been associated with a wide spectrum of infectious diseases, including typhoid fever and non-typhoid salmonellosis, which cause public health problems worldwide [3].

1.1.1 *Salmonella* nomenclature

The genus name *Salmonella* was adopted in honour of Dr. D.E. Salmon, who isolated *Salmonella* from a pig intestine [4]. Salmonellae are widely distributed but they are controversial pathogens of both humans and animals in terms of nomenclature. Despite elaborate studies, much still remains to be discovered about these organisms. Although *Salmonella* nomenclature has proved to be rather complex, in 2005, *Salmonella enterica* finally gained official approval as the type species of the genus *Salmonella*. In addition, one other species has been approved and recognised in the genus *Salmonella*, namely, *Salmonella bongori* [2].

In 2005, a new species, *Salmonella subterranea* [5] was validly published by citation on Validation List 102 [6]. However, this species is closely related to *Escherichia hermannii* and does not belong to the genus *Salmonella* http://www.bacterio.cict.fr/s/salmonella.html. Hence, according to contemporary classification, the genus *Salmonella* contains only two species: *S. enterica* and *S. bongori. S. enterica* is itself divided into six subspecies, which are referred to by a Roman numeral and a name (I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *indica*) [7]. This has now been accepted by the Judicial Commission of the International Committee on Systematics of Prokaryotes and is the nomenclature currently in use by the World Health Organisation (WHO) and other organisations [8].

S. enterica subspecies are differentiated biochemically and by genomic relatedness. More than 2,500 *Salmonella* serovars have been identified based on their somatic (O) and flagellar (H) antigens. *S. enterica* subsp. *enterica* serovars represent the majority (59%) of *Salmonella* serovars isolated from humans and warm-blooded animals [7]. Approximately 50 of all known *Salmonella* serovars are responsible for 99% of all clinical cases of *Salmonella* in humans and domestic mammals and all of these 50 serovars are in subspecies I [9]. Serovars within subspecies *enterica* show a diverse range of host specificity and disease severity, including serovar Typhimurium [7]. The other subspecies and *S. bongori* are usually isolated from cold-blooded animals and the environment but rarely from humans [7].

This work uses *Salmonella enterica* subspecies *enterica* serovar Typhimurium strain SL1344. When describing *Salmonella* serovars only *Salmonella enterica* subspecies *enterica* will be discussed from this point onwards. *Salmonella enterica* subspecies *enterica* serovar Typhimurium is abbreviated to *S.* Typhimurium.

1.1.2 *Salmonella* host specificity and types of disease

Salmonella serovars may be either host specific, in which case they are restricted to infection of only one specific host species, host adapted, which allows for the infection of a few different species, or generalists, which infect a wide range of hosts [10]. In humans, serovars Typhi, Paratyphi and Sendai cause enteric fever; however, most serovars cause enterocolitis and diarrhea [11]. Serovars Dublin, Typhimurium and Choleraesuis cause disease in both humans and animals, but even where the same serovar is involved the type of disease and symptoms vary between host species. For example, in humans, serovar Typhimurium causes gastroenteritis whereas mice do not show gastrointestinal (GI) symptoms; however, in some susceptible mouse strains *S.* Typhimurium infection results in a systemic process that mimics typhoid fever and causes the death of the affected animal. Serovar Dublin causes intestinal inflammatory disease, bacteremia and abortion in cows; and serovar Choleraesuis causes septicaemia in pigs [12].

1.1.3 *Salmonella* infections in humans

Salmonella is the most common pathogen leading to foodborne disease outbreaks in Europe according to the European Food Safety Authority (EFSA), 2009 and causes as many as 1.3 billion cases of disease annually worldwide. It is typically an orally acquired pathogen that results in one of four major syndromes: typhoid fever, enterocolitis, bacteremia and chronic asymptomatic carriage [11]. The most common syndromes associated with *Salmonella* infection in humans are typhoid fever and

enterocolitis disease [13]. The least common clinical syndrome in humans is bacteremia. This syndrome is caused by the porcine-adapted *S. enterica* serovar Choleraesuis and the bovine-adapted *S. enterica* serovar Dublin and is usually associated with the consumption of undercooked pork products and unpasteurized milk, respectively [14]. Some individuals develop an asymptomatic carrier status for both typhoidal and non-typhoidal salmonellosis that may last from weeks to years. Approximately 3% of persons infected with *S.* Typhi and 0.1% of those infected with non-typhoidal salmonellae become chronic carriers [15]. Asymptomatic carriers represent a great risk of spreading the infection, and for this reason screening of food industry workers is a common precautionary measure.

1.1.3.1 Typhoid fever

It is estimated that typhoid fever caused over 21 million illnesses and over 216.000 deaths during the year 2000 [16]. Water treatment and sanitation have reduced the incidence of typhoid fever in the developed world. In these countries, all the cases are associated with travel in developing nations where sanitation is poor and clean water is inaccessible [16].

Human typhoid is caused by *S. enterica* serovar Typhi and has an incubation period of between one and two weeks, which indicates that it has the ability to evade the host immune system at the early stages of infection [17]. It follows ingestion of contaminated water or animal products or close contact with an infected individual or carrier [18]. Most of the knowledge of typhoid pathogenesis in humans derives from the extrapolation of the infectious process in animal models, particularly in mice susceptible to *S.* Typhimurium. Typhoid fever in humans is presented with fever, general discomfort and abdominal pain with or without other symptoms such as headache, myalgias, enlarged spleen and liver, nausea, anorexia and constipation. Diarrhea has been reported in immuno-depressed individuals. If no complications arise from the process, the disease may resolve after periods of infection of variable length; however, after recovery patients can asymptomatically carry and spread the bacteria for months or years. The asymptomatic carrier state is sometimes followed by a period of acute illness. In the majority of cases treatment with antibiotics is effective and decreases the time for eliminating the bacteria and infection-associated morbidity and mortality [19].

1.1.3.2 Enterocolitis

The WHO indicates that although of considerable importance in industrialised countries, the incidence of diseases due to non-typhoidal *Salmonella* is higher in the developing world (www.who.int). *S.* Typhimurium is a principal cause of human enterocolitis [14].

A mouse model has been developed to study the pathogenic mechanisms of intestinal salmonellosis. *S.* Typhimurium causes a disease similar to typhoid fever in susceptible mice; however, streptomycin-pre-treated mice develop intestinal inflammation following infection with this serovar [20]. Prior to the availability of this model, the study of *Salmonella*-associated enterocolitis was limited to bovine infection models in which serovar Typhimurium causes non-systemic enterocolitis.

Enterocolitis caused by *S.* Typhimurium has an incubation period of between 6 and 48 h after ingestion of the pathogen. Typical symptoms include abdominal pain, headache, vomiting and diarrhea that may contain blood, lymphocytes and mucus. Fever, general discomfort and muscle pains are also common [21]. The symptoms usually last between five and seven days and the process resolves in many cases without treatment; however, in case of severe fluid loss, oral or intravenous rehydration is necessary. Antibiotics are indicated if symptoms of the infection spreading are observed [13].

1.1.4 *Salmonella* pathogenesis

Salmonella has become the preferred pathogen to identify bacterial virulence factors associated with the induction of inflammatory or immune responses in infected hosts due to the availability of both *in vitro* cell cultures and *in vivo* animal models for this infection; however, even in these models, the disease does not progress exactly as observed in humans [22]. The bacterial strain, the physiological state of the host and the kind of food ingested with the bacteria determine the infectious dose required by *Salmonella* to cause infection [21].

Salmonella is typically orally ingested and has an adaptive response to tolerate low pH levels in order to survive in the stomach environment. Once in the small intestine, *Salmonella* reaches the intestinal epithelium despite the barrier formed by the mucus layer and the inhibitory activity of digestive enzymes, bile salts, secretory IgA, antimicrobial peptides and other innate immune defences of the lumen [23]. It has been shown that *S.* Typhimurium manipulates cell apoptosis in the epithelium to its advantage, either inducing or reducing cell death, with the purpose of enhancing its

survival in the gut [24]. At the first stage of the infection, *Salmonella* reduces cell death in order to ensure intracellular proliferation. It is only after a prolonged exposure to the pathogen when the apoptosis of the intestinal epithelial cells is induced [25].

Salmonella adheres to the apical surface of the gut epithelial cells using various fimbrial adhesins and penetrates the intestinal epithelium, mainly in the distal parts of the ileum, colon and caecum, to cause an infection in the host. The infection route is via enterocytes and M-cells with no apparent preference for either route in calves [26], whereas invasion of the epithelium is only via M-cells within the Peyer's Patches in mice [27]. The implications of these differences in the invasion route remain unknown and the exact route in humans has not yet been described; some studies attribute the main route of entry in the human gut to M-cells [27], although it has been shown that there are occasions in which *Salmonella* enters via enterocytes [28].

Salmonella genes involved in the invasion of eukaryotic cells were identified by Galan and Curtiss in 1989 [29]. It was later demonstrated that these genes were part of the horizontally acquired *Salmonella* pathogenicity island 1 (SPI-1) [30]. The expression of these genes located within SPI-1 may be induced in response to several environmental cues during transit through the gastrointestinal tract (GIT) such as oxygen and pH changes, increasing salt levels, exposure to metabolites produced by the gut flora and antimicrobial peptides produced by the host [31]. SPI-1 encodes a syringe-like Type III Secretion System 1 (T3SS-1) which is a multiprotein complex formed by a basal body, a needle structure and a needle tip [32]. The inactivation of this T3SS inhibits pathogenesis, whereas its activation at the appropriate time and site leads to successful bacterial infection [32]. Furthermore, SPI-1 deficient mutants fail to cause infection following oral administration of *Salmonella* and are not able to invade enterocytes *in vitro* [33].

The function of the T3SS-1 is to transfer a set of effector proteins into the targeted eukaryotic cell by creating a pore in the host cell membrane that provides access to the host cell cytosol. SPI-1 effectors induce membrane deformation and rearrangement of the underlying actin cytoskeleton, what is known as 'membrane ruffling', which results in *Salmonella* entering intestinal epithelial cells and phagocytic cells [34]. In addition to being associated with the invasion of intestinal epithelial cells, effectors secreted by the SPI-1 enhance intestinal inflammation in infected hosts [35].

Following uptake into host epithelium, *Salmonella* cells find a relatively benign environment as they are surrounded by membrane-bound compartments, which have been called *Salmonella*-containing vacuoles (SCV). These SCV are essential for

bacterial replication, intracellular survival and progression towards systemic infection [34]. Although *Salmonella* can replicate in numerous kind of cells, it is thought to replicate mainly in macrophages and white blood cells, the function of which is to phagocytose cellular debris and pathogens and also to prompt the immune system to react against invading microbes [36]; in fact, strains impaired in macrophage replication are unable to produce disease in mouse models of infection. This highlights the relevance of *Salmonella* survival and replication in macrophages in order to cause disease [23].

An innate immune response is triggered as a consequence of *Salmonella* invasion and replication within host cells. This results in the acidification of the SCV, which might be caused by the fusion with lysosomes. This change of the pH value is sensed by *Salmonella* and leads to the expression of the bacterial virulence genes within *Salmonella* pathogenicity island 2 (SPI-2) and effector proteins transferred by the Type III Secretion System 2 (T3SS-2), through the SCV membrane, to the eukaryotic cell cytoplasm [37]. As SPI-1, SPI-2 was also horizontally acquired during the organism evolution and encodes the proteins forming the T3SS-2 [38]. The delivery of these effector proteins into and across the vacuolar membrane is critical for the bacterial survival and replication [39], as they modify the host vesicular transit in order to divert amino acids and lipids to the SCV [23]. It has been suggested that SPI-2 expression might begin in the early phases of *S*. Typhimurium infection, prior to intestinal penetration; however, there is no evidence that T3SS-2 is also responsible for intestinal colonisation [40]. Equally, it seems that T3SS-1 effectors keep being expressed after *Salmonella* penetration into the epithelial cells and take part in processes previously associated entirely with T3SS-2 [41]. These reports point out that *Salmonella* T3SS-1 and T3SS-2 might not be totally independent but work together to promote the intracellular existence of *Salmonella*.

Non-typhoidal *Salmonella* serovars such as Typhimurium lead to a localised infection of the intestinal mucosa in healthy individuals and do not extent beyond the lamina propia [42]. SPI-1 T3SS effectors, SopE, SopE2 and SopB, lead to the activation of transcription factors that regulate the production of pro-inflammatory cytokines, such as IL-8, that stimulate the recruitment of polymorphonuclear leukocytes (neutrophils); these neutrophils are major effectors of acute inflammation [43] in non-immuno compromised individuals and the inflammatory response, inducing diarrhea [23]. SopB exacerbates the intestinal disease by increasing cellular activity of the enzyme inositol phosphatase, prompting chloride secretion and fluid outflow [44]. Additionally, T3SS-1 effectors cause destabilisation of epithelial tight junctions which is an

extracellular matrix of proteins between two neighbouring cells that provides intercellular impermeability and ensures the integrity of the GI barrier. This leads to paracellular fluid leakage, movement of neutrophils from the basolateral to the apical surface and access of bacteria to inner layers of the intestinal wall [23].

Other serovars such as Typhi survive within the macrophages by avoiding fusion or minimising interaction of SCV with cell lysosomes [42, 45]; it has also been reported that it is the capacity of *Salmonella* to endure lysosomal contents such as antimicrobial peptides and nitric oxide and oxidative killing what confers the bacteria the survival in the macrophage and its virulence [23]. These serovars are then carried by the lymphatic and blood stream to the liver, spleen, lymph nodes and throughout the reticuloendothelial system causing a systemic infection [42, 46]. The systemic involvement of *S*. Typhi facilitates the colonisation of the gall bladder from which *Salmonella* can gain access to the intestine intermittently, leading to a chronic carrier status of the affected individual [17].

Macrophages containing *S*. Typhimurium are destroyed by the bacteria; this provides a way for *Salmonella* to disseminate from the affected macrophages to neighbouring cells. However, it is unclear what happens with macrophages that are persistently infected with *Salmonella*. The T3SS-2 responsible for host cell death is thought not to be active in these macrophages. They may carry the bacteria for their lifetime and then *Salmonella* infects another macrophage [42].

1.1.5 *Salmonella* epidemiology

According to the British National Health Service (NHS), the main human illnesses associated with *Salmonella* in the UK before the middle of the last century were typhoid and paratyphoid fever. These diseases are transmitted by the faecal-oral route, normally by ingesting contaminated water, food, or by person-to-person contact. Improvements in sanitation during the last decades have led to a great decline in cases of these diseases in the UK.

Reported cases of enterocolitis caused by *Salmonella* have decreased in the last decade in the UK mainly due to the introduction, in 1998, of compulsory vaccination against *Salmonella* Enteritidis of the UK egg-laying flock; however, greater public awareness of food safety may have also contributed to the reduction of the disease. The highest incidence of salmonellosis is seen in the summer months as a result of faster bacterial growth in contaminated products that are not refrigerated (www.wales.nhs.uk).

1.1.5.1 Typhoid fever

According to the WHO (www.who.int), although typhoid fever has practically disappeared from industrialised countries, it remains a serious threat to public health in several Asian regions of the former USSR and in parts of South and South-East Asia, Africa and South America. Studies reported an incidence of >100/100,000 cases/year in south central Asia and south-east Asia, whereas in Europe, Australia and New Zealand and North America the incidence was <10/100,000 cases/year [16].

Although the number of typhoid fever cases appears to be rising mainly due to the expanding world population, differences in the methods used for obtaining the estimates make it difficult to assess. In fact, there are studies that indicate a declining incidence in countries such as Chile, Egypt, India, the former Soviet Union and Vietnam as a result of economic development and improved sanitation in the last decades [16].

Humans are the only reservoir for *S.* Typhi and *S.* Paratyphi. The infection is normally transmitted by the ingestion of food and water contaminated with faecal material [47]. *S.* Paratyphi has been associated with the consumption of food obtained from street vendors [48]. *S.* Paratyphi was thought to be responsible for a smaller proportion of enteric fever in the past, *S.* Typhi being the main problem worldwide; however, the incidence of *S.* Paratyphi has increased around the globe in the last 10 years, particularly in south-central Asia and south-east Asia where it has been involved in 50% of the reported typhoid cases [49].

In order to eradicate typhoid fever the implementation of rapid, accurate and affordable diagnostic systems and epidemiological surveillance, in areas where the disease is endemic, is imperative [50]. Another challenge for public health is the emerging antibiotic resistance in *Salmonella* serovars. It is essential that unnecessary use of drugs effective against *Salmonella* is restricted in order to maintain this efficiency [51].

1.1.5.2 Non-typhoidal salmonellosis (NTS)

Estimations show that *Salmonella* spp. are responsible for 93.8 million cases of gastroenteritis worldwide, of which 80.3 million cases are thought to be foodborne, with 155,000 of those resulting in deaths each year [52]. A NTS incidence of 690 cases per 100,000 inhabitants per year is estimated in Europe, although it varies depending on the region [52]. Data obtained from the Health Protection Agency (www.hpa.org.uk) show that 9133 GI infections were attributed to *Salmonella* in 2009

in England and Wales. This figure is likely to be substantially underreported due to the self-limiting nature of the disease [53]. In the United States, NTS was the foodborne disease with the greatest mortality, with 39% of fatal cases, from 1996 to 2005. The highest mortality was detected in adults older than 65 years and the highest incidence in children younger than 5 years [54]. Currently, in sub-Saharan Africa, cases of NTS, often resulting in bacteremia, outnumber the cases of typhoid fever [17].

Transmission of *Salmonella* infections to humans occurs by consumption of contaminated food. However, contaminated water, direct human to human and animal to human contact can also be the cause of NTS. In developed countries farm animals are a major reservoir of non-typhoidal *Salmonella* and the transmission is caused by contaminated meat [55]. Products most commonly involved in salmonellosis are poultry and eggs but *Salmonella* has also been found in beef and pork [56]. Vegetables and fruits are of increasing concern; they are usually eaten without cooking and can be contaminated during production, storage or by food handlers that are carriers of the bacteria [57].

The most common contributing factors associated with foodborne salmonellosis are: cross-contamination, inadequate cooling or refrigeration, inadequate heat treatment or contamination from food handlers [58]. Transmission of *Salmonella* is not uncommon in households where reptiles are kept as pets. The mode of transmission, in these cases, is unclear and the serovars may vary; however, exposure to reptiles is a risk factor, particularly in children [59].

1.1.6 *Salmonella* genome sequences

S. Typhimurium LT2 and *S*. Typhi CT18 were the first *Salmonella* genomes to be published [60, 61]. Later publications include the genome sequences of *S*. Choleraesuis SC-B67, *S*. Typhi Ty2 and *S*. Paratyphi A ATCC 9150 [62-64]. Since then, another nine serovars have been sequenced, including *S.* Enteritidis PT4, *S. bongori* 12419 ATCC 43975 and *S.* Typhimurium SL1344. The sequence of *S.* Typhimurium DT2 is in progress (Sanger Institute, 2013).

The strain used in this work is *S*. Typhimurium SL1344. It is a histidine auxotroph derived from *S*. Typhimurium 4/74 [65]. The strain ST4/74 was originally isolated from a calf bowel [66]. The complete and annotated genome sequence of *S*. Typhimurium SL1344 was published in 2012 [67]. This strain shares a similar GC ratio (52.3%) with other *S. enterica* serovars. SL1344 genome contains 4530 chromosomal coding

sequences. In addition, 3 plasmids (pSLT^{SL1344}, pCol1B9^{SL1344} and pRSF1010^{SL1344}) encode 212 genes [67].

1.2 Human gut microbiota

1.2.1 Diversity of the intestinal microbiota

The human GIT is approximately 7 m long and has a surface area, amplified by folds, villi and microvilli, although less marked in the colon, that can reach ca. 300 m^2 in adults, an area comparable to the size of a tennis court [68]. It is a nutrient-rich environment that harbours up to 100 trillion (10^{14}) microbes; the vast majority of them reside in the colon where densities approach 10^{11}–10^{12} cells/ml, the highest recorded for any microbial habitat [69]. The number of microbial cells within the gut lumen is about 10 times larger than the number of eukaryotic cells in the human body and they weigh 1-2 kg [68]. These bacteria contain at least 100 times as many genes as our own genome and more than half of them have never been cultured [70].

The diversity of the GIT microbiota is described not only in sheer numbers or abundance of bacteria but also in terms of its richness in the number of species present [71]. Its composition is relatively stable in healthy adults over time as long as factors such as diet, health status and environment are not drastically changed [72, 73]. Commensal gut organisms play an important role on human health, yet the complete microbial composition and species diversity remain unknown because of cultivation failure; between 40-80% of the total microscopic counts are unrecoverable by culture in laboratory media [74]. Culture-independent approaches based on 16s rRNA gene sequences and molecular finger-printing methods are currently being used to explore the diversity of the gut microbiota [75].

1.2.1.1 Bacterial composition and physiology along the GIT in adults

The gut is sterile at birth, however, it is immediately colonised and a stable bacterial community is developed. This gut microbiota is host specific and there are considerable variations in its composition between individuals [76] that are particularly marked among infants [77]. The gut bacteria composition is stable at different anatomic locations along the gut, but absolute numbers range from 10^{11} cells/g content in the proximal colon to 10^{7-8} cells/g in the distal ileum and 10^{2-3} cells/g in the proximal ileum and jejunum [76]. Low bacterial concentration in the upper segments of the GIT can be attributed to the composition of the luminal medium (acid, bile, pancreatic secretion), which kills most of the ingested microorganisms, and to the

peristaltic movements towards the ileal end, which prevent stable colonisation of bacteria in the lumen [74].

Regardless of the anatomical site, two bacterial phyla, the Firmicutes (49%) and the Bacteroidetes (23%), commonly dominate the ecosystem, as they do in the gut of at least 60 mammalian species, followed by Proteobacteria (21%) and Actinobacteria (5%) [78-80]. Within the Firmicutes, 95% are members of the Clostridia class [75]. Anaerobic bacteria outnumber aerobic bacteria by a factor of 100-1000. In human beings, the genera *Bacteroides, Bifidobacterium, Eubacterium, Clostridium, Peptococcus, Peptostreptococcus*, and *Ruminococcus* are predominant, whereas aerobes (facultative anaerobes) such as *Escherichia, Enterobacter, Enterococcus, Klebsiella, Lactobacillus* and *Proteus* are among the subdominant genera [74].

The microbial composition varies along the different segments of the GIT. *Bacillus* subgroup of Firmicutes, primarily *Streptococcaceae*, and Actinobacteria prevail in the small intestine. The bacterial population in the jejunum, dominated by the *Streptococcus* genus, is the least diverse and significantly different from that of the distal ileum, ascending colon and rectum, where the order Bacteroidetes and the family *Lachnospiraceae* (which comprises the *Clostridium*), are the prevalent genera [78, 81]. Other studies have reported that, quantitatively, *Bacteroides* and bifidobacteria are the most important bacterial genera in the colon of animals and man and represent 30% and 25% of the total anaerobic counts respectively [82]. The strict anaerobic environment, physical exclusion and bacterial waste products are factors that inhibit the growth of other bacteria in the large bowel [83]. Comparative studies between the microbiota of the human caecum and the distal colon, based on faecal samples, reveal that in the caecum *Escherichia coli*, lactobacilli, and enterococci achieved higher densities and strict anaerobes, bifidobacteria, *Bacteroides* and *Clostridium* were less prevalent than in the distal colon [84].

The differing luminal gut microbiota patterns, depending on the anatomical region of the GIT, are explained by the changes in ecological conditions, such as pH and concentration of fermentation products, found along the digestive tract. For example, carbohydrate fermentation in the caecum and ascending colon produces short chain fatty acids (SCFA), mainly lactate and ethanol; as a consequence the content of this GIT region maintains pH values between 5-6 [85]. In more distal segments of the colon, carbohydrates are scarce and proteins become the most available substrate for the microbiota which leads to slower growth of bacterial populations; products of anaerobic fermentation of amino acids, such as NH_3, branched chain fatty acids and

phenolic compounds, progressively increase from the right to the left colon, resulting in pH values of gut contents closer to neutral [74].

Focusing now on the tissues rather than on the location along the gut, the bacterial community found in faeces is thought to represent the luminal microbiota of the distal colon; for that reason, due to its accessibility, most of the studies carried out to determine the bacterial diversity of the human GIT have been based on the analysis of faecal bacteria [86]. In fact, the faecal microbiota is close to the bacterial community isolated from the colon, with the same dominant phyla. Over 90% of sequences found in human faeces belong to Firmicutes and Bacteroidetes which is similar to the data obtained from colonic bacteria [80]. However, the microbiota in direct contact with the intestinal epithelium is different from the microbiota found in the gut lumen and the bacteria embedded in the mucus layer that separates the epithelial cells from the lumen [87]. The degree of this difference remains unclear and it is thought that the faecal microbiota is formed by a combination of mucosal and non-adherent luminal bacteria [75]. *Bacteroides, Bifidobacterium, Streptococcus, Enterococcus, Clostridium, Ruminococcus, Lactobacillus* and members of *Enterobacteriaceae*, are present in faeces, whereas only *Clostridium, Lactobacillus* and *Enterococcus* are found in the mucus layer and epithelial crypts of the small intestine [87].

It has been reported that the prevalent mucosa-adherent bacteria are uniformly distributed along the colon and are host specific, which indicates that the bacterial community in the mucosa is influenced by host factors [81, 86]. This suggests that luminal faecal bacteria are influenced by diet, while the more stable mucosa-adherent community consists of dense cohesive microbial communities that adhere to surface-associated polysaccharide matrices, have a close interaction with the mucosal immune system and are maintained in spite of the epithelium cell turnover [88].

To explain how so many different microbial species cohabit in the gut, a nutrient-niche theory postulates that the anatomical segment colonised by each bacterial species in the intestine is dependent on its nutrient availability [89]. Therefore, the intestinal substrate distribution highly influences the microbial community structure in the gut [90]. Interaction studies of gut commensals in gnotobiotic mice have demonstrated that *Lactobacillus johnsonii colonises* the stomach whereas *Bifidobacterium longum,* an anaerobic bacterium, targets the anoxic environment of the colon; *L. johnsonii is* unable to synthesize amino acids and relies on taking them from the environment for its survival. It is therefore logical that these species reside predominantly in the stomach, where the amino acids have not yet been absorbed by the host. On the

other hand, *B. longum* has a much higher capacity for processing complex carbohydrates than lactobacilli; therefore the environment of the distal GIT is appropriate for the fermentation of these components by the bifidobacteria [91]. Gut bacteria do not always compete for substrates and colonisation sites of the GIT; there is also cooperation in the digestion of complex substrates, where the end product of one bacterium becomes the food of another [92], which contributes to the microbial distribution in the gut. *Lactobacillus* spp. and *Eubacterium dolichum* are examples of these ecological networks in the gut. These bacteria obtain amino acids that they are unable to produce, from other residents of the gut microbiota; it has also been reported that obligate anaerobes produce hydrogen as a by-product which is used by methanogens as a source of energy [90].

1.2.2 Microbiota-host crosstalk in the gut

Microorganisms and humans have been linked by co-evolution in an intricate relationship that results in a highly specific and stable human microbiome [93]. The human gut and its complex and diverse resident bacterial community are in continuous interaction and establish a symbiotic relationship. The microorganisms are provided with a supply of substrates and an immediate environment, while the host benefits from a more versatile metabolome to digest complex polysaccharides and the exclusion of incoming pathogens. The host immune system is responsible for regulating bacterial numbers and diversity, whereas the gut bacteria, in turn, influences the development of the immune system and have the means to modify host processes.

There are established crosstalk mechanisms between the microbiota and the host in order to maintain equilibrium in this symbiotic relationship. This cross-talk is conveyed through multiple signalling pathways that involve different molecules and extend beyond the immune system. [76, 94]. In fact, bacteria produce and detect numerous extracellular signalling molecules that have an effect on the host, and *vice versa,* as well as on other members of the gut community [95]. Studies with mice have demonstrated that *Bacteroides thetaiotaomicron*, a component of the intestinal microflora of mice and humans, controls the production of some sugars by enterocytes. *B. thetaiotaomicron* uses fucose as a substrate and induces epithelium fucosylation only when the carbohydrate is not present in the intestinal tract, whilst simultaneously stopping enzymes involved in the fucose catabolic pathway. After the production of this carbohydrate by enterocytes is triggered, the induction of the host fucose production ceases and those *B. thetaiotaomicron* genes involved in this

substrate degradation are expressed [96]. It has been reported that the use of fucose by gut bacteria increases host pathogen resistance and disease tolerance as this carbohydrate is metabolised by the commensals providing them a survival advantage in competitive environments [97]. It has also been demonstrated that other gut bacteria, in particular segmented filamentous bacteria, induce epithelium fucosylation. This process has also been reported after the injection of lipopolysaccharides (LPS) - simulating infection [97, 98]; further, these studies demonstrated that innate lymphoid cells participate actively in epithelium fucosylation. Commensal bacteria induce the lymphoid cells to produce interleukins, which stimulate the expression of *Fut2*, the gene that mediates fucosylation in the epithelial cells. On the other hand, it has been reported that mice lacking *Fut2* suffered more inflammatory damage after *S.* Typhimurium infection, which suggest that epithelial fucosylation promotes host defence against *S.* Typhimurium [97]. Phenomena such as this prove the importance of the crosstalk between host immune and epithelia cells and commensal microbiota.

Another example of the microbiota influence on the host is a polysaccharide produced by *Bacteroides fragilis*, a gut commensal, that has been shown to be involved in the maturation of the developing immune system by activating CD4+ T cells and cytokine production in experiments with mice [99]. On the other hand, it has been described that hormones produced by the host have an effect on the gut microbiota [100]. Norepinephrine, a hormone released during trauma, causes a substantial increase in aerobic and facultative anaerobic bacteria, mainly due to the growth of *E. coli* [101]. It has also been shown that genes involved in Enterohemorrhagic *E. coli* (EHEC) virulence react to the hormone epinephrine released by the host eukaryotic cells. Furthermore, β- and α-adrenergic antagonists inhibit the EHEC response to this hormone [102]. Norepinephrine also stimulates the cytotoxic activity of *Vibrio parahaemolyticus* by affecting the expression of its T3SS [103].

It is thought that the host influences the colonisation process of some of the indigenous bacteria of the gut microbiota, since the named commensal colonisation factors (CCF) genes of *B. fragilis* and *B. thetaiotaomicron* are up-regulated in the presence of host mucus components such as N-acetyl-D-lactosamine [104, 105]. CCFs are genes, studied in bacteria of the genus *Bacteroides*, involved in the utilisation of specific polysaccharides and are expressed mainly in the presence of mucosal tissue; this helps *Bacteroides* colonise specific niches of the intestinal tract. The close association of *B. fragilis* to the colonic crypts is also attributed to these CCF

[105]. The same studies describe how animals colonised by *B. fragilis* were resistant to super-colonisation by bacteria of the same species but receptive to *Bacteroides* of different species. It was demonstrated that CCF are involved in this 'colonisation resistance' by *Bacteroides* to bacteria of the same species. These findings reveal that *Bacteroides* have developed species-specific physical interactions with the host that moderate a stable colonisation and that the establishment of *Bacteroides* in the intestine takes place in a saturable manner [105].

1.2.3 Main roles of the gut microbiota derived from host-bacteria crosstalk in the gut

Given that there are more bacterial cells in the GIT than eukaryotic cells in the human body, the whole metabolic activity of the gut microbiota acts as a virtual hidden organ, comparable to the activity of the liver, making the microbiota an integral part of the human physiology [106]. Multiple mechanisms have been established during the co-evolution of the gut microbiota and its eukaryotic host that have a generally beneficial influence on the latter [76]. The most important functions of the gut microbiota involve metabolic activities that lead to energy savings and nutrient absorption, important trophic effects, influence on the normal structural and functional development of the mucosal immune system and protection of the host against invasion by pathogens [74].

1.2.3.1 Protective functions: Maintenance of the GI barrier

Resident gut bacteria are a crucial luminal barrier against colonisation by exogenous microbes. They stimulate epithelial turnover and biosynthesis of mucin which are two important components of the line of resistance and, therefore, essential in preventing the invasion of tissues by pathogens [107].

The intestinal epithelium, covered by a mucus layer of variable thickness, forms the first barrier between the gut lumen and the body. The epithelial cell monolayer lining the small intestine has a complex architecture, with invaginations into the intestinal wall, called crypts, located between finger-like projections into the lumen, called villi. Several crypts surround a villus forming a crypt-villus unit; each crypt is involved in more than one unit, providing cells to more than one villus. Intestinal stem cells located at the base of each crypt proliferate and give rise to epithelial cells, which migrate to the tip of the neighbouring villi, from where they are shed into the gut lumen. In the healthy intestine, the dimensions and cell number on this crypt-villus unit remain remarkably constant during adult life. This implies that the rate of cell shedding from the villus tip is balanced by the rate at which new cells produced within the supporting

crypts migrate from these crypts onto the villus. This renewal activity is essential for the intestinal homeostasis [108]. The integrity of the gut barrier may be affected when cell shedding from the villus is not compensated by cell proliferation within the crypts. Equally, an increase in proliferation of cells without the adequate cell apoptosis/anoikis may lead to an altered epithelium. Mechanisms of signalling between the commensal gut microbiota and the epithelium to control cell proliferation, differentiation and exfoliation have been reported [109]. SCFA, such as butyrate, produced by commensal bacteria promote cell differentiation and apoptosis in the intestinal epithelium [110]. Epithelial cell death may be a host immune defence system to limit bacterial colonisation. However, there are pathogenic microbes that cause apoptosis to gain access to deeper tissues; once the bacteria has disseminated within the epithelium, cell death may help the spread to other tissues [108].

To restrict the commensal bacteria to the intestinal lumen, the gut epithelium is also equipped with protein complexes called tight junctions (TJ) that seal off gaps between epithelial cells. The TJ are regulated by an intricate network of signal pathways and establish intestinal permeability. However, several physiological and pathological conditions leading to the release of inflammatory cytokines can compromise the gut barrier integrity by altering these TJ; for example, an inflammatory response can be triggered when invasive bacteria manage to circumvent the host front line defences such as the epithelial cells and mucus layer and cross the epithelial barrier, reaching the phagocytes and lymphocytes of the lamina propia – which are the next defence system responsible for carrying out antimicrobial activities. Inflammatory situations and metabolic stresses of the intestine have been reported to result in an increase of commensal gut bacteria disseminating to extraintestinal tissues, what is known as bacterial translocation [109]. This phenomenon has been reported in the presence of pathogens; *Campylobacter jejuni* penetrates into epithelial cells and promotes the translocation of non-invasive *E. coli* [111]. Gut permeability is, therefore, influenced by incoming pathogens as they disrupt signal pathways to break down TJs in order to penetrate into deeper tissues. A damaged epithelium leads to an increase of the permeability and the progress of the disease [108]. Pathogens such as *S.* Typhimurium and *V. parahaemolyticus* deliver effectors to disrupt the epithelial cell-cell adherence to increase the permeability and facilitate the uptake of bacteria by the gut epithelial cells. Consequently, epithelial integrity is essential for the host protection against pathogenic microbes [108].

The mucus layer, that covers the intestinal epithelium, acts as a frontline defence barrier and prevents direct interaction between the host and the gut microbiota and

incoming pathogens; it is formed mainly of heavily glycosylated proteins called mucins that protect the epithelium against toxins, acids and bacterial invasion [112]. The mucus is formed by two layers: a loose mucus layer and an inner one, firmly adhered to the epithelium, which is free from bacteria [113]. Goblet cells, found along the intestinal epithelium, are responsible for the production of mucin which contains digestive enzymes, immunoglobulins and antimicrobial peptides and help remove gut contents and exogenous bacteria. An unregulated low-level of mucin secretion takes place continuously under physiological conditions [24, 114]; not surprisingly, bacterial products of the gut microbiota favour mucus secretion, as mucus confers ecological advantages to them such as the provision of a direct source of carbohydrates and peptides and other external substrates like vitamins and minerals. However, the secretion of mucin can be regulated by inflammatory mediators, hormones and infectious bacteria. LPS and flagellin A from Gram-negative bacteria and lipoteichoic acids from Gram-positive bacteria are the most common modulators that are recognised by the host and trigger inflammatory pathways that induce the transcription of mucin genes [108, 114].

The inner mucin layer prevents pathogen colonisation of the intestinal epithelium; microbes are eliminated due to rapid mucin secretion and mucus shedding; however, some intestinal pathogens have evolved mechanisms to elude the mucus barrier. Flagella and chemotaxis systems are tools used by pathogens to trespass the mucus layer and invade the intestinal epithelial cells [24]. Bacteria that manage to colonise the mucus avoid expulsion from the intestine by peristaltic movements and can take advantage of underlying signalling pathways to increase or reduce mucin secretion; it has been reported that glycosylation can be altered as a consequence of mucosal infection and inflammation, which would be an important pathogenic mechanism for invasive bacteria [114].

Another front line defence barrier in the mucus layer is the presence of secretory IgA (SIgA) which is a dominant antibody produced in the mucous membranes, particularly in the intestine of mammals. It contributes to the mucosal immunity of the gut as it is involved in pathogen neutralisation, known as immune exclusion, and it prevents infection dissemination in the gut epithelium, preserving local homeostasis of the GIT. The secretion of SIgA is induced by intestinal dendritic cells following contact with commensal bacteria. Studies with mice show how low levels of SIgA increase after inoculation with luminal bacteria [115, 116].

Although restricted to the small intestine, Paneth cells, located at the base of the invaginations of the intestinal epithelium or crypts of Lieberkühn, are specialised secretory epithelial cells that produce defensins, antibiotic peptides and other proteins. They play a role in the defence against pathogenic microbes and influence the composition and numbers of the luminal gut flora in the small intestine. In addition, they help maintain sterility in the inner mucus layer of the small intestine [117, 118].

The dense and host-dependent microbial community establishes an ecological mutualism with the host that confers a powerful barrier against pathogenic invaders, referred to as colonisation resistance [119]. The efficiency of this barrier is such that for *S.* Typhimurium to colonise gut infection animal models, the normal microbiota has to first be disrupted by antibiotics [120] or by mucosal inflammation [121]. Numerous studies reveal the importance of colonisation resistance and the interactions between pathogens and the gut microbiota [122]; however, the mechanisms of bacterial interplay are still poorly understood. It has been demonstrated that some pathogens manage to circumvent the gut barrier by altering the microbiota composition through the host response. Triggering inflammation changes the balance between the protective microbiota and the pathogen in favour of the latter. Host-mediated inflammation alters the gut microbiota and provides a platform for the growth of either resident or exogenous aerobic bacteria, especially of the *Enterobacteriaceae* family [123].

1.2.3.2 Immunological functions

The intestinal mucosa is an important site of interaction between the host immune system and bacteria, both commensals and pathogens. Colonisation of the GIT by microorganisms, soon after birth, influences the composition and development of the host intestinal immune system by inducing the maturation of the gut-associated lymphoid tissue [124]. When the intestinal lumen is exposed to microbes, the number of intraepithelial lymphocytes grows, germinal centres containing immunoglobulin-producing cells appear in follicles and in the lamina propia, and circulating immunoglobulin levels increase [74]. Molecular exchange involving bacterial signals that are recognised by the host enhances the GI immune system and influences T cell differentiation [125]. It has been demonstrated that germ-free mice have reduced gut secretory IgA, poor development of lymphoid cells in the intestinal mucosa and smaller Peyer's patches and mesenteric lymph nodes [126].

The host mucosal immune system has been reported to distinguish between commensal and pathogenic bacteria; the equilibrium between the host and the gut

microbiota is regulated via immune-mediated pathways that allow resident microorganisms to establish into a non-hostile environment. Disruption of these pathways results in an altered bacterial community in the gut [124]. In the case of *Enterobacteriaceae*, a single plasmid can make the difference between symbiotic and pathogen. The host is equipped with receptors along the GIT that are capable of identifying the incoming pathogens and generate inflammatory defensive reactions. The defensive response must be tightly regulated so it does not eliminate the commensal microbiota and the benefits it offers [76].

The innate immune system in the gut relies on extracellular and intracytoplasmic receptors that recognise and bind microbial-associated molecular patterns (MAMP), which are molecular signatures like LPS, peptidoglycan and flagellin that have been conserved in a group of microbes [127, 128]. Transmembrane receptors such as Toll-like receptors (TLR) scan the extracellular or vacuolar space for MAMPs and induce pro-inflammatory gene expression, whereas cytosolic detectors such as Nod-like receptors (NLR) identify MAMPs in the cytoplasmic compartment and induce either transcriptional or post-translational responses [129]. TLRs lead to the release of cytokines and chemokines, which are secreted following stimulation; other factors such as pro-IL-1β and pro-IL-18 are held in reserve, awaiting a second signal [129]. Through interaction with TLR, the resident microflora promote immunological tolerance and does not trigger inflammatory response [130]. NLRs lead to the production of inflammasomes, which are multi-protein complexes involved in the activation of Caspase-1 and serve as sensors of damage-associated patterns. Activated Caspase-1 cleaves other proteins, such as the precursor forms of the inflammatory cytokines pro-IL-1β and pro-IL-18, into active mature peptides. In addition, Caspase-1 activation induces pyroptosis or programmed cell death [129]. In summary, the intestinal mucosa is in contact with great amounts of bacteria and has to establish tolerance or immunity depending on the nature of the antigens that are found [113]. Bacterial MAMPs stimulate host TLR or NLR; the intensity, duration and anatomical origin of the resultant signalling response will regulate if MAMP perception involves a “low gain” cytoprotective response, a “medium gain” inflammatory reaction or “high gain” programmed cell death result [76].

Dysbiosis and disturbances in the gut microbiota can lead to disorders or chronic inflammatory intestinal diseases and the development of extraintestinal immune-mediated conditions [126, 131]. Other studies conclude that an altered intestinal microflora, as a result of antibiotics, hygiene or life style, can have an important effect

not only on the immunology of the intestinal mucosa but also on the systemic immune system [132].

1.2.3.3 Metabolic/Nutritional functions

The fermentation of carbohydrates that escape digestion in the upper digestive tract is a major metabolic function of the colonic microbiota. The gene diversity of these bacteria encodes biochemical pathways that allow the fermentation of otherwise indigestible carbohydrates to SCFA [133] which represent 40-50% of the available energy of the carbohydrates [134]. SCFA are also the end product of the bacterial anaerobic metabolism of dietary proteins, pancreatic enzymes and GI secretions. The main SCFA are acetate, propionate and butyrate; they are metabolised by the muscle, liver and colonic epithelium respectively. Their absorption in the colon is an efficient process and only a small proportion is eliminated in the faeces [135]. SCFAs provide between 5-15% of human energy requirements [76]. *Clostridium* and *Bifidobacterium* species are the gut microbes that produce SCFA most effectively and therefore extract more energy from the diet than other commensals. These species have been found to be more predominant in obese mice and humans [136]. SCFAs can also be used as substrate by the gut microbiota that has produced them. However, this process generates potentially toxic compounds such as NH_3, amines, phenols, thiols and indols [74]. In addition to SCFA production, gut microbes are also involved in vitamin synthesis and absorption of minerals such as calcium, magnesium and iron [74]. Vitamin K, which is required for blood coagulation and metabolic pathways in bone tissue, is synthesised by gut commensals such as *B. fragilis* and *E. coli;* yet, it still needs to be supplied in the diet [137]. Vitamin B_{12} is also produced by the colonic bacteria [138]; however, it is absorbed in the small intestine and not in the colon and therefore it must be obtained from the diet [139].

1.2.4 Disorders related to the gut microbiota

An ecological disorder of the microbiota or dysbiosis has been observed in different diseases some of which are related to the GIT. Since there is large inter-individual and intra-individual variability in the composition of the microbial population of healthy individuals, it is difficult to associate the presence or relative abundance of specific bacterial communities to human health [71]. The human gut is colonised by multiple microorganisms; hence, it is thought that host disorders might be the consequence of various microbial disturbances rather than caused by a single microbe. Diseases that have been associated with dysbiosis of the gut flora include allergies and autoimmune diseases, obesity, inflammatory bowel disease (IBD) and diabetes [127].

1.2.4.1 Obesity

All reports coincide in saying that a relevant factor in the development of obesity is carbohydrate metabolism and the amount of SCFA produced by the microbiota; however, the bacterial groups linked to obesity remain controversial. The Firmicutes/Bacteroides ratio of the human microbiota, which are the two main bacterial groups found in the colon, has been linked to obesity. An increase of Firmicutes, and a corresponding decline of Bacteroides, has been observed in both obese mice and humans [140, 141]. On the other hand, some studies show a change in the Firmicutes/Bacteroides ratio in favour of the Bacteroides in overweight and obese individuals [142].

It is thought that obesity is associated with slight changes in the bacterial composition rather than changes at phylum level and that it is a reduction of the diversity of the bacterial composition that leads to physiological disorders such as obesity [79]. The obesity-associated microbiome contains more genes responsible for the production of enzymes involved in the digestion of dietary polysaccharides. As a result, obese individuals harvest energy from food more efficiently than lean individuals. In addition, it has been observed that the faeces produced by lean individuals have a lower energy content than obese individuals due to the higher capacity of their gut microbiota for extracting energy from the diet [136].

It is still unclear if obesity is a direct consequence of an altered intestinal microbiota or if the changes in the gut bacteria of obese individuals are an adaptation to a disturbed host environment [71].

1.2.4.2 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a generic name for complex chronic inflammatory disorders, such as Crohn's disease (CD) and ulcerative colitis (UC) that affect the intestinal mucosa. Although the cause is not fully known, an abnormal response of the host immune system against intestinal microbial antigens has been reported as the origin of this condition [143]. Gut microbiota, therefore, plays an important role in the development of IBD in genetically predisposed individuals [144], as supported by studies that show that: bacteria are necessary to trigger inflammation in animal models, antibiotics have an alleviating effect in CD and UC, probiotics are beneficial in the treatment of UC and removing faecal content from affected segments of the intestine has a curing effect in CD, whereas its reinfusion generates re-occurrence of the condition [124].

The dysbiosis associated to IBD involves a reduction of gut bacterial diversity and a decline of Firmicutes coincidental with an increase of Bacteroides and Enterobacteria [143]. In particular, CD is associated with a reduced diversity of Firmicutes, especially the *Clostridium leptum* subgroup [124]. Additionally, it has been observed that individuals suffering from CD and UC present bacteria that are not part of the usually dominant phylogenetic groups in their faecal-associated microbiota. On the other hand, in the mucosa-associated microbiota, CD patients present increased concentrations of total bacteria and total facultative anaerobes, whereas UC patients show increased concentrations of total bacteria, total anaerobes and aerobes. It has also been reported that *Fusobacteria* increase in faecal bacteria in patients suffering from UC and that bifidobacteria and lactobacilli presence in individuals suffering from CD is reduced [124].

There are studies that strongly link the gut microbiota to IBD and suggest faecal microbiota transplantation as an alternative effective treatment, when conventional treatments have failed [145]. Infusion of donor faeces has already been successfully used for the treatment of recurrent *Clostridium difficile* infection [146]. It remains to be elucidated if dysbiosis is a direct cause of IBD or is the consequence of a disturbed environment in the GIT [124].

1.2.4.3 Allergy

The intestinal mucosa confines millions of harmless antigens and MAMPs, derived from food and bacteria, to the gut lumen, separating them from deeper tissues. An inflammatory immune response following penetration of low levels of antigens and MAMPs would be damaging to the host; for that reason, the intestinal immune system is equipped with innate and adaptive tolerance mechanisms towards these structures [147]. The “microbiota hypothesis” indicates that the use of antibiotics and changes in the diet lead to a reduced bacterial allergen exposure that results in the establishment of an immature microbiota in the gut. As a consequence, there is a delay in the development of the immune system, disrupting the chain of events responsible for establishing mucosal immunological tolerance, which leads to an increase of allergic hypersensitivities [148].

IgA-secreting B cells and antigen-specific regulatory T cells, which are favoured by the intestinal immune system, lead to the neutralisation of antigens with no inflammatory reaction. On the contrary, IgE and various interleukins induce pro-inflammatory signalling cascades and the recruitment of effector cells to sites of inflammation [147, 149]. Studies using antibiotics to target the commensal microbiota

have demonstrated that an altered gut flora results in an increase of serum IgE and basophile concentrations and susceptibility to inflammation and allergic disease [149]. High levels of intestinal IgA in children are associated with reduced intestinal inflammation and indicate low risk for IgE-associated allergic diseases [150].

In general terms, clinical data indicate that gut microbiota influence the development and virulence of allergic processes. Other studies conclude similarly, reporting that the increase of allergic conditions in the industrialised world is associated with a reduced microbial exposure and alterations of microbial communities in different body sites; children growing up in farms, in contact with livestock and drinking raw milk, are less vulnerable to allergic diseases [147, 151]. Recently, it has been reported that the microbiota of allergic individuals is different from that of non-allergic ones, suggesting that a disturbed gut microbiota might be the origin of the development of allergic conditions [152, 153]. Evidence from research in humans suggests that individuals suffering allergic diseases such as rhinitis and atopic eczema have been found to have a disturbed composition of the gut microbiota [147]. In addition, reduced faecal microbiota diversity in one-week old babies has been linked with the development of allergic diseases at later stages in life [71]. Reinstating a “healthy” microbiota by administrating probiotics may prevent the appearance of allergies or even treat existing disease [148]. However, conflicting results have been found in clinical trials with probiotics, using various bacterial strains. No beneficial effects have been described in some studies, whereas a reduction in the incidence and severity of allergies following treatment with probiotics has been reported in others [147].

1.2.5 Studying the gut microbiota

1.2.5.1 Culture-based techniques

The characterisation of the gut microbiota has traditionally been done using culture-based techniques, which consist of using different selective media for specific bacterial groups based on their metabolic needs and resistance to antimicrobial compounds. However, ca. 80% of the gut microbiota cannot be cultured under laboratory conditions or in the current defined media [75]. Thus, these techniques are very limiting and in addition, they do not inform on bacterial phylogenetic groups [95].

1.2.5.2 Culture-independent techniques

Currently, molecular approaches, based on 16S ribosomal RNA (rRNA) and its corresponding gene, are used for the phylogenetic analysis and classification of gut microbes. This 1.5 kb gene, present in bacteria, has enough variations to distinguish

between different species and strains and enough similarities as to identify microbes belonging to the same phylogenetic group [154].

The methods known as 'fingerprinting' include techniques such as denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient gel electrophoresis (TTGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP) and ribosomal intergenic spacer analysis (RISA). These techniques allow comparison of the different samples by producing DNA profiles of the bacterial community in each of them. They do not provide an analysis of microbial composition but can provide information about the predominant taxa [124].

DGGE consists of extracting the bacterial DNA from the sample and denaturing the PCR-amplified 16S rRNA gene. The products obtained are dispensed in an acrylamide gel and migrate according to their G+C content. This creates a distinct banding pattern that represents the bacterial diversity of the sample [95].

Fluorescence *in situ* hybridisation (FISH) and real-time PCR, also known as quantitative PCR (qPCR), are useful for identifying specific bacterial taxonomic groups. A disadvantage of these techniques is that since they use fewer and specific probes, they cannot identify novel strains of bacteria [95].

DNA microarrays are another technique used for the characterisation of gut microbiota. It uses specific probes to detect species of the human gut bacterial communities. Limiting factors of microarrays are that the design of the probes is restricted to known sequences and that it is not quantitative [124].

All these techniques are helpful in providing knowledge about microbial composition and bacterial identity of the resident gut microbiota; however, in order to obtain an insight into potential functions and activities of these bacteria, the use of the meta-"omic" approaches is necessary [155]. Metagenomics provide comprehensive information about the genes that exist in a particular community; Metaproteomics is based on the identification of the diversity and abundance of proteins that have been formed by the bacteria; Metabolomics studies the functions of bacterial populations by analysing the metabolites produced and surveying their metabolic profiles; Metatranscriptomics studies consist of the characterisation of RNA content in a given sample [95].

1.2.6 Bacterial interactions

Although bacteria are often viewed as independent units, they display numerous cooperative and competitive behaviours that are essential for establishing multicellular communities [156]. The structure of bacterial populations in natural environments and during multimicrobial infections is determined by the interplay of different species [157]. In the gut, the largest reservoir of resident bacteria in the human body, there is a balanced relationship between all the commensal species and a symbiotic association between them and the host; this equilibrium, however, can be altered by the presence of exogenous pathogens [122]. The interaction and communication mechanisms used by these microbial communities are very diverse and have recently become the focus of multiple studies [158].

1.2.6.1 Metabolic/Nutritional interactions

Bacterial communities work in a way in which resources are used very efficiently and the acquisition of the nutrients that they require to survive is strongly dependent on the rest of the neighbouring bacterial cells. One of the most important forms of bacterial communication consists of obtaining the substrates for metabolism and releasing the end products [159].

Studies with *B. thetaiotaomicron* and *Eubacterium rectale* reveal how these two members of the dominant gut bacterial phyla, Bacteroides and Firmicutes, can adapt their substrate utilisation in response to one another and to host dietary changes [160]. Other experiments involving colonisation of germfree mice with *B. thetaiotaomicron* and *B. longum*, which is commonly used as a probiotic, have revealed similar findings. The presence of *B. longum* results in *B. thetaiotaomicron* widening the diversity of polysaccharides targeted for degradation. This expanded capacity to utilise polysaccharides occurs independently of host genotype, and is also observed when *B. thetaiotaomicron* is associated with other bacteria, for example, *Lactobacillus casei* [161]. Combining *B. thetaiotaomicron* with *Methanobrevibacter smithii* has also been demonstrated to result in changes in *B. thetaiotaomicron* genes responsible for carbohydrate metabolism. In addition, the combination of *B. thetaiotaomicron* and *M. smithii* results in a more successful colonisation of germfree mice than using one species alone [92].

In terms of bacterial competition, SCFAs such as acetate, propionate, formate or butyrate are produced by the microbiota and have an important influence on prevention of gut colonisation by pathogens [162]. For instance, butyrate is known to down-regulate invasion genes in *Salmonella* [163]. This colonisation resistance also

involves growth restriction to opportunistic resident bacteria such as *C. difficile* [164]. Commensals also inhibit the growth of pathogens by competing with them for nutrients. It has been reported that *B. thetaiotaomicron* coordinates its substrate requirements with the hosts' production of an energy source. *B. thetaiotaomicron* uses epithelial fucosylated glycans as a substrate; however, this bacterial species use a repressor, FucR, to control the genes of intestinal enterocytes responsible for the production of fucosylated glycans. This means that there is minimal energy cost to both the host and the microorganism [96]. Additionally, this symbiotic relationship maintains an environment devoid of excess nutrients that could be utilised by microbial intruders with potential pathogenicity to the host [74].

Some enteropathogens develop 'ecological strategies' to escape from the action of the microbiota by targeting niches different to those used by competing commensals. For example, *Citrobacter rodentium* expresses an adhesion molecule that permits the bacterium to lodge in the gut epithelial surface during replication; this way it avoids being outcompeted by the commensals with similar nutritional requirements, present in the gut lumen [165]. On the other hand, there are cases where resident mucosa-adherent bacteria can physically occupy pathogen attachment sites and prevent the adherence of pathogens into intestinal epithelial cells; for instance, it has been reported that a strain of *Lactobacillus plantarum* inhibits the cell attachment of the pathogen *V. parahaemolyticus* by obstruction mechanisms [166].

Differences in substrate utilisation between bacteria are exploited by enteropathogens to cause infection in the GIT. Pathogenic strains of *E. coli* present a higher flexibility in their fermentative metabolism than commensal strains, which provides them with an advantage to outcompete the microbiota and colonise the intestine. EHEC is able to use galactose, hexuronates, mannose and ribose as carbon sources, whereas these substrates cannot be used by commensal *E. coli;* sugar metabolism is therefore involved in the virulence modulation of extraintestinal pathogenic *E. coli* strains [167]. In addition to this, EHEC has a *eut* (ethanolamine utilisation) gene cluster that regulates the use of ethanolamine as a nitrogen source, whereas most species in the mammalian gut microbiota lack this *eut* operon and therefore cannot utilise ethanolamine as a substrate [168].

1.2.6.2 Bacterial signalling: Quorum sensing (QS)

Chemical signals are utilised by numerous bacteria to communicate and regulate gene expression. QS is a process used by bacteria to communicate with their own and other species that is based on diffusible extracellular hormone-like molecules called autoinducers. The production, release, detection and response to these signals are necessary for chemical communication [169]. N-acyl-L-homoserine lactone (AHL) is a QS signalling component used by Proteobacteria, whereas peptide-based signals have been described in Gram-positive bacteria [158]. The purpose of QS is to monitor the population density or the time a closed environment has been populated by the signal producing cells, or a combination of both, by quantifying molecules that have diffused from a distance. This information allows bacteria to prepare an appropriate collective response, such as the synchronisation of gene expression, to act in unison [170, 171]. QS-dependent processes, undertaken simultaneously by a high number of bacterial cells, result in bacterial populations resembling multicellular organisms [169]. For example, the formation of biofilms, structures of surface-associated aggregated bacteria embedded in a self-produced biopolymer matrix, is a QS regulated process that provides advantages to the bacterial population; biofilms have been demonstrated to provide tolerance to antibiotics and disinfectants by bacteria such as *Staphylococcus* spp. and *Pseudomonas aeruginosa* [172].

Using QS, pathogens entering the intestine can receive signals from gut microbiota that trigger the expression of colonisation genes. Studies with EHEC demonstrate that sterile supernatants of gut bacteria cultures can induce the expression of genes encoding T3SS in the pathogen [102]. It has been reported that *Salmonella* can detect other species, using QS, to affect pathogenesis [122]. Although *Salmonella* does not produce AHL, it can recognise AHLs generated by other bacterial species. AHL produced by *Yersinia enterocolitica* in mouse Peyer's patches is detected by *Salmonella* and leads to the activation of the genes encoding a Type III secreted effector [173].

It has been suggested that since QS is an essential process used by pathogens for colonising the host and spreading disease, intervention in this form of bacterial communication can be an alternative to current antimicrobial treatments [174].

1.2.6.3 Bacteriocins and other inhibitory products

The production of bacteriocins is widely distributed among the bacterial community of the intestine. These mechanisms are relevant for the interference of commensals with foreign microorganisms [175]. It has been demonstrated that several *Bifidobacterium* strains isolated from the human gut exert antimicrobial activity against pathogens, which suggests that they play an important role in the "barrier effect" produced by the indigenous microflora [176]. In addition, other secreted extracellular soluble components are thought to play an important part in bacterial competition. For example, the probiotic *E. coli* Nissle strain 1917 has been proved to be effective against intestinal invasion of pathogens such as *S*. Typhimurium, *Shigella flexneri, Legionella pneumophila* and *Listeria monocytogenes* through a mechanism based in soluble molecule production [177]. Further, there are cases that illustrate that commensals can have a major impact on the virulence of intestinal pathogens; it has been shown that the production of Shiga toxin 2 by *E. coli*, which is one of the main virulence factors of this species, is repressed by molecules of a molecular mass below 3 kDa produced by *B. thetaiotaomicron*, a microbe commonly found among human gut bacteria [178].

1.2.6.4 Cell-cell contact

The secretion, and subsequent detection, of soluble components into the environment is not the only form of bacterial communication. There are interaction mechanisms that require cell contact or close proximity between the bacterial cells.

Gram-negative bacteria generate outer membrane vesicles (MV) that are used for the transfer of toxins, proteins and DNA to other bacterial cells in close proximity. The MV are double-layered structures of a diameter between 50-250 nm formed of lipopolysaccharide, outer membrane proteins and phospholipids [157]. It has been demonstrated that *P. aeruginosa* produces MV that contain antimicrobial substances capable of killing other Gram-negative and Gram-positive species [179]. This antimicrobial activity is not limited to MV produced by *P. aeruginosa;* there is evidence of MV from 15 other Gram-negative bacteria that have similar antimicrobial activity [180]. Conversely, MV are also involved in the transport of proteins between bacterial cells in order to share beneficial properties. Antibiotic resistance protein β-lactamase has been reported to be conveyed among *P. aeruginosa* bacteria via MV [181]. There is evidence that MV are also involved in the transfer of DNA in species such as *E. coli* O157:H7. The MV are an efficient vehicle for the traffic of DNA from one strain of *E.*

coli to another, as they provide protection against degradation by extracellular DNases [182]. Further, MV have also been associated with the transport of QS signalling molecules within *P. aeruginosa* communities. Given the hydrophobic nature of these chemical signals, the MV offer a good tool for their diffusion in the extracellular environment [179].

Other reported mechanisms of bacterial communication involve cytoplasmic connections between neighbouring cells. The development of filaments for the exchange of metabolites and small molecules such as sugars and amino acids has been demonstrated in cyanobacteria. These filaments consist of individual cells, each one with its own plasma membrane and cell wall, surrounded by a common outer membrane. The diffusion of molecules takes place non-specifically between the cytoplasm of all the cells in the filament and is an essential form of intercellular cooperation [183, 184].

Conjugation is another intercellular exchange process that requires physical interaction between bacterial cells. It involves a translocation channel that spans through the layers of the cell surface to permit the transit of macromolecular DNA from a donor to a recipient cell. It works in conjunction with either a tube-like structure, called pilus in Gram-negative bacteria, or surface-linked adhesins in Gram-positive bacteria [185]. Conjugation mechanisms belong to the family of Type IV Secretion Systems (T4SS) whereby hereditary genetic information is transferred. It is commonly found in a wide spectrum of bacteria including *Bacillus subtilis*, *Haemophilus* spp., *Pseudomonas* spp., *S.* Typhi and others [186].

A similar mechanism, the Type VI Secretion System (T6SS), has recently been found in Gram-negative bacteria. As T4SS and T3SS, T6SS apparatus comprises a membrane-located subassembly and a bacteriophage-like subassembly (cell-puncturing device) involved in the secretion and translocation of proteins directly into prokaryotic and eukaryotic cells in a contact-dependent manner [187]. The highly clustered genes that regulate T6SS have been identified in over 25% of the sequenced Gram-negative bacteria genomes and are widespread in Proteobacteria [187, 188]. The inputs that regulate the expression and activity of T6SS are chemical or physical environmental signals such as cations, temperature, pH, quorum sensing and cell contact [189]. T6SS functions have normally been studied in the context of pathogenic bacterial interactions [190], similar to the processes described in section 1.1.4 for T3SS. However, T6SS are also involved in bacterial cooperative or competitive interactions and even as anti-pathogenesis factors. Studies of the Gram-

negative *Myxococcus xanthus* suggest T6SS is used for interspecies cooperation processes; additionally, cell-cell contact of these bacterial species is required for the formation of cooperative feeding colonies when the substrate is abundant, and the formation of spore-filled fruiting bodies when the nutrients are scarce [191]. On the other hand, T6SS has been reported to be responsible for antagonistic bacterial interaction; *P. aeruginosa* produces a protein, Tse2, which can halt the growth of various eukaryotic and bacterial cells. Despite the interaction with multiple cells, T6SS has been found to specifically target Tse2 to bacteria, which demonstrates the antimicrobial competition function of T6SS [192]. In *S.* Typhimurium, T6SS has been found to exert an anti-pathogenesis function. The gene *sciS* regulates part of a T6SS and is responsible for controlling intracellular bacterial levels and attenuating the virulence of *S.* Typhimurium; it, therefore, promotes a prolonged colonisation. *sciS* mutants show an increased replication of *S.* Typhimurium in macrophages at 24 h postinfection and have been proven to be hypervirulent in mice [193].

Other secretion systems have also been associated with contact-dependent growth inhibition. Type V Secretion System has been reported to be used by *E. coli* EC93 to stop the growth of *E. coli* K-12 [194]. *E. coli* EC93 regulates a toxin-immunity protein pair, encoded by the *cdiBAI* gene cluster, which is used to outcompete other *E. coli* strains. CdiB is a β-barrel protein that transports CdiA, responsible for growth inhibition activity, through the outer membrane. CdiA attaches to BamA, its receptor in *E. coli* K-12; although BamA can be found in all Gram-negative bacteria, suggesting E*. coli* EC93 could inhibit other species, unrelated bacteria are not sensitive to *E. coli* EC93. To protect itself from inhibition, *E. coli* EC93 regulates the *cdiI* gene, which is associated with *cdiA* and encodes an immunity protein [156].

Recent reports have revealed the existence of nanotubes that originate from the cell membrane and enable the transfer of cytoplasmic molecules, organelles and even viruses over long distances between eukaryotic cells [195]. Nanotubular channels of a similar nature have been found to be used by *B. subtilis* for the exchange of intracellular content with other bacteria. Physical interaction of adjacent cells is required for the formation of these nanotubes that cannot only connect *B. subtilis* with cells of its own species but also with other Gram-positive bacteria like *Staphylococcus aureus* and even evolutionary distant Gram-negative bacteria like *E. coli* [196]. This work demonstrates that nanotube networks are structures whereby bacteria can transfer cytoplasmic molecules and genetic materials to adjacent inter and intra species bacterial cells [196]. All these systems suggest that contact-dependent interactions are an important part in the shaping of bacterial communities.

Interaction of microorganisms also involves viruses. Although not involving direct bacterial interaction, DNA can also be transferred between cells through bacteriophages. This phenomenon is known as transduction and it is found in species such as *S. aureus*. Resident phages provoke that DNA fragments excise and replicate and are also responsible for their enclosure in a capsid and passage from one cell to another [186]. Other example of viral-bacterial interaction is the herpes virus, which can remain in latent state in humans following the resolution of acute infections. Studies with mice have revealed that this virus confers protection against *L. monocytogenes* and *Yersinia pestis* to the host by increasing the levels of the interferon-gamma cytokine and the systemic activation of macrophages [197].

Gut bacteria can also interact with eukaryotic parasites. It is known that the resident bacterial community protects the host against the development of disease caused by *Giardia* [127]. Lactobacilli particularly, have been associated with growth inhibition of the parasite by releasing a compound that has not yet been identified [198].

Although some of the mechanisms of gut bacterial interactions remain elusive and they can be of a metabolic nature rather than bacterial communication as such, it is clear that the complex relationships within bacterial communities are important for the maintenance of the ecosystem they inhabit.

1.3 Outline of the thesis

1.3.1 Background

The WHO states that S*almonella* is a ubiquitous bacterium responsible for widely spread foodborne diseases around the globe. Tens of millions of human cases take place worldwide yearly resulting in more than a hundred thousand deaths. *Salmonella* is a major public health burden and involves tremendous cost to society. On the other hand, the International Food Safety Authorities Network (INFOSAN) alerts of the rapidly emerging problem of antimicrobial resistance and the challenge of treating human infections. In addition, given the protection that the gut microbiota confers on the host, the use of antibiotic treatments increases the risk of salmonellosis [199]. It therefore seems imperative that alternative ways of dealing with enteropathogens without the use of antibiotics are found.

Salmonella is an intracellular pathogen that colonises the distal parts of the ileum, cecum and proximal colon [13]. Along the GIT, the gut microbiota interferes with enteropathogens and can even inhibit their growth, what is known as ‘colonisation resistance’ [200]. However, once the infection has taken place, it has not yet been

discerned how these microbes are excluded from the gut lumen, what is known as 'pathogen clearance' [201]. After the clinical signs of NTS infection have abated, *Salmonella* is normally excreted for 5 weeks, although this can be extended to several months and even to a year in 1% of the affected population [202]. Between 1 and 6% of individuals suffering from enteric fever become chronic carriers of *S.* Typhi for periods ranging from one year to their entire lives. In both types of the disease, the shedding of *Salmonella* by asymptomatic patients is a risk to public health as these individuals are reservoirs for the transmission of bacteria [42].

1.3.2 Aims

The aim of this study was to investigate the interactions between *S.* Typhimurium and GI bacteria, to search for ways in which the gut microbiota influences the pathogenicity and survival of this pathogen in the gut. Using batch and continuous culture systems [203], this work aimed to investigate the impact of GI bacteria, obtained from culture collections, and of faecal bacteria of human origin, on growth, survival and molecular activity of *S.* Typhimurium in the gut environment. This project intended to contribute to the understanding of interactions between *S.* Typhimurium and GI bacteria and the mechanisms that eliminate the pathogen from the gut lumen. Understanding this can help limit infections and define better treatments when diseases occur.

1.3.3 Question driving this research

What kind of interactions between *S.* Typhimurium and the GIT bacteria affect the concentration of this pathogen in the gut environment?

2 Chapter 2 – General methods: Bacterial strains, media and colon model systems

2.1 Bacteriology

2.1.1 Strains used

The strain of *Salmonella* used in the study was *Salmonella enterica* subsp. *enterica* serovar Typhimurium SL 1344. The selected GI tract strains, which are microbes commonly found in the gut, were *Enterococcus faecalis* GB 122, *Bacteroides fragilis* NCTC 9343, *Bacteroides ovatus* V975, *Bifidobacterium bifidum* NCIB 8807, *E. coli* K12 MG1655, *Clostridium perfringens* DSM 11780 and *Lactobacillus gasseri* DSM 20243.

2.1.2 Inocula preparation

Stock cultures of each bacterial strain were maintained in 40% glycerol at -80°C. Each strain was recovered by transferring 10 µl of the stock culture into 15 ml of pre-reduced growth media under oxygen-free conditions at 37°C for 24 h, or for the time required to observe turbidity. Two more subcultures in pre-reduced growth media were made immediately prior to the experiments.

2.1.3 Isolation of bacteria from fresh human faeces

Faecal samples were obtained immediately prior to the experiments from healthy adults, kept in an anaerobic cabinet (10% H_2, 10% CO_2 and 80% N_2) and used within a maximum of 10 min after collection. Faecal inocula were prepared by homogenising 25 g of fresh faecal samples and 250 ml of oxygen-free 0.1 M phosphate-buffered saline solution, pH 7.4, in a Stomacher 400 (Seward, UK) at 230 rpm for 45 s.

2.2 Media and supplements

2.2.1 Media for culturing commensal bacteria

Various commensal bacterial groups of the gut were enumerated by culture using the selective media shown in Table 2.1.

Xylose lysine deoxycholate (XLD) agar (Oxoid, UK) was used for *S.* Typhimurium counts and incubated in aerobic conditions at 37°C for 48 h.

Media	Bacteria	Incubation time (days)	Temperature
Nutrient agar	Total aerobes	2	37℃ Aerobic conditions
Mac Conkey No 3	*Enterobacteriaceae*	1	
Slanetz & Barley	Gram-positive cocci	3	45℃ Aerobic conditions
Wilkins-Chalgren	Total anaerobes	3	37℃ Anaerobic conditions
Bacteroides	*Bacteroides* spp.	5	
Clostridia	*Clostridium* spp.	5	
Rogosa	*Lactobacillus* spp.	3	
Beeren's	*Bifidobacterium* spp.	5	

Table 2.1. Media and conditions for culturing commensal gut bacteria.

2.2.1.1 Brain, heart infusion (BHI) agar (Oxoid, UK)

A suspension of 37 g of BHI (Oxoid, UK) broth and 15 g of agar base (Oxoid, UK) in 1 litre of deionised water was prepared. The medium was autoclaved at 121℃ for 15 min. Plates of 20 ml were poured at 50℃.

2.2.1.2 De Man, Rogosa, Sharpe (MRS) agar (Oxoid, UK)

A suspension of 62 g MRS (Oxoid, UK) broth, 15 g of agar base (Oxoid, UK) and 1 litre of deionised water was prepared. The medium was autoclaved at 121℃ for 15 min. Plates of 20 ml were poured at 50℃.

2.2.1.3 Xylose lysine deoxycholate (XLD) agar (Oxoid, UK)

A suspension of 53 g XLD agar (Oxoid, UK) and 1 litre of deionised water was prepared and boiled for 15 min. Plates of 20 ml were poured at 50℃.

2.2.1.4 Rogosa agar (Oxoid, UK)

A suspension of 82 g Rogosa medium (Oxoid, UK) and 1 litre of deionised water was prepared and autoclaved at 121℃ for 15 min. Plates of 20 ml were poured at 50℃.

2.2.1.5 Beeren's agar

As previously described [204]: A suspension of 15.6 g Columbia base agar (Oxoid, UK), 2 g glucose (Sigma, UK), 0.2 g cysteine HCl (BDH Prolabo, Belgium), 2 g agar base (Oxoid, UK), 2 ml propionic acid (Sigma, UK) and 16 ml 1 M NaOH (Sigma, UK) in 400 ml of deionised water was prepared and autoclaved at 121℃ for 15 min. Plates of 20 ml were poured at 50℃.

2.2.1.6 Bacteroides mineral salt (BMS) agar

As previously described [205]: A suspension of 11.2 g Brucella broth (Oxoid, UK) and 6 g agar base (Oxoid, UK) in 400 ml deionised water was prepared and autoclaved at 121℃ for 15 min. The medium was cooled to 50℃ and 4 ml 0.5 mg/ml hemin solution (Sigma, UK), 80 µl 5% v/v vitamin K solution (Sigma, UK), 1.2 ml 25 mg/ml kanamycin (Sigma, UK), 3 ml 1 mg/ml vancomycin (Sigma, UK) and 20 ml laked horse blood (Oxoid, UK) were added. Plates of 20 ml were poured.

2.2.1.7 MacConkey No 3 (Oxoid, UK)

A suspension of 52 g MacConkey agar (Oxoid, UK) in 1 litre of deionised water was prepared and autoclaved at 121℃ for 15 min. Plates of 20 ml were poured at 50℃.

2.2.1.8 Wilkins-Chalgren agar (Oxoid, UK)

A suspension of 43 g Wilkins-Chalgren agar (Oxoid, UK) in 1 litre of deionised water was prepared and autoclaved at 121℃ for 15 min. Plates of 20 ml were poured at 50℃.

2.2.1.9 Nutrient agar (Oxoid, UK)

A suspension of 13 g Nutrient broth (Oxoid, UK) and 15 g agar base (Oxoid, UK) in 1 litre of deionised water was prepared and autoclaved at 121℃ for 15 min. Plates of 20 ml were poured at 50℃.

2.2.1.10 Clostridia agar

A suspension of 17.2 g Wilkins-Chalgren agar (Oxoid, UK) in 400 ml of deionised water was prepared and autoclaved at 121℃ for 15 min. The media was cooled to 50℃ and 3.2 ml 1 mg/ml novobiocin (Sigma, UK) and 3.2 ml 1 mg/ml colistin (Sigma, UK) were added, to have a concentration of 8 µg/ml of each antibiotic. Plates of 20 ml were poured.

2.2.1.11 Slanetz and Bartley agar (Oxoid, UK)

A suspension of 16.8 g Slanetz-Bartley agar (Oxoid, UK) in 400 ml of deionised water was prepared, autoclaved at 121℃ for 15 min and cooled to 50℃. Plates of 20 ml were poured.

2.2.2 Batch fermentation medium (growth medium)

The growth medium was prepared as shown in Table 2.2. The components were dissolved in 1 litre deionised water and autoclaved at 121℃ for 15 min.

Component	/litre
Peptone water (Oxoid, UK)	2 g
Yeast extract (Oxoid, UK)	2 g
NaCl (Sigma, UK)	0.1 g
Carbohydrate D-Glucose (Fisher Chemical, UK)	10 g
K_2HPO_4 (Fisher Scientific, UK)	0.04 g
KH_2PO_4 (Fisher Scientific, UK)	0.04 g
$MgSO_4.7H_2O$ (Fisher Scientific, UK)	0.01 g
$CaCl_2.2H_2O$ (Fisher Scientific, UK)	0.01 g
$NaHCO_3$ (Fisher Scientific, UK)	2 g
Tween 80 (Sigma, UK)	2 ml
Vitamin K 5% v/v aqueous solution (Sigma, UK)	10 µl
Cysteine. HCl (BDH Prolabo, Belgium)	0.5 g
Bile salts (Oxoid, UK)	0.5 g
Hemin (Sigma, UK) dissolved in 300 µl 1M NaOH (Sigma, UK)	0.02 g

Table 2.2. Composition of the growth medium.

2.2.3 Colon model medium

The composition of the medium used for the colon model system was made as shown in Table 2.3. The components were dissolved in 1 litre deionised water and autoclaved at 121℃ for 15 min.

Component	/litre
Starch (BDH Ltd, UK)	5 g
Peptone water (Oxoid, UK)	5 g
Yeast extract (Oxoid, UK)	4.5 g
Mucin, porcine gastric III (Sigma, UK)	4 g
NaCl (Sigma, UK)	4.5 g
KH_2PO_4 (Fisher Scientific, UK)	0.5 g
K_2HPO_4 (Fisher Scientific, UK)	0.5 g
$MgSO_4.7H_2O$ (Fisher Scientific, UK)	1.25 g
$CaCl_2.6H_2O$ (Fisher Scientific, UK)	0.15 g
$NaHCO_3$ (Fisher Scientific, UK)	1.5 g
KCl (Sigma, UK)	4.5 g
$FeSO_4.7H_2O$ (BDH Ltd, UK)	0.005 g
Tryptone (Oxoid, UK)	5 g
Casein (BDH Ltd, UK)	3 g
Pectin, citrus (Sigma, UK)	2 g
Xylan, oatspelt (Sigma, UK)	2 g
Arabinogalactan, larch wood (Sigma, UK)	2 g
Tween 80 (Sigma, UK)	1 ml
Vitamin K 5% v/v aqueous solution (Sigma, UK)	10 µl
Cysteine HCl (BDH Prolabo, Belgium)	0.8 g
Bile salts (Oxoid, UK)	0.4 g
Hemin (Sigma, UK) dissolved in 300 µl 1M NaOH (Sigma, UK)	0.05 g

Table 2.3. Composition of the colon model medium.

Batch fermentation and colon model media were adjusted to pH 7.0 using 1 M HCl (Sigma, UK) and were pre-reduced by boiling under oxygen-free nitrogen for 15 min and cooling in ice under $N_2/CO_2/H_2$ (80:10:10) atmosphere. They were dispensed under anaerobic conditions.

2.2.4 Preparation of the batch model (one compartment system)

Water-jacketed fermenter vessels (Soham Scientific, UK) were filled with 270 ml of batch fermentation media (Fig. 2.1). Each vessel was magnetically stirred and the temperature set at 37°C by a circulating water bath (Jencons-Pls, UK). Culture pH was maintained at pH 6.8-7.2 using automated pH controllers (Electrolab Ltd, UK) and the addition of either 0.5 M NaOH or HCl. Anaerobic conditions were maintained by continuously sparging the vessels with oxygen-free nitrogen [206].

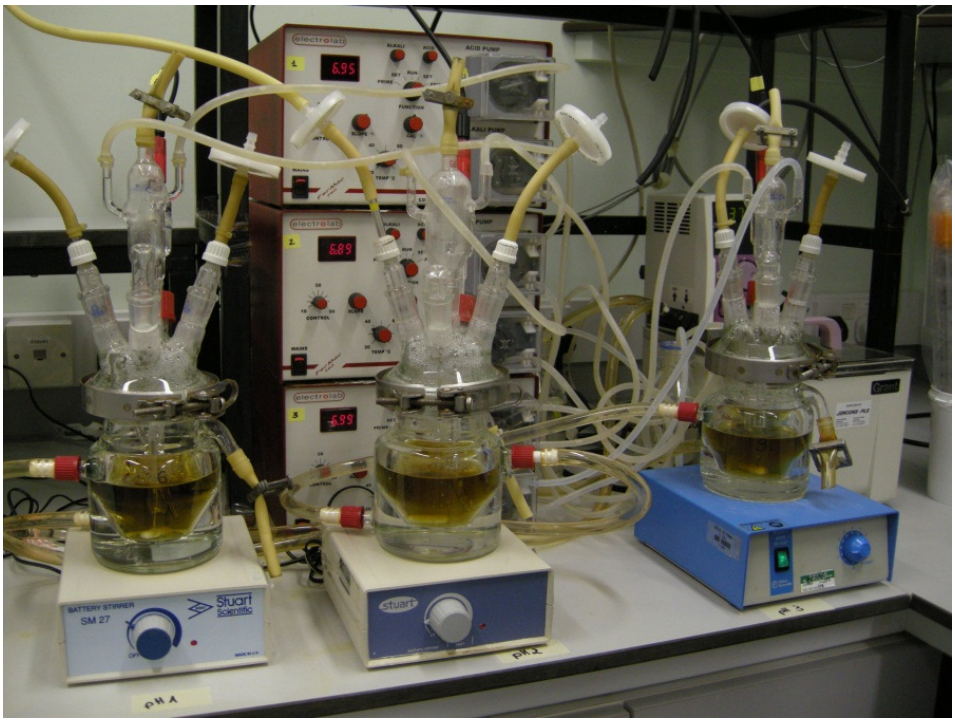


Figure 2.1. Batch fermenter vessels

3 Chapter 3 – Bacterial interactions of *S.* Typhimurium and gut bacteria

3.1 Abstract

The impact of selected GI bacteria obtained from culture collections on the survival of *S.* Typhimurium was initially evaluated in a two-compartment culture system separated by a 0.45 µm pore membrane and in stirred, pH-controlled, oxygen-free batch cultures mimicking the human colon environment. Subsequently, interactions between *S*. Typhimurium and total human faecal bacteria were quantified in both batch and continuous culture systems simulating the human colon.

Growth inhibition was detected in the two-compartment culture system inoculated with *S.* Typhimurium and GI bacteria. The exponential growth of *S.* Typhimurium was halted when the population of *E. coli* inoculated in the second compartment reached the maximum population density. On the other hand, the growth of some GI bacteria such as *L. gasseri* and *B. bifidum* was inhibited when paired with *S*. Typhimurium that was inoculated in the other compartment. The survival of *S.* Typhimurium was severely affected by the presence of fresh human faecal samples in a batch culture system in a single compartment under conditions simulating the colon environment; a reduction of ca. 10^3-10^4 cfu/ml in the concentration of *S.* Typhimurium was observed in these mixed cultures. However, under the same culturing conditions, no effect on *S.* Typhimurium survival was observed in defined mixed cultures that included seven bacteria of GI origin. The growth of *S*. Typhimurium was also affected when inoculated with human faecal bacterial samples in a three-stage continuous culture model of the human colon. Under these conditions, faecal bacteria inhibited the growth of *S*. Typhimurium, which was then 'washed out' of the system.

The experiments described in this chapter illustrate various ways of interaction between gastrointestinal commensal bacteria and pathogens as well as variation in the bacterial behaviour depending on the bacterial origin, i.e. culture collection bacteria or fresh faecal bacterial samples.

3.2 Introduction

The human GIT is colonised by a vast community of symbionts and commensals that have important effects on the immune function, nutrient processing and a broad range of other host activities [207]. The oral route links the digestive tract to the outside environment and food intake involves the entry of exogenous microbes and supports growth and survival of gut microbiota [109]. Taxonomic diversity at the species and strain level is remarkably extensive. More than 7000 unique strains or species units in the human gut have been estimated in metagenomic studies of colonic human microbiota [75]. Nevertheless, the Firmicutes and Bacteroidetes represent the predominant phyla making up 90-99% of total intestinal microbiota in both humans and mice [208].

An important function of the microbiota is to provide an efficient barrier against exogenous pathogens and indigenous pathobionts that could overgrow as the result of a disrupted microbial community [90]. Commensal microbiota suppresses the proliferation and/or attenuates the virulence of pathogens through different mechanisms such as production of bacteriocins, SCFA and other metabolites that modify the bacterial composition and levels of residual oxygen in the gut content [162]. Pathogens, in turn, have developed systems to circumvent luminal microbiota-mediated host defense. The disruption of the commensal-mediated resistance to colonisation by mucosal inflammation has been used by *S.* Typhimurium to cause infection [121]. Avirulent *S.* Typhimurium, which does not induce inflammation because it lacks both T3SS, was outgrown by the microbiota within four days post infection. By contrast, wild type *S.* Typhimurium induced gut inflammation which altered the microbiota composition and allowed the pathogen to outgrow the commensals [121]. It has also been reported that neutrophils which infiltrate the inflamed mucosa generate reactive oxygen species that oxidise thiosulphate, a sulphur compound produced by the epithelium, resulting in the production of tetrathionate, which is an alternative electron acceptor. The production of this respiratory electron acceptor produced by the host facilitates the growth of *S.* Typhimurium by anaerobic respiration, providing the pathogen a growth advantage over the fermenting gut bacteria [209].

There are reports that demonstrate that *S.* Typhimurium is effectively outcompeted by gut bacteria in healthy mice [122]. The work described in this chapter is based on the hypothesis that, similarly, human gut bacteria could have an effect on the population dynamics of *S.* Typhimurium. Culture systems with two compartments

separated by a membrane as well as mixed cultures in batch and continuous systems were used here to investigate the interactions between *S*. Typhimurium and human faecal bacteria.

Batch and three-stage continuous culture systems have been developed to simulate the colon conditions and provide intestinal components such as gut microbiota, their substrates and metabolic products, which represent the ideal platform for experiments of this nature [210]. These experimental models are frequently used for studies involving faecal bacteria [203]. Batch fermentations present a simple preliminary *in vitro* model system for observing interaction between bacteria from the human large intestine [211]. These fermentation systems have also been used for screening of the *in vitro* utilisation of certain carbohydrates by the human gut microbiota [212], measuring the symbiotic effects on the human faecal composition [213] and assessing the effect of prebiotics in the survival of pathogens such as *Salmonella* spp [214]. Although some studies have used single-stage continuous cultures, there are differences between proximal and distal sections of the colon, both in terms of the environmental conditions and substrate availability, which cannot be mimicked with a single-vessel system [215]. Multistage continuous culture systems, on the other hand, provide a more complex environment as they allow for spatial and temporal heterogeneity microflora [216]. The three-stage continuous culture system represents the conditions of the ascending, transverse and descending sections of the human colon; it was validated based on chemical and microbiological measurements on material from the intestine of human sudden death cases [203]. These culture systems mimicking the colon conditions are often used in studies involving intestinal pathogens such as *Campylobacter jejuni* and *Salmonella enterica* [210, 217].

In this and following chapters, the terms “growth inhibition” or “loss of culturability” are preferably used to “killing” or “inactivation”. It was observed that *S.* Typhimurium cells lost the ability to form colonies on agar but it could not be proved that they were effectively dead in mixed cultures with faecal bacteria. In the conditions of the experiments that were carried out, non-culturable cells never recovered their ability to form colonies on agar. Whether viable but non-culturable bacteria are dormant or undergoing cell death is controversial [218].

3.3 Objectives

- To quantify the response of *S.* Typhimurium in competition with bacteria of GI origin obtained from culture collections in the following experimental

conditions: (I) Co-cultures of *S.* Typhimurium with each of the selected bacteria in exponential growth and in stationary phase using a two-compartment culture system; (II) Mixed cultures of *S.* Typhimurium and a cocktail containing all the selected GI bacteria in batch cultures with one unique compartment incubated at the temperature, pH and anoxic conditions characteristic of the human colon.

- To quantify the response of *S.* Typhimurium in mixed cultures with fresh faecal samples from different human donors: (III) in the batch system described in (II) and (IV) by using a three-stage continuous fermentation experimental colon model.

3.4 Methods

3.4.1 Bacterial strains, growth media and preparation of inocula

Intestinal and *Salmonella* strains used were kept and activated as previously described in section 2.1.2. Batch fermentation media and colon model media preparation are described in 2.2.2 and 2.2.3.

The bacterial cocktail including all strains was prepared by mixing 1ml of an early stationary phase culture of each of the selected GI bacteria with concentrations ranging between 10^8-10^9 cfu/ml. Faecal samples were obtained from 4 healthy donors, two females and two males; they were taken immediately prior to experiments and prepared as described in section 2.1.3.

Batch fermentation media was used for maintaining and subculturing strains, for experiments in the two-compartment culture system and for experiments mimicking the colon conditions in batch cultures. Colon model media was used in the continuous culture system colon model. When required, bacterial and faecal inocula were diluted in batch fermentation or colon model media according to the experiment.

3.4.2 Competition studies of *S.* Typhimurium and GI bacteria obtained from culture collections

3.4.2.1 Co-culture of *S.* Typhimurium and individual bacteria of GI tract origin using a two-compartment culture system

S. Typhimurium and each of the selected GI bacteria were co-cultured using a six-well Transwell system (24 mm Transwell® (#3412), Corning®, NY, USA). As shown below (Fig. 3.1) each well consists of an upper chamber or "*insert"* and a lower chamber or "*well"* separated by a polycarbonate membrane containing randomly

distributed 0.45 µm-pores. The membrane allows diffusion of the medium soluble components but prevents bacterial cells from changing compartment.

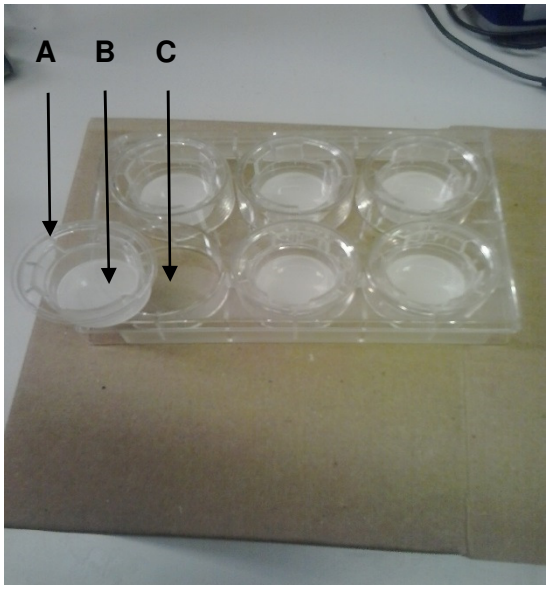


Figure 3.1. The six-well Transwell system. (A) is the upper chamber or "insert" that has been taken out, (B) is the polycarbonate membrane with 0.45 µm-pores that separates the two compartments and (C) is the lower chamber or "well".

To investigate possible interactions during exponential growth phase, bacterial co-cultures were prepared by dispensing into the upper chamber 1.5 ml of a *S.* Typhimurium inoculum diluted in batch fermentation media to a final concentration of ca. 10^2 cfu/ml. The lower chamber was filled with 2.6 ml of the inoculum of the selected GI bacteria diluted in batch fermentation media to a final concentration of ca. 10^4-10^5 cfu/ml. Volumes were chosen following the manufacturer's guidelines.

To study the response of *S*. Typhimurium in co-culture with GI bacteria in stationary phase, 4.1 ml of a stationary culture of the selected GI bacteria with ca.10^8-10^9 cfu/ml was dispensed into the lower chamber. This volume is the total recommended volume by the manufacturer for both chambers. The upper chamber was rapidly filled with bacteria-free medium that diffused through the membrane from the lower chamber. An inoculum of 10 µl of *S*. Typhimurium was added to the medium in the upper chamber to give a final concentration of ca.10^5 cfu/ml. Control cultures for *S*. Typhimurium were prepared by filling the lower chamber with growth medium and inoculating *S*. Typhimurium in the upper chamber after diffusion.

The pH of the co-culture was regularly monitored by using pH indicator papers (Whatman^{R}) and adjusted to ca. 7 when required.

The Transwell plates were incubated in oxygen-free conditions at 37°C. To prevent the evaporation of the culture medium, plates were kept in sealed boxes with sterile water in the base. Experiments were carried out twice with independently prepared inocula.

3.4.2.2 Mixed cultures of *S.* Typhimurium and a cocktail containing a defined mix of gut bacteria in a one-compartment system (batch model) mimicking the colon conditions

The batch model system was prepared as described in section 2.2.4. The vessels were inoculated with 30 ml of the bacterial cocktail and/or *S.* Typhimurium which were appropriately diluted to reach final concentrations ranging between 10^4 and 10^5 cfu/ml. Control cultures were prepared by inoculating alone either the GI bacterial cocktail or *S.* Typhimurium. Vessels were incubated at 37°C for 72 h. To enumerate bacteria, samples were plated out every two or four hours during the first day and once a day thereafter. Experiments were carried out twice with independently prepared inocula.

3.4.3 Competition studies of *S.* Typhimurium and fresh human faecal bacterial samples

3.4.3.1 Mixed cultures of *S.* Typhimurium and human faecal samples in a batch model system mimicking the colon conditions

The fermenter vessels were arranged as explained in 2.2.4 and inoculated with the faecal slurry, prepared by homogenising 10% (w/v) freshly voided faecal material in 0.1 M phosphate-buffered saline, and *S.* Typhimurium inoculum appropriately diluted to reach final concentrations ranging between 10^4 and 10^5 cfu/ml. Control cultures were prepared by inoculating alone either the faecal sample or *S.* Typhimurium. Experiments were carried out three times with independently prepared inocula and faecal material from different donors.

3.4.3.2 Mixed cultures of *S.* Typhimurium and human faecal samples in a continuous model system mimicking the colon conditions

The continuous culture system consisted of three linked vessels, V1, V2 and V3 representing the ascending, transverse and the descending sections of the colon with operating volumes of 0.28, 0.3 and 0.3 litres and pH values of 5.8, 6.2 and 6.8, respectively. Colon model media was continuously sparged with oxygen-free N_2 and fed by peristaltic pump to V1. Effluent from V1 flowed into V2, which in turn flowed

into V3 via a series of weirs. Effluent from V3 was collected in a waste container (Fig. 3.2).

Figure 3.2. The colon model system. The model consists of three vessels: Vessel 1 (V1), vessel 2 (V2) and vessel 3 (V3); Each vessels is pH-controlled (P), maintained at 37℃ by a circulating water bath (W) and kept oxygen-free via a continuous N_2 purge (N). Media (A) is fed into vessel 1 and waste is collected at the end of the system (B).

The medium container and each vessel were magnetically stirred. The pH value was maintained at the desired levels using automated pH controllers as above. Oxygen-free nitrogen was continuously sparged into the vessels to maintain anoxic conditions and the temperature was held at 37℃ by a circulating water bath [219]. The system was operated at a flow rate of 26 ml/h, which resulted in a total retention time (R) of 33.8 h estimated as the flow rate divided by the total volume in the system, 880 ml.

Each vessel was half filled with anaerobic fermentation medium. The fermentation system was equilibrated overnight before the medium pump was activated. The remaining volume of each vessel was made up with a freshly prepared faecal inoculum. The continuous faecal culture was stabilised for eight days prior to the addition in V1 of the inoculum of *S.* Typhimurium. The initial concentration of *S.* Typhimurium in the vessels was in average 3.8 x 10^6 cfu/ml. A control continuous faecal culture without *S.* Typhimurium was also monitored. Samples were plated out once a day for one week.

3.4.4 Plate counts

Samples from the two-compartment culture system were plated on Brain, heart infusion (BHI) agar (Oxoid, UK) and incubated aerobically at 37°C for 24 h for all bacteria, except for *B. bifidum* and *L. gasseri* which were plated on De Man, Rogosa, Sharpe (MRS) agar (Oxoid, UK) and *B. fragilis* and *B. ovatus* which were plated on Wilkins-Chalgren (WK) agar (Oxoid, UK) and incubated in anoxic conditions at 37°C for 48 h. Samples from the mixed cultures in batch and continuous systems were plated as described on Table 2.1.

3.4.5 Modeling of population dynamics and statistical analysis

In the experiments carried out in this work, the number of cfu/ml have been quantified over time while replication or death of the bacterial cells within the population has not been directly assessed. For that reason, suitable models have been fitted in order to estimate a "net specific rate". When positive, this rate is referred to as "net specific growth rate" while "net specific decay rate" is the term used when this rate is negative.

The model of Baranyi and Roberts [220] was fitted to the generated bacterial curves showing increasing concentration over time. The net specific growth rate, μ, and the maximum reached bacterial concentration, C_{max}, were the parameters used to investigate growth kinetics during exponential growth phase.

Population decay curves were fitted assuming a linear relationship between the natural logarithm of the bacterial concentration and time, with δ net specific decay rate.

Curves showing growth without lag phase followed by decay were fitted with a biphasic model:

$$\frac{d\ln C}{dt} = \mu \qquad \text{if } C < C_{\max}$$
$$\frac{d\ln C}{dt} = \delta \qquad \text{if } C \geq C_{\max} \tag{1}$$

Where *t* is the time, *C* the bacterial concentration (cfu/ml) and C_{max} (cfu/ml) is the maximum concentration reached at the end of exponential phase.

Using the explicit solution of equation (1), the model coefficients were estimated by non-linear regression using the NLIN procedure of the software package SAS version 9.3.

An *F* tests [221] was used to compare the net specific growth rate, the logarithm of the maximum concentration and the net specific decay rate of the populations in mixed and pure cultures.
The F statistics was estimated as:

$$F = \frac{(SS_1 - SS_2)/(df_1 - df_2)}{SS_2 / df_2} \tag{2}$$

Where SS_1 is the residual sum of squares for a model with a unique fitted value for the parameter in comparison, i.e. the maximum specific growth rate, the logarithm of the maximum concentration and the specific decay rate, for both populations in mixed and pure culture; SS_2 is the residual sum of squares for a model with a fitted value of that parameter for each population; df_1 and df_2 are the degrees of freedom associated to SS_1 and SS_2, respectively. Differences were considered significant if the *p* value associated to the *F* statistic was smaller than 0.05.

3.5 Results

3.5.1 Competition studies of *S.* Typhimurium and GI bacteria obtained from culture collections

3.5.1.1 Co-cultures of *S.* Typhimurium and each GI bacterial species in a two-compartment system

Interactions during the exponential growth of *S.* Typhimurium and GI bacteria were investigated by inoculating relatively low concentrations of each of the GI bacteria, 10^4 - 10^5 cfu/ml, and *S.* Typhimurium, ca. 10^2 cfu/ml, in a two-compartment system so that exponential growth could be observed in both populations. This advantage in inoculum size aimed to compensate the poor growth performance of most of the selected GI bacteria when compared with *S.* Typhimurium.

The net specific growth rate of *S.* Typhimurium was not affected in co-culture with any of the selected GI bacteria (Table 3.1 and Appendix 1, Fig. A.1.1).

	Pure cultures				Growth parameters of GI bacteria in co-culture with *S.* Typhimurium				Growth parameters of *S.* Typhimurium in co-culture with GI bacteria			
Bacteria	μ	*se*[a]	$\log_{10}C_{max}$	*se*	μ	*se*	$\log_{10}C_{max}$	*se*	μ	*se*	$\log_{10}C_{max}$	*se*
E. coli	1.21	0.129	9.44	0.167	1.88	0.293	9.32	0.213	1.76	0.191	6.27*	0.219
E. faecalis	1.05	0.124	9.45	0.734	1.13	0.630	8.37	0.299	2.19	0.159	7.93	0.186
C. perfringens	1.64	0.102	8.54	0.129	1.87	0.506	7.80	0.284	2.10	0.185	7.51	0.150
L. gasseri	1.30	0.344	8.17	0.495	0.250*	0.120	5.84*	0.174	1.99	0.0986	9.61	0.210
B. bifidum	0.882	0.344	7.32	0.203	0.636	0.133	5.86*	0.270	1.93	0.106	8.48	0.210
B. fragilis	0.414	0.0689	7.54	0.211	0.384	0.0595	7.37	0.390	2.02	0.0732	8.01	0.0768
B. ovatus	0.944	0.131	8.42	0.194	0.645	0.113	7.16	0.175	2.19	0.112	8.21	0.131
S. Typhimurium[b]	1.72	0.125	8.57	0.444								

[a] Standard error of the fitted parameter
[b] Results from four independent curves
* Underlined if significantly different from parameter in pure culture ($p < 0.05$)

Table 3.1. Growth parameters of *S.* Typhimurium and GI bacteria in the two-compartment system in exponential phase. Net specific growth rate, μ (h^{-1}), and maximum concentration, C_{max} (cfu/ml), of *S.* Typhimurium and of culture collection bacterial strains of GI origin in exponential growth in a two-compartment system separated by a 0.45 μm pore membrane and in pure cultures.

However, the maximum concentration reached by *S.* Typhimurium was significantly affected ($p < 0.05$) in co-culture with *E. coli*. The maximum concentration reached by *S.* Typhimurium was 3.7 x 10^8 cfu/ml in pure cultures, whereas it was 1.8 x 10^6 cfu/ml in co-cultures with *E. coli*, because the growth of *S.* Typhimurium was halted when *E. coli* entered stationary phase (Table 3.1, Fig. 3.3 and Appendix 1, Fig. A.1.2).

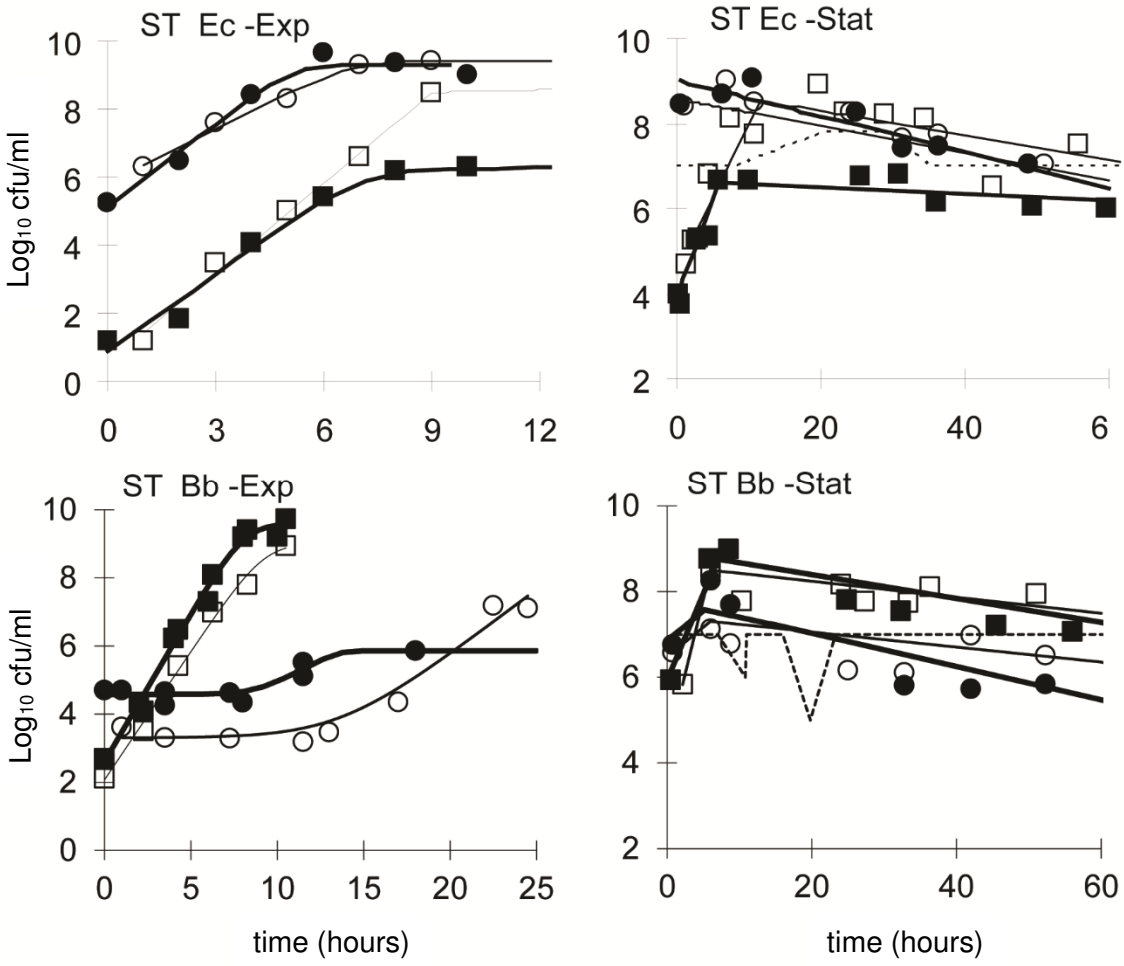


Figure 3.3. Concentration of *S.* Typhimurium, *E. coli* and *B. bifidum* in the two-compartment system. Population response of *S.* Typhimurium (square), *E. coli* (circle, upper panels) and *B. bifidum* (circle, lower panels) in exponential phase (left panels) and stationary phase (right panels) in a two-compartment system separated by a 0.45 µm pore membrane. Filled symbols and thick lines represent measurements from co-cultures and fitted models, respectively. Empty symbols and thin lines show measurements from pure cultures and fitted models, respectively. Discontinuous line represents the pH.

The final concentration of *S.* Typhimurium was also affected in co-culture with *E. faecalis* and *C. perfringens.* The growth of *E. faecalis* and *C. perfringens* was slower than that of *S*. Typhimurium but due to a higher initial inoculum they also entered stationary phase before *S*. Typhimurium and inhibited its growth. However, when this happened, the maximum concentration of *S*. Typhimurium was already close to that observed in pure cultures (Table 3.1, Fig. 3.4 and Appendix 1, Fig. A.1.2).

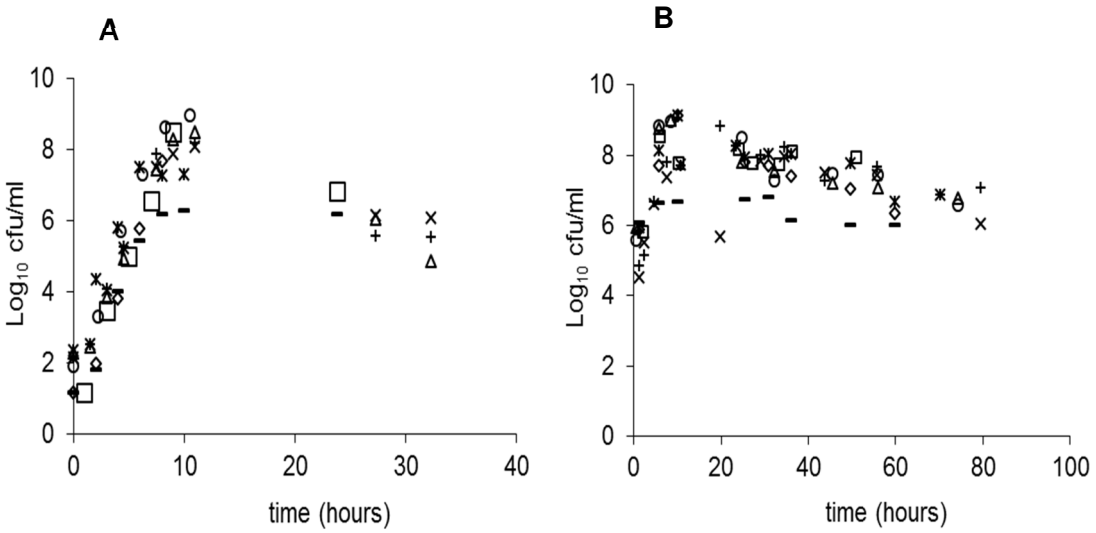


Figure 3.4. Concentration of *S.* Typhimurium in co-cultures with GI bacteria in the two-compartment system. Concentration of *S*. Typhimurium during exponential (A) and stationary (B) phase in co-cultures with *E. coli* (**-**), *E. faecalis* (◊), *C. perfringens* (✳), *B. fragilis* (x), *B. ovatus* (+), *B. bifidum* (△), *L. gasseri* (○) and in pure culture (□).

On the other hand, inhibition of the growth of some GI bacteria was also observed in co-cultures with *S.* Typhimurium. The growth of *B. bifidum* was inhibited when *S*. Typhimurium entered stationary phase, therefore, the maximum concentration reached by *B. bifidum* was significantly reduced (Table 3.1, Fig. 3.3 and Appendix 1, Fig. A.1.2); *B. bifidum* reached 2 x 10^7 cfu/ml when inoculated alone but only 7.2 x 10^5 cfu/ml when *S*. Typhimurium was in the other compartment. In co-culture with *S*. Typhimurium, *L. gasseri* exhibited a significantly slower net specific growth rate, 0.25 h^{-1} ($p < 0.05$), and lower maximum population density, 6.9 x 10^5 cfu/ml ($p < 0.05$), than in pure cultures, where these growth parameters had values equal to 1.30 h^{-1} and 1.5 x 10^8 cfu/ml, respectively (Table 3.1 and Appendix 1, Fig. A.1.1 and Fig. A.1.2).

Growth and survival of *S*. Typhimurium was also monitored in co-cultures with each of the selected GI bacteria in stationary phase in the two-compartment system (Fig.3.4). Stationary phase cultures of GI bacteria at their maximum population density were added into one compartment of the system. Approximately 10^5 cfu/ml of *S*. Typhimurium were added to the other compartment, which was previously filled with diffused cell-free supernatant from the stationary phase GI bacteria culture. Initially, *S*. Typhimurium grew exponentially to reach stationary phase. The maximum concentration reached by *S.* Typhimurium during this initial exponential growth was significantly smaller in co-culture with *E. coli*, 1.1 x 10^6 cfu/ml ($p < 0.05$), than in pure culture, 3.6 x 10^8 cfu/ml, or with the other selected GI bacteria, which did not affect *S.* Typhimurium (Table 3.2, Fig. 3.4 and Appendix 1, Fig. A.1.3). A slow decay of the population was observed in stationary phase in all cases (Fig. 3.4). None of the selected GI bacteria had any effect on the decay rate of *S.* Typhimurium (Fig.3.4) and similarly *S.* Typhimurium did not affect the decay rate of any of the GI bacteria in stationary phase (Table 3.2 and Appendix 1, Fig. A.1.4). This indicates that the survival of *S.* Typhimurium during stationary phase in one compartment was not affected by the presence of any of the GI populations in the other compartment and *vice versa*. The decay of *L. gasseri* was significantly ($p < 0.05$) faster in pure culture than in co-culture with *S.* Typhimurium (Table 3.2 and Appendix 1, Fig. A.1.4); this could be attributable to the combination of difficulties in manually maintaining the pH value in the two-compartment system which could have failed while culturing *L. gasseri* alone and the low resistance of *L. gasseri* to acid stress [222].

	Pure cultures		Parameters of stationary phase GI bacteria in co-culture with *S.* Typhimurium		Parameters of *S.* Typhimurium in co-culture with stationary phase GI bacteria			
Bacteria	δ	*se*[a]	δ	*se*	$\log_{10}C_{max}$	*se*	δ	*se*
E. coli	-0.0728	0.0120	-0.0974	0.0144	6.06*	0.314	-0.0480	0.0232
E. faecalis	-0.0533	0.0103	-0.0515	0.00778	8.93	0.186	-0.0955	0.0101
C. perfringens	-0.0284	0.0124	-0.0145	0.00856	8.96	0.250	-0.0935	0.0192
L. gasseri	-0.292	0.0293	-0.0385*	0.0170	8.86	0.228	-0.0769	0.0140
B. bifidum	-0.0403	0.0218	-0.0716	0.0261	8.87	0.204	-0.0739	0.0111
B. fragilis	-0.0231	0.0106	0.00348	0.0637	8.30	0.149	-0.0727	0.0128
B. ovatus	-0.0524	0.0210	-0.0376	0.00779	8.46	0.180	-0.0624	0.0128
S. Typhimurium in pure culture[b]					8.56	0.137	-0.0699	0.0108

[a] Standard error of the fitted parameter
[b] Results from 4 independent curves
* Underlined if significantly different from parameter in pure culture ($p < 0.05$)

Table 3.2. Growth parameters of *S.* Typhimurium and GI bacteria in the two-compartment system in stationary phase. Maximum concentration, C_{max} (cfu/ml), and net specific decay rate, δ (h^{-1}), of *S.* Typhimurium in co-culture, in a two-compartment system separated by a 0.45 µm pore membrane, with stationary phase cultures of collection bacterial strains of GI origin and in pure cultures.

3.5.1.2 Mixed cultures of *S.* Typhimurium and a cocktail containing a defined mix of GI bacteria in a batch model mimicking the colon conditions

S. Typhimurium and a cocktail containing all the selected GI bacteria were inoculated in a stirred, pH-controlled, oxygen-free batch culture at initial concentrations ranging between10^4 and 10^5 cfu/ml. All bacterial groups exhibited an initial growth phase followed by survival in stationary phase (Fig. 3.5).

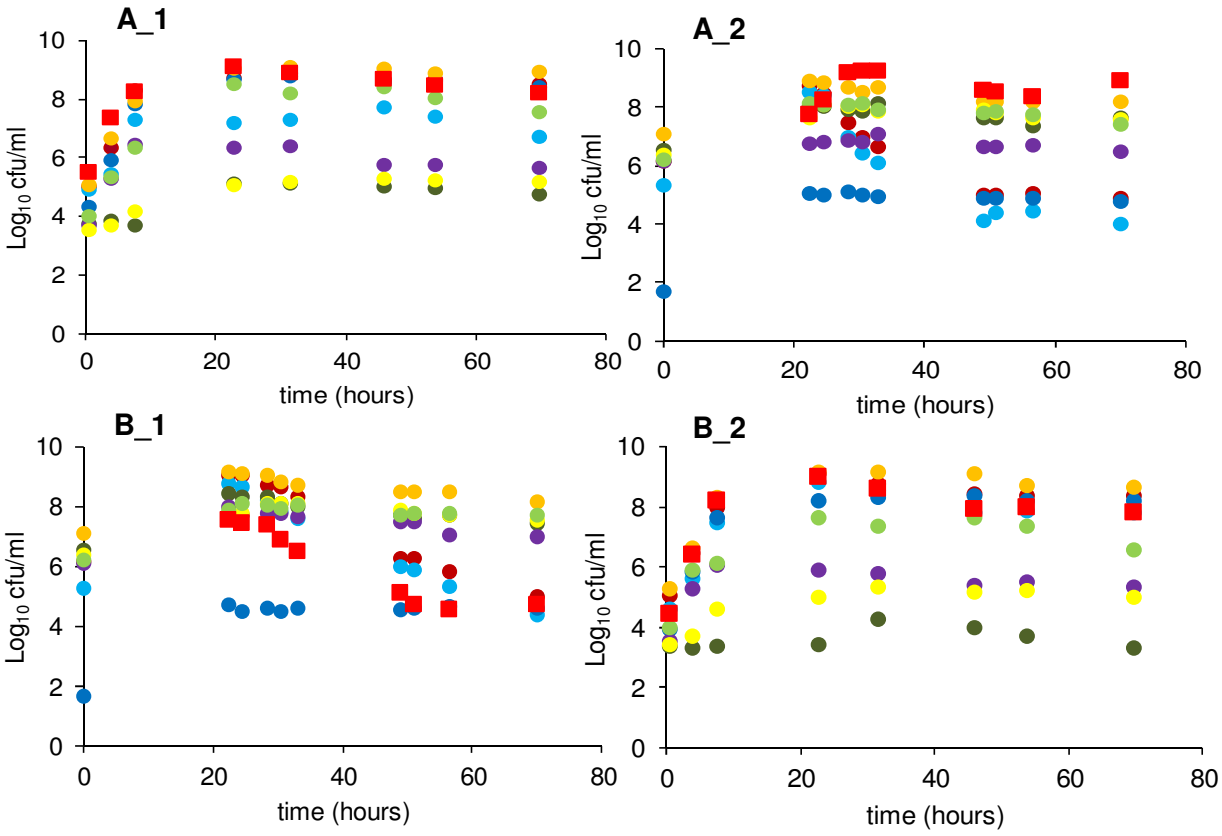


Figure 3.5. Population kinetics of *S.* Typhimurium and GI bacterial groups in mixed and separated cultures. GI bacteria from culture collections are represented on the top panels (A) whereas faecal bacteria is in the bottom panels (B). Mixed cultures of *S.* Typhimurium and GI bacteria are represented in the left panels (1) whereas separated cultures of *S.* Typhimurium and GI bacteria are represented on the right panels (2). *S.* Typhimurium (■), total anaerobes (●), total aerobes (●), *Enterobacteriaceae* (●), *Clostridium* spp (●), *Bacteroides* spp (●), *Bifidobacterium* spp (●), *Lactobacillus* spp (●), Gram + cocci (●).

During the initial exponential growth, the concentration of *S*. Typhimurium reached values of 1.5 x 10^9 and 6.4 x 10^8 cfu/ml in pure and mixed culture with the GI bacterial cocktail, respectively (Fig. 3.5). These concentrations remained fairly constant during the experimental period in both pure and mixed cultures.

Regarding the GI bacterial groups, both in the presence or absence of *S.* Typhimurium, Gram positive cocci, *Enterobacteriaceae* and *Bifidobacterium* spp. were the dominant bacteria reaching concentrations of ca. 10^8-10^9 cfu/ml while *Bacteroides* spp., *Lactobacillus* spp and *Clostridium* spp. reached concentrations lower than 10^6 cfu/ml. These concentrations remained practically unchanged over the experimental period (Table 3.3, Fig. 3.5 and Appendix 1, Fig. A.1.5). Thus, no differences were observed between the population response of *S.* Typhimurium when inoculated in a mixed culture with a cocktail containing all the selected GI bacteria and when inoculated alone (Table 3.3, Fig. 3.5 and Appendix 1, Fig. A.1.5 and Fig. A.1.6). Neither the maximum concentration reached by the microbial groups included in the GI bacterial cocktail nor their decay rates during stationary phase were affected by *S.* Typhimurium.

	S. Typhimurium and GI bacteria cocktail inoculated together in mixed culture				*S.* Typhimurium and GI bacteria cocktail inoculated in separated cultures			
Bacteria	$\log_{10}C_{max}$	se	δ	se	$\log_{10}C_{max}$	se	δ	se
Aerobes	8.94	0.123	-0.0358	0.0103	8.82	0.0599	-0.0135	0.00501
Enterobacteriaceae	8.69	0.18	-0.0535	0.0151	7.74	0.434	-0.0391	0.0325
Gram positive cocci	8.87	0.0718	-0.0226	0.00538	8.77	0.0641	-0.016	0.00537
Anaerobes	9.22	0.0919	-0.0271	0.00769	9.08	0.0476	-0.00832	0.00399
Bacteroides spp	4.51	0.062	-0.0572	0.00464	5.16	0.0328	-0.0174	0.00275
Clostridium spp	5.87	0.0873	-0.0294	0.0073	6.37	0.133	-0.0408	0.0111
Lactobacillus spp	5.4	0.0666	-0.0186	0.00498	5.23	0.0831	-0.00114	0.00622
Bifidobacterium spp	7.72	0.238	-0.0431	0.0199	8.51	0.159	-0.0397	0.0133
Salmonella spp	8.81	0.16	-0.057	0.0134	9.08	0.0218	-0.0428	0.00182

Table 3.3. Population kinetic parameters of *S.* Typhimurium and GI bacterial groups (culture collection) in batch culture. Maximum concentration, C_{max} (cfu/ml), and net specific decay rate, δ (h^{-1}), of *S.* Typhimurium and of the GI bacterial groups from culture collections in mixed and separated cultures were measured.

3.5.2 Competition studies of *S.* Typhimurium and fresh human faecal bacteria samples

3.5.2.1 Mixed cultures of *S.* Typhimurium and human faecal samples in a batch system mimicking the colon conditions

Vessels containing fresh colon model media were inoculated with ca. 10^5-10^6 cfu/ml of faecal bacteria and/or a similar concentration of *S*. Typhimurium. Both faecal bacterial groups and *S*. Typhimurium initially grew in order to reach stationary phase. However, following this initial growth phase, the concentration of *S*. Typhimurium decreased sharply from 3.6 x 10^7 to 3.4 x 10^4 cfu/ml; thus, following the initial growth a reduction of 3 decimal logarithmic units in *S*. Typhimurium concentration was detected within 24 h when inoculated together with faecal bacteria (Fig. 3.5, Table 3.4 and Appendix 1, Fig. A.1.7 and Fig. A.1.8). Conversely, in pure cultures, the concentration of *S*. Typhimurium reached ca. 10^8 cfu/ml and this concentration was fairly maintained during the experimental period (Fig. 3.5, Table 3.4 and Appendix 1, Fig. A.1.7 and Fig. A.1.8).

	S. Typhimurium and faecal sample inoculated together in mixed culture				*S.* Typhimurium and faecal sample inoculated in separated cultures			
Bacteria	$\log_{10}C_{max}$	se	δ	se	$\log_{10}C_{max}$	se	δ	se
Facultative aerobes	9.2	0.125	-0.221	0.012	8.45	0.206	-0.324	0.0383
Enterobacteriaceae	8.69	0.0754	-0.22	0.00726	8.22	0.265	-0.381	0.0494
Gram positive cocci	4.58	0.0421	0.00097	0.00406	5.05	0.0233	-0.0131	0.00224
Anaerobes	9.08	0.0464	-0.0452	0.00447	8.8	0.0695	-0.0394	0.0067
Bacteroides spp	8.34	0.0565	-0.048	0.00544	8.02	0.0748	-0.0286	0.0072
Clostridium spp	7.9	0.0581	-0.0449	0.0056	6.89	0.0603	-0.018	0.00581
Lactobacillus spp	7.99	0.0833	-0.0168	0.00803	7.97	0.0825	-0.0165	0.00795
Bifidobacterium spp	8.01	0.0493	-0.0163	0.00475	8.16	0.0386	-0.0323	0.00372
Salmonella spp	7.56*	0.124	-0.242*	0.0208	9.14	0.187	-0.038	0.0193

*Significantly different from parameters in separated culture ($p < 0.05$)

Table 3.4. Population kinetic parameters of *S.* Typhimurium and GI bacterial groups (faecal samples) in batch culture. Maximum concentration, C_{max} (cfu/ml), and net specific decay rate, δ (h^{-1}), of *S.* Typhimurium and of the bacterial groups from a faecal sample in mixed and separated cultures were measured.

Donors were free of *Salmonella* spp. which was not detected when only faecal bacteria were inoculated. The magnitude of the difference in the logarithm of the concentration of *S.* Typhimurium, when inoculated alone and in co-culture with faecal bacteria, is greater than 3 times the standard deviation of the observations, which has an average value of ca. 0.5 Log_{10} cfu/ml, and then further statistical assessment was not necessary (Fig. 3.6).

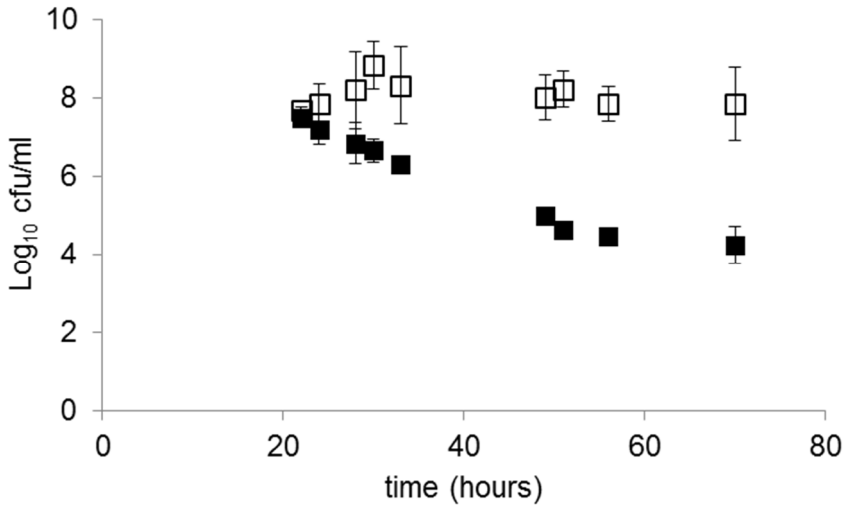


Figure 3.6. *S*. Typhimurium in mixed culture with fresh human faecal samples and in pure culture. Mixed cultures (■); pure culture (□). Values are means of triplicates ± SD.

S. Typhimurium did not have any effect on the population dynamics of any of the bacterial groups of faecal origin when inoculated together (Fig. 3.5, Table 3.4 and Appendix 1, Fig. A.1.7 and Fig. A.1.8).

Fig. 3.7 shows the population kinetics of the main faecal bacterial groups in cultures inoculated with faecal bacteria only and in mixed cultures of *S*. Typhimurium and faecal bacteria. These are the results of 3 independent experiments inoculated with samples from different faecal donors. The standard deviation ranged from 0.002 to 2, with an average of 0.5.

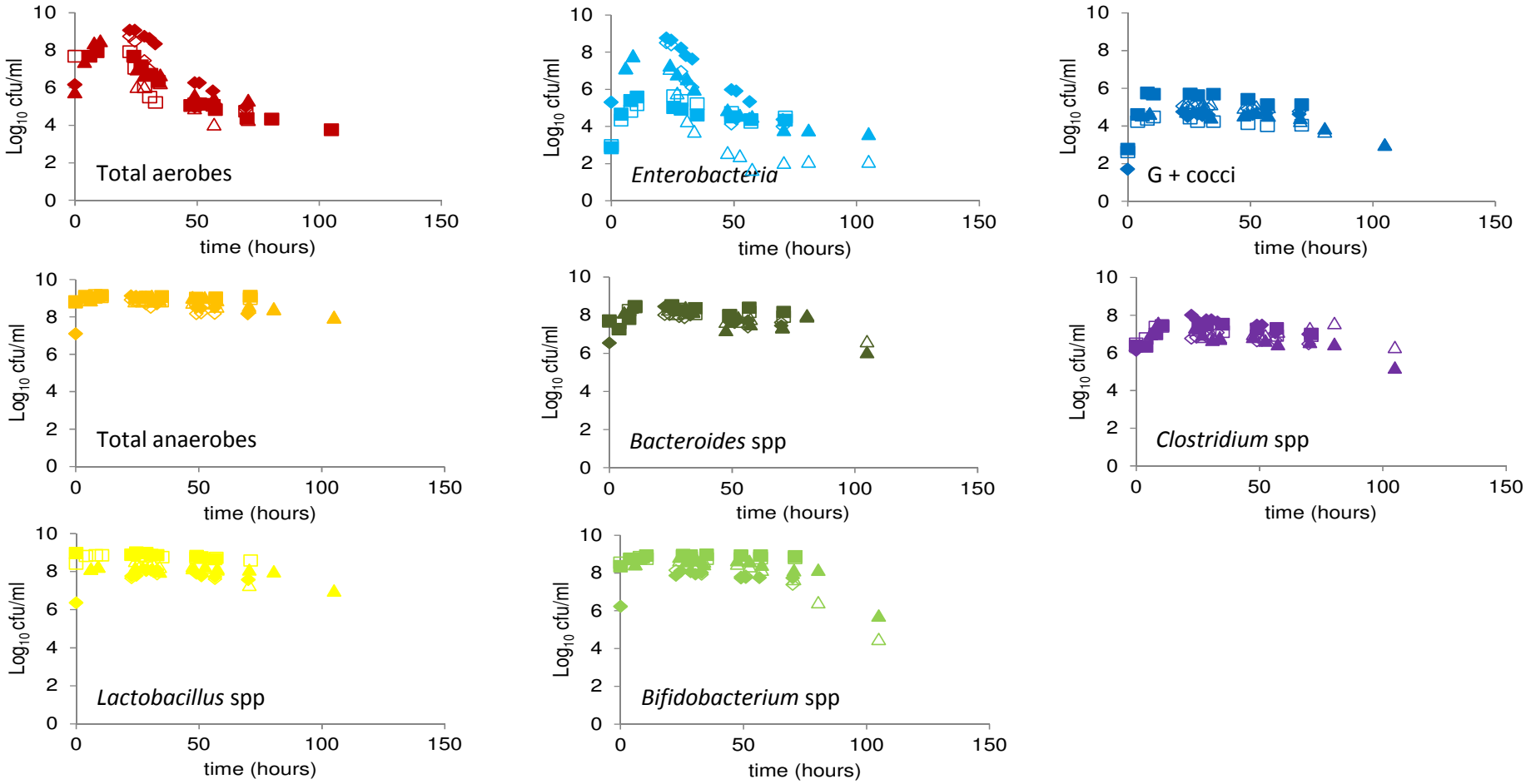


Figure 3.7. Gut bacterial groups in batch cultures. The different symbols correspond to different replicas. Filled symbols represent the bacterial groups in mixed cultures of *S.* Typhimurium and faecal samples. Empty symbols represent the control without *S.* Typhimurium.

The dominant bacterial groups in the faecal culture were *Bacteroides* spp, *Clostridium* spp., *Lactobacillus* spp and *Bifidobacterium* spp. with concentrations of about 10^8 cfu/ml which were kept fairly constant during the observation time (Fig. 3.5, Table 3.4 and Appendix 1, Fig. A.1.8). Gram positive cocci did not exceed 10^5 cfu/ml while *Enterobacteriaceae* reached a concentration greater than 10^8 cfu/ml. However, the growth of *Enterobacteriaceae,* as well as the growth of the total facultative aerobes, was followed by an inactivation phase comparable to that of *S.* Typhimurium. The concentration of the total facultative aerobes decreased from 1.6 x 10^9 to 9.7 x 10^4 cfu/ml and from 2.8 x 10^8 to 7.2 x 10^4 cfu/ml within 24 h in cultures with and without *S*. Typhimurium, respectively (Fig. 3.5, Table 3.4 and Appendix 1, Fig. A.1.8). Similarly *Enterobacteriaceae* numbers decreased from 4.9 x 10^8 to 2.4 x 10^4 cfu/ml and from 1.9 x 10^8 to 10^4 cfu/ml in cultures with and without *S*. Typhimurium, respectively, within 24 h after the initial growth phase (Fig. 3.5, Table 3.4 and Appendix 1, Fig. A.1.8).

3.5.2.2 Mixed cultures of *S.* Typhimurium and human faecal bacteria in a continuous model system mimicking the colon conditions

An *in vitro* three-stage continuous culture system was used to simulate the composition and dynamics of the ascending, transverse and descending sections of the human colon. Two parallel culture systems were inoculated with faecal bacteria and stabilised for eight days prior to the inoculation of *S.* Typhimurium in one of them.

The addition of *S.* Typhimurium did not affect the concentration of any of the faecal bacterial groups. All faecal bacterial groups maintained constant levels over the experimental period which implies that they grew at a constant rate, equal to the flow rate in each vessel (Fig. 3.8, Table 3.5 and Appendix 1, Fig. A.1.9). The specific flow rate in each vessel, i.e. total flow per hour divided by the total vessel volume, was 0.093, 0.087 and 0.087 h^{-1} in the ascending, transverse and descending sections of the colon model, respectively.

Bacteroides spp. was the predominant genus, with counts of ca. 10^9 cfu/ml, followed by *Clostridium* spp. and *Bifidobacterium* spp. with concentrations of ca. 10^8 cfu/ml. *Enterobacteriaceae, Lactobacillus* and Gram positive cocci had lower counts, ranging between 10^6-10^7 cfu/ml. However, the population kinetics of Gram positive cocci varied between the parallel continuous cultures, in that after the stabilization period and prior to the inoculation of *S*. Typhimurium the population of Gram positive cocci did not exceed 10^4 cfu/ml in any of the three vessels of one of the systems whereas

it was greater than 10^7 cfu/ml in the three vessels of the second system (Table 3.5, Fig. 3.8 and Appendix 1, Fig. A.1.9).

	Steady state faecal culture inoculated with *S.*Typhimurium						Control steady state faecal culture					
	ascending		*transverse*		*descending*		*ascending*		*transverse*		*descending*	
	$log_{10}C$	sd	$log_{10}C$	sd	$log_{10}C$	sd	$log_{10}C$	sd	$log_{10}C$	sd	$log_{10}C$	sd
Facultative aerobes	7.63	0.0718	7.57	0.172	7.38	0.27	7.51	0.484	7.05	0.574	6.82	0.699
Enterobacteriaceae	7.19	0.233	6.78	0.489	6.66	0.306	7.26	0.456	6.83	0.577	6.53	0.643
Gram positive cocci	7.29	0.105	7.23	0.229	7.04	0.0848	4.08	0.961	3.92	0.733	3.67	0.733
Anaerobes	9.74	0.078	9.53	0.146	9.38	0.192	9.27	0.16	9.2	0.116	9.14	0.253
Bacteroides spp	9.61	0.117	9.36	0.252	9.16	0.374	8.64	0.229	8.61	0.285	8.55	0.261
Clostridium spp.	8.13	0.154	8.24	0.211	8.52	0.436	7.8	0.53	7.85	0.282	7.9	0.184
Lactobacillus spp	6.73	0.279	7.18	0.415	7.08	0.41	7.07	0.382	6.89	0.635	6.85	0.488
Bifidobacterium spp.	8.27	0.447	8.45	0.249	8.36	0.204	8.27	0.505	8.39	0.355	8.26	0.484

Table 3.5. Concentration of the gut bacterial groups in the continuous culture system. Average values and standard deviation, sd, of the concentration of bacterial groups in samples obtained during seven days from a steady state faecal culture in a continuous system with three vessels representing the ascending, transverse and the descending sections of the colon after the inoculation of *S.* Typhimurium and in a control culture not inoculated.

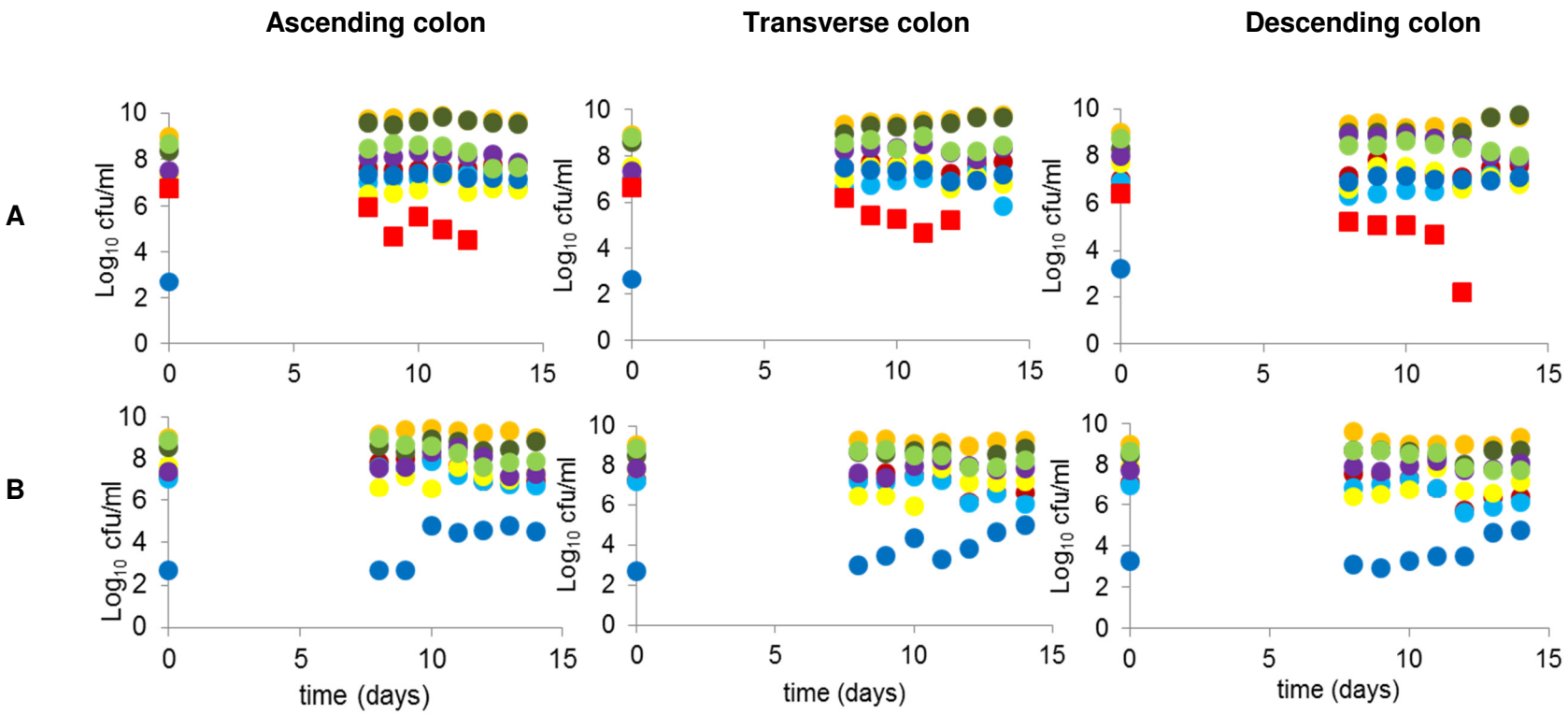


Figure 3.8. Concentration of the gut bacterial groups and *S.* Typhimurium in the continuous culture system. Bacterial concentration in a continuous faecal culture system with three vessels representing the ascending, transverse and the descending section of the colon in steady state added with *S.* Typhimurium (A) and in a control experiment (B). *S.* Typhimurium (■), total anaerobes (●), total aerobes (●), *Enterobacteriaceae* (●), *Clostridium* spp (●), *Bacteroides* spp (●), *Bifidobacterium* spp (●), *Lactobacillus* spp (●), Gram + cocci (●).

Faecal bacteria inhibited the growth of *S.* Typhimurium in this continuous culture system. The concentration of *S.* Typhimurium decreased from 3.9 x 10^6 cfu/ml to 9.5 x 10^3 cfu/ml during the entire period of observation. (Fig. 3.8, Fig. 3.9).

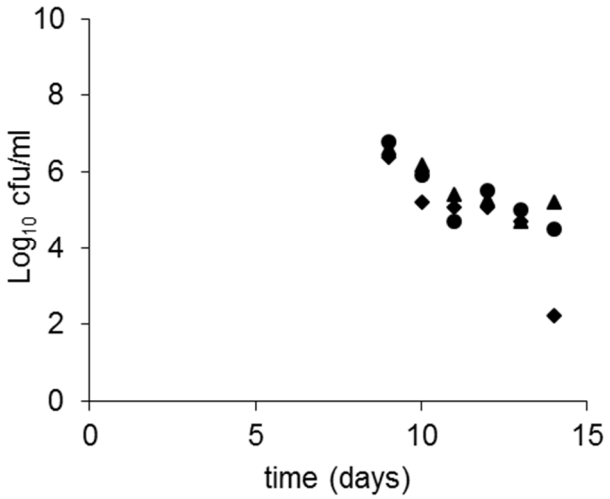


Figure 3.9. *S.* Typhimurium concentration in the continuous culture system. Symbols represent the first (●), second (▲) and third (♦) stage of the continuous system.

The exact estimates of the net specific decay rates of *S.* Typhimurium were -0.036, -0.032 and -0.062 h^{-1} in the ascending, transverse and descending sections of the colon model, respectively (Fig. 3.9). An F test indicated that these rates were significantly different from zero with *p* values equal to 0.041, 0.021 and 0.024, respectively. Thus, in the continuous steady state culture of faecal bacteria, the growth of *S.* Typhimurium was inhibited so that it grew at slower rate than required to maintain its concentration and therefore was removed from the system by the constant flow of medium (Figure 3.9). When compared with the specific flow rates of the vessels, -0.093, -0.087 and -0.087 h^{-1}, respectively, the decay rates of *S.* Typhimurium seem to be slower which indicates that the growth of *S.* Typhimurium may not be completely halted but inhibited or only part of the population is affected.

3.6 Discussion

Different ways of interaction between *S.* Typhimurium and gut bacteria were observed. These interactions were detected at high bacterial concentrations, greater than 10^8 cfu/ml in all cases and they can be summarized as follows: i) in a two-compartment system where two populations separated by a permeable membrane shared the growth medium, the exponential growth of one of the populations was

halted in some cases when the population in the other compartment reached its maximum density; ii) in batch cultures mimicking the colon conditions, the survival of *S.* Typhimurium in stationary phase was severely affected when inoculated together with fresh faecal bacterial samples but no effect was detected if inoculated with a bacteria of GI origin; iii) in a continuous colon model system simulating the transit and content of the human colon, the growth of *S.* Typhimurium was inhibited resulting in its exclusion from the system.

In a two-compartment culture, with each one of the selected GI bacteria, *S.* Typhimurium reached a maximum concentration of ca. 10^8-10^9 cfu/ml except when in co-culture with *E. coli*. Because of a higher initial concentration, *E. coli* reached its maximum population density and entered stationary phase earlier than *S.* Typhimurium, which resulted in *S.* Typhimurium going into growth arrest (Fig. 3.3). Growth inhibition of *S.* Typhimurium was not detected when inoculated at the same initial concentration as the GI bacteria in a cocktail including *E. coli*. In that case, *S.* Typhimurium was one of the bacteria reaching first the maximum population density and inhibiting other bacterial groups (Fig. 3.5). The exponential growth of *B. bifidum* also ceased prematurely in co-culture with *S.* Typhimurium, in the two-compartment system (Fig. 3.3), when the latter reached stationary phase. A phenomenon referred as the 'Jameson Effect', has been previously described as an unspecific growth suppression of multi-species bacterial populations in batch cultures by the single 'dominant' strain that reaches first the maximum concentration [223-226]. The deceleration and eventually cessation of the growth of the populations have been attributable to the competition for common limiting resources [226] and to accumulation of end metabolites [227]. In these experiments, the populations were separated by a permeable membrane and thus growth inhibition is also mediated by a modification of the soluble components of the medium.

The assumption of the Jameson effect is that the dominating microbiota inhibits the growth of the other population in the same way that they inhibit their own growth when reaching their maximum population density. Accordingly, one would expect the inhibition of the growth of *S*. Typhimurium when inoculated together with stationary phase cultures of GI bacteria at their maximum population densities. However, growth inhibition of *S.* Typhimurium is only observed in co-culture with *E. coli* while no such effect was observed in co-culture with any of the other selected GI bacteria at maximum population density. This result suggests that the observed growth inhibition in the two-compartment culture system resulted from something more specific than just the density of the populations. Reaching the maximum population

density was a necessary condition but it seemed not to be sufficient to cause growth cessation of the other population. Interestingly, it has been reported that the effect of high levels of the natural pork microbiota on the growth of *Salmonella* was temperature dependent so that below 15°C the growth of *Salmonella* ceased when the natural microbiota reached its maximum population density, whereas above 15°C, growth inhibition was not detected [228]. In addition, the observed effect of *E. coli* on the growth of *Salmonella* could be the result of a more specific inhibition mechanism mediated by toxin production [119, 229].

The survival of *S.* Typhimurium in mixed cultures with fresh human faecal samples was very different from its survival in the defined mixed cultures with selected GI bacteria (Fig. 3.5). The survival of *Salmonella* during stationary phase was not affected either in the two-compartment system with each of the selected GI bacteria or in a mixed culture including all GI bacteria in a one-compartment batch model system mimicking the human colon conditions. Remarkably, a reduction of the culturable population of *S.* Typhimurium in the order of ~10^4 cfu/ml was observed in batch mixed cultures with faecal samples from different human donors.

The inactivation curve of *S.* Typhimurium in mixed cultures with faecal bacteria has a first phase of log-linear decay of the concentration followed by a tail of survival (Fig. 3.6.). The lack of log-linearity or heterogeneous behaviour could be due to an increase of resistance of survival cells of *S.* Typhimurium because of adaptive evolution, but it could be also attributed to the faecal bacteria. For instance, behavioural differences have been associated to the modification of the interaction of competing strains due to the different community structure of the surrounding strains [230]. What this implies is that the density of the bacterial group affecting *S.* Typhimurium could be affected by a third party species and/or that a third species could be changing the 'communication' between *S.* Typhimurium and its interacting bacteria due to phenotypic alterations. Interestingly, the mechanism of inactivation observed in batch cultures with faecal bacteria was not specific for *S.* Typhimurium because the counts of facultative aerobic bacteria and *Enterobacteriaceae* also decreased at the same rate as the counts of *S.* Typhimurium (Fig. 3.5).

Differences in the population composition dynamics in the GI bacterial cocktail and the faecal sample were observed under the same culture conditions. In batch cultures initiated with the GI bacterial cocktail, the dominant bacterial groups were Gram positive cocci, *Enterobacteriaceae* and *Bifidobacterium* spp. while *Bacteroides* spp., *Lactobacillus* spp and *Clostridium* spp. were in much lower concentrations. Results

were different in batch cultures initiated with faecal samples, where *Bacteroides* spp., *Lactobacillus* spp., *Clostridium* and *Bifidobacterium* spp. were the dominant bacterial groups, whereas the *Enterobacteriaceae* group was in lower numbers and Gram positive cocci had variable results. These results are in agreement with previously reported dominant bacterial groups in batch faecal cultures [231, 232]. This disparate behaviour between the results obtained with selected GI bacteria originated from culture collections and from human faecal bacteria may be the result of the difficulty in isolating and growing *in vitro* 'fastidious' organisms of faecal origin whose nutritional requirements are not well understood and that sometimes require complex nutritional interactions with other bacteria [233]. A recovery of 70% of the microscopic count of the colonic microbiota has been reported in chicken samples [234] while only 58% of the colonic microbiota has been recovered in samples of human origin [235]. However, the results on the population dynamics of the culture initiated with a cocktail of selected bacteria may be also specific to the experimental conditions described here. Other researchers working in batch culture systems inoculated with *B. thetaiotaomicron*, *C. perfringens*, *B. longum* and various *E. coli* strains have reported different populations dynamics to that one found here [230]. In the mentioned study, *Bifidobacterium* spp. was not among the bacterial groups with the highest bacterial counts and *C. perfringens* was the dominant bacteria after ten days of incubation.

A different way of interaction affecting not the survival but the growth of *S.* Typhimurium was observed in the three stage human colonic model. All the faecal bacterial groups, including facultative aerobic bacteria and *Enterobacteriaceae*, maintained constant concentrations in this continuous culture system, which imply that they grew at a growth rate equal to the wash out rate of the system. However, *Salmonella* was eliminated from this system by the continuous flow of the medium indicating that its growth was inhibited. Production of SCFA and/or broad spectrum natural antibiotics by the colonic microbiota is the most usually described mechanism with antibacterial efficiency involved in the inhibition of colonisation of enteropathogenic bacteria [214, 217, 236]. Several serovars of *Salmonella* and strains of *E. coli* have been reported to be rapidly excluded from a chemostat system simulating the porcine colon [237]. In other study involving human faecal continuous cultures, the counts of *S.* Typhimurium diminished immediately after inoculation but were essentially maintained thereafter [238]. Other pathogenic bacteria, such as *S. aureus*, have been demonstrated to be able to compromise the colonisation resistance of the colonic microbiota by growing and successfully establishing as a dominant organism in a continuous human colonic model system [239].

The dense and host-dependent microbial community establishes an ecological mutualism with the host that confers a powerful barrier effect against pathogenic invaders, referred to as colonisation resistance [119]. The efficiency of this barrier is such that gut infection animal models have first to get the normal microbiota disrupted by antibiotics [120] for *S.* Typhimurium infection to take place [121]. The mechanisms of colonisation resistance of the gut microbiota are still poorly understood. Highly complex bacterial interactions taking place in the gut environment were found and several interactions between faecal bacteria and *S.* Typhimurium that result in compromising either growth or survival of *S.* Typhimurium were observed in these experiments. It is unclear if these effects were caused by specific species or genera and the mechanisms involved remain unknown but the understanding of mechanisms of these interactions can lead to the development of new treatments for infectious gut diseases. From the results in this chapter, the relatively rapid and drastic reduction of *S.* Typhimurium population when mixed with faecal bacteria in batch cultures is very interesting. The next two chapters are focussed on the study of that phenomenon.

4 Chapter 4 – Growth inhibition of *S.* Typhimurium in direct contact with faecal bacteria

4.1 Abstract

In the previous chapter, a reduction of the population of culturable *S.* Typhimurium in the order of approximately 10^3-10^4 cfu/ml was observed in batch anaerobic mixed cultures with faecal samples from different human donors. The aim of the work gathered in this chapter was to explore that phenomenon; it was concluded that the loss of culturability of *S.* Typhimurium was observed in mixed cultures with anaerobic faecal bacteria under conditions that allow local interaction between cells such as cell contact. Culturability was not affected either in supernatants collected at several times from faecal cultures, when separated from faecal bacteria by a membrane of 0.45 µm pore size or when in contact with inactivated faecal bacteria cells. Metabolomic analysis of the supernatant obtained from the two compartments separated by this membrane, containing *S.* Typhimurium alone and the mixed culture of faecal bacteria and *S.* Typhimurium, did not show any significant difference. Numerous tubular and vesicular structures were observed between cells of apparently different morphology in both separated and mixed cultures of faecal bacteria and *S.* Typhimurium by electron microscopy. These structures could facilitate local interactions between bacteria. Loss of culturability kinetics was characterised by a sharp reduction of several logarithmic units followed by a pronounced tail. A mathematical model was developed to describe the rate of loss of culturability as a function of the frequency of encounters between populations and the probability of inactivation after encounter. The model term $F(S \cdot F)^{1/2}$ quantifies the effect of the concentration of both populations, faecal bacteria, *F*, and *S.* Typhimurium, *S,* on the loss of culturability of *S.* Typhimurium by cell contact with faecal bacteria. When the value of $F(S \cdot F)^{1/2}$ decreased below ca. 10^{15} $(cfu/ml)^2$, the frequency of encounters sharply decreased, leading to the deceleration of the inactivation rate and to the tailing off of *S.* Typhimurium population; the probability of inactivation after encounter*, P,* was constant with an estimated value of ca.10^{-5} for all datasets. *P* might be characteristic to the mechanism of growth inhibition after cell encounter.

4.2 Introduction

Interactions between microorganisms such as symbiosis or competition shape microbial ecosystems. In the intestine, foreign bacteria and commensals have developed exclusion mechanisms against each other since both of them compete for similar ecological niches [90]. The resident microbiota control incoming pathogens by stimulating host immunity [126]; however, the commensal microbiota also prevent the proliferation of invading bacteria through direct interactions such as production of bacteriocins and SCFA and by using specific substrates required by the pathogens. Metabolites generated by the commensals may not only directly inhibit virulence genes of the pathogens but also modify the conditions, such as pH, required for virulence activity [90]. Some examples of these protective mechanisms of the gut microbiota are: The growth of EHEC is curbed by bacteriocins produced by commensal *E. coli* [240]; *Bifidobacterium* ssp. hinders pathogenic *E. coli* gut colonisation by acidifying the environment [241]; commensal *E. coli* strains served as a barrier against to EHEC infection as they compete for similar substrates [242]; the depletion of residual oxygen by commensal facultative anaerobes, such as *Enterobacteriaceae*, can have an impact on the activity of T3SS of *S. flexneri*, as the expression of this virulence factor is determined by the oxygen concentration of the environment [243]. However, secretion and detection of extracellular molecules or changing the surrounding environmental is not the only form of bacterial interaction. Recently cell-cell contact between bacteria has been demonstrated to be required for some bacterial interactions [194, 196, 244-247]. Furthermore, cell-cell contact has been demonstrated to take place between *S*. Typhimurium and gut commensals [108].

It is important to understand bacterial interactions in the gut as the interplay between commensals and pathogens is critical for controlling infection and disease. Given the literature in the field and the results obtained in the experiments presented in the previous chapter, a first step to understand the mechanisms of growth inhibition of *S*. Typhimurium in mixed cultures with faecal bacteria is to investigate if loss of culturability of *S*. Typhimurium is caused by soluble diffusible particles or by local bacterial interaction - which refers to any type of interaction requiring close proximity such as cell contact. Moreover, it is very important to investigate the interactions between enteric pathogens and the gut microbiota in conditions mimicking those *in vivo* as the vast number and range of intestinal microbes, metabolites, proteins and surfactants may interfere with bacterial responses; therefore the experiments were

carried out in pH-control, oxygen-free vessels (Fig. 2.1) using fresh faecal samples of human origin.

4.3 Objectives

- To investigate the nature of the interactions between *S.* Typhimurium and human commensal gut microbiota in batch cultures by studying *S.* Typhimurium response in the following experimental conditions:
    - 1) In mixed culture with faecal bacteria under different inoculation protocols and environment oxygenation during incubation.
    - 2) In supernatants obtained from faecal cultures at various times during incubation.
    - 3) In mixed cultures with faecal bacteria with a 0.45 µm pore filter separating both populations.
    - 4) In mixed culture with inactivated faecal bacterial cells.
- To quantify the interactions between *S.* Typhimurium and human commensal gut microbiota with a mathematical model. The purpose of developing a model is to test the interaction hypothesis and to identify the parameters describing the inactivation kinetics.

4.4 Methods

4.4.1 Bacterial strain, faecal sample, cultures preparation and bacterial counts

S. Typhimurium was maintained and cultured as previously described in section 2.1.2. Batch fermentation media was prepared as previously described in section 2.2.2. Faecal samples were obtained immediately prior to experiments and prepared as described in section 2.1.3. Culture conditions were set up as described in section 2.2.4. The vessels were then inoculated with 30 ml of processed faecal samples and/or 1 ml of *S.* Typhimurium inoculum, diluted when required in growth medium to obtain the targeted initial concentrations. Samples from the cultures were plated as per Table 2.1.

4.4.2 Inoculation protocols

The following inoculation protocols were used to set up mixed cultures: i) Faecal inoculum added first to the vessel and incubated for 24 h prior to the inoculation of *S.* Typhimurium; ii) *S.* Typhimurium and faecal samples inoculated at the same time into the vessels containing fresh medium and at similar concentrations of ca. 10^6 cfu/ml;

iii) *S.* Typhimurium inoculated first into the vessels and incubated for 24 h in order to reach a maximum density of ca. 10^9cfu/ml prior to the addition of the faecal inoculum; iv) re-inoculation of *S.* Typhimurium in a mixed culture prepared according to protocol i) and incubated for 72 h after the first inoculation of *S.* Typhimurium.

4.4.3 Supernatant collection

Supernatants were collected from mixed cultures of *S.* Typhimurium and faecal samples prepared according to the protocol of inoculation i). At several sampling times (4.5 h, 11 h, 19 h, 26.5 h, 33.5 h and 72 h) the content of the vessels was centrifuged at 6000 x *g* for 20 min and the supernatant filtered in vacuum-driven Stericup filters units (Millipore, UK) with 0.22 µm pore size in an oxygen-free cabin.

4.4.4 Two-compartment culture system

A two-compartment culture system was set up by placing a closed container made from 0.45 µm pore size membrane (Millipore, UK) in a vessel containing 300 ml of a faecal culture incubated at 37°C for 24 h under standard incubation conditions (Fig. 4.1). A volume of 1 ml of *S.* Typhimurium inoculum was added in the cell-free medium diffused within the container which had a volume of ca. 25 ml; 10 ml of *S.* Typhimurium inoculum was added outside of the container in contact with faecal bacteria.

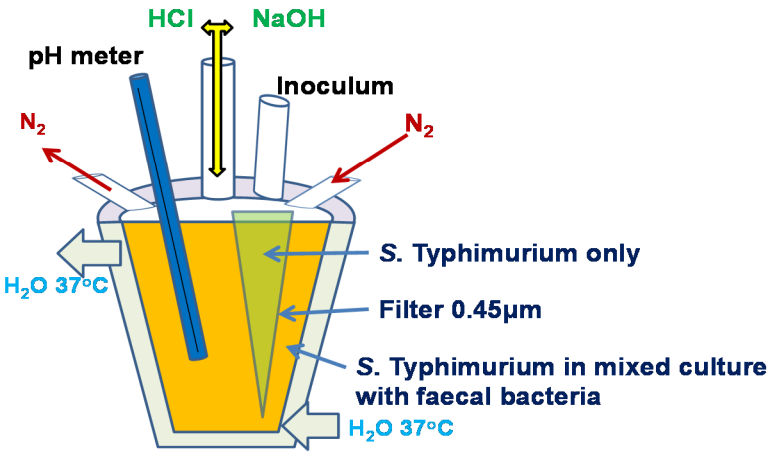


Figure 4.1. Two-compartment system. A filter of a 0.45 µm pore size membrane allows having a mixed culture of faecal bacteria and *S.* Typhimurium (orange) and *S.* Typhimurium in isolation from faecal bacteria (green).

4.4.5 Inactivated faecal bacteria

Inactivated faecal bacteria cells were obtained by centrifuging 300 ml of a faecal culture incubated at 37℃ for 24 h in oxygen-free conditions and pH 6.8-7.2. The supernatant was filtered (0.45 µm pore size membrane, Millipore, UK) and maintained at the standard incubation conditions during the cell inactivation process in order to be used later as medium for the co-culture. Faecal bacterial cells were inactivated in sodium cacodylate buffer containing 2.5% glutaraldehyde at room temperature for 1 h. After three successive washes with fresh growth medium, the cells were resuspended in 150 ml of the initial supernatant, which was a reduced volume to compensate cell loss during the cell inactivation process. By assuming 50% cell loss, it was ensured that the concentration of inactivated faecal bacteria would be sufficient to result in high frequency of cell encounters with *S.* Typhimurium. The pathogen was inoculated into this medium to evaluate the effect of cell-cell contact with inactivated faecal bacteria.

The inactivation of faecal bacteria exposed to glutaraldehyde was checked by plating out samples on Wilkins-Chalgren agar for total anaerobic counts and on Nutrient agar for total facultative aerobic counts.

4.4.6 Metabolomic analysis

Nuclear Magnetic Resonance (NMR) was used to identify the presence, absence and concentration of several metabolites in cultures of *S*. Typhimurium alone and mixed cultures of *S.* Typhimurium and faecal bacteria in the two-compartment system. Supernatant samples were thawed at room temperature and prepared for ^{1}H NMR spectroscopy by mixing 400 ml of spent medium with 200 ml phosphate buffer (0.2 M Na_2HPO_4 and 0.038 M NaH_2PO_4, pH 7.4) made up in 100% D_2O and containing 0.06% sodium azide, 6 mM DFTMP (difluoro (trimethylsilyl) methylphosphonic acid) and 1.5 mM DSS (sodium 2, 2-dimethyl-2-silapentane-5-sulfonate) as a chemical shift reference. The sample was mixed, and 500 ml transferred into a 5 mm NMR tube for spectral acquisition. ^{1}H NMR spectra were recorded at 600 MHz on a Bruker Avance spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) running TOPSPIN 2.0 software and fitted with a cryoprobe and a 60 slot autosampler. Each ^{1}H NMR spectrum was acquired with 256 scans, spectral width 12500 Hz and an acquisition time 2.62 s. The 1D NOESY with presaturation (noesypr1d) sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay. Spectra were transformed with 0.3 Hz line broadening, manually phased, baseline corrected and referenced by setting the DSS methyl signal to 0 ppm.

4.4.7 Electron microscopy

4.4.7.1 Scanning electron microscopy (SEM)

Two hundred microlitres of fresh cultures were gently deposited onto an isopore membrane polycarbonate filter (Millipore, UK) and incubated for 10 min at room temperature. The process was repeated three times before adding 200 µl of 2.5% glutaraldehyde (Agar Scientific, Stansted, UK) in 0.1 M sodium cacodylate buffer (pH 7.2) and incubating the filter for 1 h at room temperature. The filter was transferred to a vial containing 3 ml of 2.5% glutaraldehyde in sodium cacodylate buffer and after 15 min, three successive 10-min washes were carried out with sodium cacodylate buffer. Specimens were dehydrated by exposure to a graded series of ethanol washes (from 10 to 100% by 10% increase) for 15 min with two final steps of 100% ethanol. The dried specimens were coated with gold-palladium (8 nm cluster size) with an agar high resolution sputter coater and cells observed with a FEG HR-SEM (Zeiss Supra 55 VP). Cells were not centrifuged during sample processing in any case.

4.4.7.2 SEM-parcel method

Samples were prepared by transferring 1 ml of turbid bacterial suspension into a sterile 1.5 ml microtube. Immediately, 110 µl 25% glutaraldehyde (Agar Scientific, Stansted, UK) was added and mixed well to produce a final concentration of 2.5% glutaraldehyde. Samples were left to fix for 1 h before being submitted for SEM analysis.

4.4.7.3 Negative staining method for transmission electron microscopy (TEM)

Formvar/carbon grids (Agar Scientific, Stansted, UK) were picked up using tweezers and bent slightly at the edge by holding the dark shiny (coated) side against the bottom of a petri-dish and turning the tweezers. This ensured that the grids were levelled and were held in place in an upright position. After gently mixing the bacterial suspension to achieve homogeneity, a small drop of ca. 30 µl was pipetted onto each grid and incubated for 1 min at room temperature. Excess liquid was removed from the edge of the grid using a piece of filter paper and each was placed face-up onto a filter paper in a Petri dish, taking note of the position relating to each sample. When all the grids were prepared, the dish was transferred to a fume cabinet and 5 ml 25% glutaraldehyde (Agar Scientific, Stansted, UK) was pipetted into a plastic cap placed in the middle of the Petri dish. The lid of the dish was immediately replaced and the edge of the dish was sealed with parafilm. The grids were left to fix in the glutaraldehyde vapour for 2 h. After removing the parafilm and the cap containing the glutaraldehyde, the grids were submitted to the electron microscopy department for analysis.

4.4.8 Model description

The pattern of loss of culturability of *S.* Typhimurium in co-culture with faecal bacteria is characterised by a rapid reduction of several decimal logarithmic units of the culturable population followed by a gradual tailing off pattern. The hypothesis of the model is that the rate of loss of culturability of *S.* Typhimurium due to local interaction with faecal bacteria is dependent, through a scaling function $G(x)$, on the product of the frequency of encounters between the two populations, Z, and the probability of loss of culturability after an encounter, P, leading to the expression:

$$\frac{dS}{dt} = -\left(r \cdot S + G\left(Z \cdot P\right)\right) \quad (1)$$

$$\frac{dF}{dt} = -r \cdot F \quad (2)$$

Where *S* and *F* denote the concentration (cfu/ml) of *S.* Typhimurium and anaerobic faecal bacteria at a time *t*, respectively; *r* denotes the rate of decay of the population in a batch culture in stationary phase, which is assumed to be equal for both populations as suggested by the results of my experiments, however, in other conditions they could exhibit different decay rates and require an extra model parameter. This decay rate accounts for the loss of cell viability in stationary phase as well as for the reduction in plate counts due to the aggregation of bacteria in clusters.

The number of encounters per volume and unit time, *Z*, is dependent on the concentration (cfu/ml) of the two populations, *S.* Typhimurium, *S*, and anaerobic faecal bacteria, *F*, the cross section or effective encounter area, $\pi \cdot D^2$ with *D* denoting the average bacteria diameter, and the average velocity of bacteria, *v*, as follows.

$$Z = \frac{1}{2} \cdot \pi \cdot D^2 \cdot v \cdot S \cdot F \quad (3)$$

The factor ½ avoids counting each encounter twice. The cell diameter and the cell velocity were assumed to be constant. A cell diameter of two µm was derived from the average of cell length and width and the cell velocity, seven µm/s, was estimated as the average of the published bacterial velocities measured by using a three dimensional particle tracking technique [248].

This theory assumes that cell encounter is required but not sufficient for cell inactivation. After cell-cell contact the process leading to cell inactivation occurs with a certain probability, *P*.

The chosen scaling function, $G(x)$, was the function $G(x) = x^n$. If $n = 1$, equation 1 describes a first order kinetics for the loss of culturability of *S.* Typhimurium by cell-cell contact with faecal bacteria. The resulting expression follows collision theory and it can be rearranged to obtain the Arrhenius equation. However, this is not appropriate to describe the observed tailing pattern of loss of culturability of *Salmonella* spp. in mixed cultures with faecal bacteria. First-order kinetics implies that when the population of faecal bacteria has a decay rate close to zero, which is often the case, the inactivation kinetics of *Salmonella* spp. is practically log-linear which is in

disagreement with the observed tails in the inactivation curves of *Salmonella* spp. in mixed cultures. First order kinetics expressions and linear models in general are not capable of describing appropriately the behaviour of complex biological systems [221]. For $n > 1$, equations 1, 2 and 3 describe a non-linear system of differential equations. Similar expressions have been used to describe predator-prey systems [221]. In predator-prey models, the prey rate of consumption is dependent on the predator population whereas the predator rate of growth is dependent on the prey population leading to non-linear differential equations that generally cannot be solved. The interaction phenomenon observed in my experiments differs from a predator-prey system in that the faecal bacteria population, which could be identified with the predator, is not affected by *Salmonella* spp. which would be the prey.
The explicit solution for the equations 1 and 2 of the model can be found as follows:

The solution of equation 2 for an initial value $F(0) = F_0$ is equal to:

$$F(t) = F_0 \cdot e^{-rt} \tag{4}$$

For $n > 1$, with an initial value $S(0) = S_0$ and substituting in equation 1 the expressions in equations 3 and 4, equation 1 has solutions:

$$\left(S(t)\right)^{1-n} = \frac{n-1}{r(-2n+1)} CF_0^n e^{-nrt} + \left(S_0^{1-n} + \frac{1-n}{r(-2n+1)} CF_0^n \right) e^{-(1-n)rt} \qquad r \neq 0 \tag{5}$$

Where $C = \left(\frac{1}{2} \cdot \pi \cdot D^2 \cdot v \right)^n \cdot P^n$

The value of the exponent, *n*, governs the transition from the phase of sharp decrease of the population caused by the encounters with faecal bacteria to the decay phase of a stationary population in batch culture. With my experimental data, the most suitable value for *n* was 1.5, i.e. $G(x) = x\sqrt{x}$.

4.4.8.1 Model parameters estimation and model simulation

Parameters *r* and *P* from equations 4 and 5 were estimated by fitting the model to experimental measurements.

The decay rate of the population in stationary phase, *r*, was fitted by linear regression using the logarithm of the explicit solution of equation 2 and the concentration of total anaerobic bacteria measured in time. The estimated value of *r* was constant and equal to ca. 0.01 h^{-1} in all assays.

The probability of loss of culturability after encounter, which is the parameter *P* in equation 5, was estimated by non-linear regression using the measurements of the concentration of *S.* Typhimurium from the three datasets. An *F* test was used to test whether the fitted value of *P* differs significantly in any of the three datasets [221].

4.5 Results

4.5.1 Reduction of the culturable population of *S.* Typhimurium in batch mixed culture with anaerobic faecal bacteria in several protocols of inoculation

As explained in the previous chapter, a reduction of culturable *S.* Typhimurium in the order of approximately 10^3-10^4 cfu/ml was observed in co-cultures with faecal bacteria from different human donors; the concentration of *S.* Typhimurium in pure cultures under identical conditions did not show that reduction (Fig. 3.6). In subsequent experiments, the fate of *S*. Typhimurium was explored using various protocols of inoculation. The protocol of inoculation varied in the experiments as shown in Fig. 4.2. In three experiments, faecal samples were incubated for 24 h prior to the inoculation of *S.* Typhimurium. The reduction of the population of *S.* Typhimurium was detected immediately after inoculation (Fig. 4.2A). When the faecal sample and *S.* Typhimurium were inoculated at the same time in fresh medium at similar concentrations, ca. 10^6 cfu/ml, both *S.* Typhimurium and faecal bacteria increased to reach a maximum concentration of ca. 10^9cfu/ml in the first 12 h. This increase was followed by a sharp reduction of the culturable population of *S.* Typhimurium *(*Fig. 4.2B). When *S.* Typhimurium was inoculated 24 h prior to the faecal sample, the loss of culturability was detected ca. 12 h after the inoculation of the faecal sample (Fig. 4.2C).

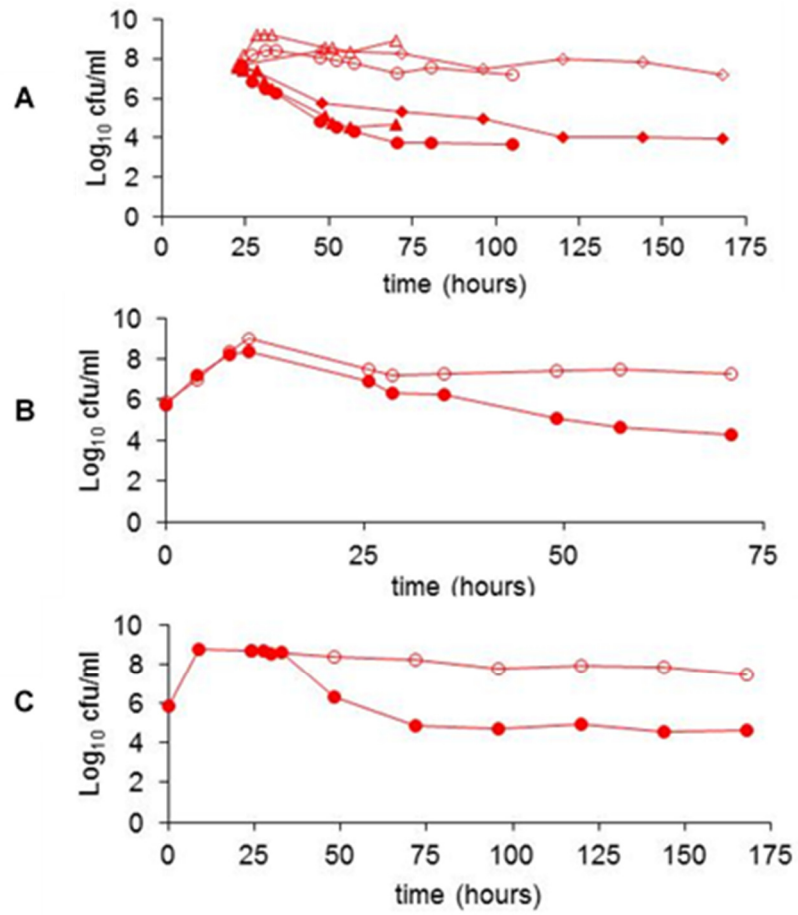


Figure 4.2. Concentration of *S.* Typhimurium in mixed cultures with faecal bacteria in three inoculation protocols. Comparison between the concentration of culturable *S.* Typhimurium in anoxic mixed cultures with faecal bacteria (filled symbols) and the concentration of *S.* Typhimurium in pure cultures under identical conditions (empty symbols). Different inoculation protocols were assayed: **A)** Faecal inoculum added into the vessels 24 h prior to the addition of *S.* Typhimurium. Different symbols show experiments set up with different faecal donors; **B)** *S.* Typhimurium and faecal sample inoculated at the same time; **C)** *S.* Typhimurium added into the vessel and incubated for 24 h prior to the addition of the faecal inoculum.

The loss of culturability of *S.* Typhimurium was detected in anoxic conditions only. The populations of *S.* Typhimurium and facultative aerobic bacteria decreased abruptly in mixed cultures in anoxic conditions; however, the inactivation of both populations ceased when oxygen was introduced in the atmosphere. After 24 h under oxic conditions the number of faecal anaerobic bacteria decreased, while facultative aerobic bacteria and *S.* Typhimurium increased (Fig. 4.3).

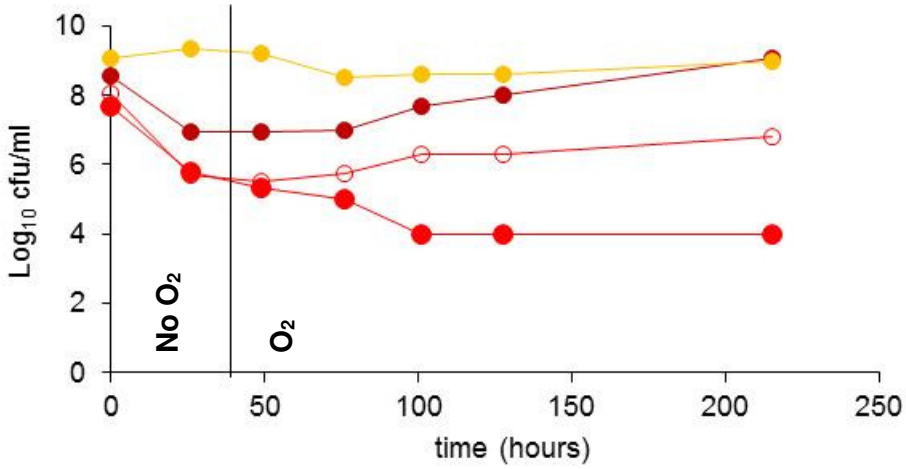


Figure 4.3. Population kinetics of *S.* Typhimurium and of faecal bacteria in co-culture for the first 24 h under anoxic conditions and under an oxygen-rich atmosphere afterwards. The symbols denote total anaerobes (●), total facultative aerobes (●) and S. Typhimurium (○). *S.* Typhimurium counts in co-culture with faecal bacteria under oxygen-free conditions throughout the experimental period (●) is also represented.

4.5.2 Loss of culturability of *S.* Typhimurium requires cell-cell contact with living anaerobic faecal bacteria

Supernatants were collected and processed under oxygen-free conditions from co-cultures of *S.* Typhimurium and faecal samples at several sampling times during the inactivation of *S.* Typhimurium. After processing, supernatants were immediately re-inoculated with *S.* Typhimurium. The concentration of *S.* Typhimurium was not affected in any of the supernatants, being not significantly different from that in fresh medium (Fig. 4.4). Therefore, the inactivation of *S.* Typhimurium is not mediated by soluble components present in the supernatant of faecal cultures.

To prove further that the reduction in the population of *S.* Typhimurium requires local interaction with faecal bacteria cells, a closed container made from 0.45 µm pore size filter material was placed into a 24 h faecal culture. Soluble components could diffuse into the container but cell trespassing was prevented by the membrane. *S.* Typhimurium was inoculated in the cell-free medium diffused within the container as well as outside of the container, where cell-cell contact with faecal bacteria was possible. Thus, *S.* Typhimurium was exposed to non-processed faecal culture medium in communicated compartments that only differed by the presence of faecal bacteria cells in one of them. The concentration of *S.* Typhimurium in contact with faecal bacteria decreased abruptly in the first 24 hours of incubation whereas this

decrease was not observed in the population of *S.* Typhimurium separated from faecal bacteria by the membrane (Fig. 4.5). The terms cell-cell contact and local interaction are used indistinctly as the degree of proximity between cells required for the loss of culturability of *S.* Typhimurium is not known.

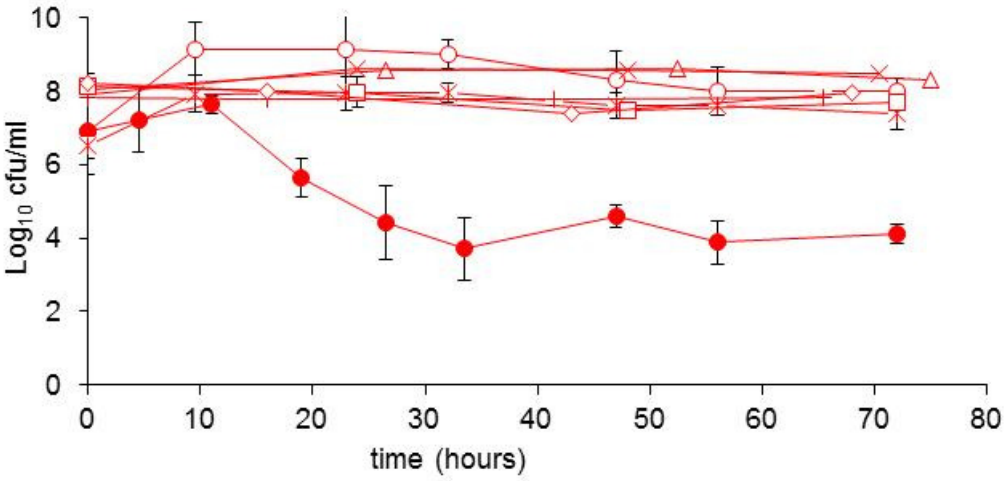


Figure 4.4. *S.* Typhimurium concentration in supernatants. The supernatants were collected anaerobically from co-cultures of *S.* Typhimurium and faecal sample after 4.5 (△), 11 (x), 19 (+), 26.5 (□), 33.5 (◊) and 72 (✳) hours of incubation. The concentration of *S.* Typhimurium was also monitored in a pure culture in fresh medium (○) and in co-culture with of *S.* Typhimurium and faecal bacteria (●).

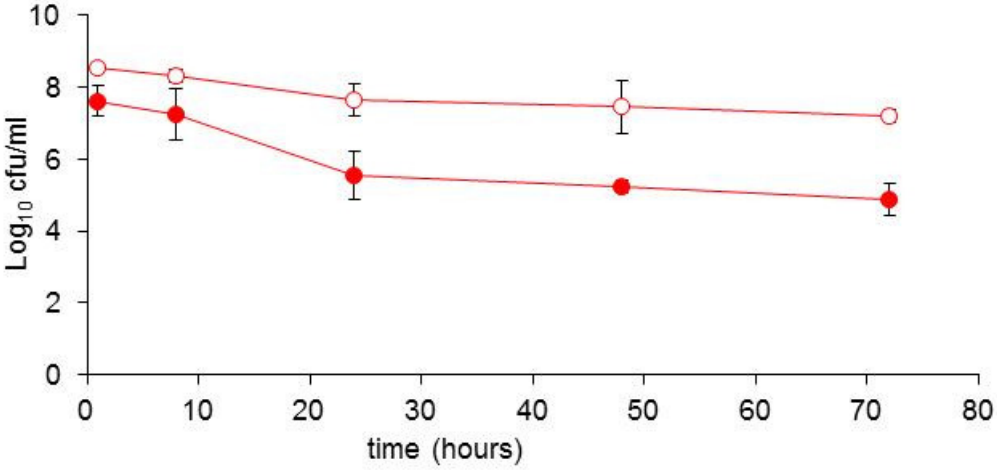


Figure 4.5. Concentration of *S.* Typhimurium in direct contact with faecal bacteria and in free-cell media. A closed container made of 0.45 µm pore size membrane was placed in a faecal culture in order to create a compartment with all soluble components but free of faecal bacteria. *S.* Typhimurium was inoculated into the faecal bacterium-free bacteria container (○) and outside the container (●) in direct contact with faecal bacteria. Results are averages of 4 independent experiments set up with samples from different donors.

In addition, it was investigated if cell-cell contact in itself could be sufficient to cause loss of culturability of *S.* Typhimurium. To do this, the response of *S.* Typhimurium in contact with inactivated faecal bacterial cells was investigated. A faecal culture incubated for 24 h was centrifuged to collect both cells and supernatant. Faecal bacteria cells were inactivated and resuspended into the supernatant prior to the inoculation of *S.* Typhimurium. Results in Fig. 4.6 show that the culturability of *S.* Typhimurium was not affected by contact with inactivated faecal bacteria cells.

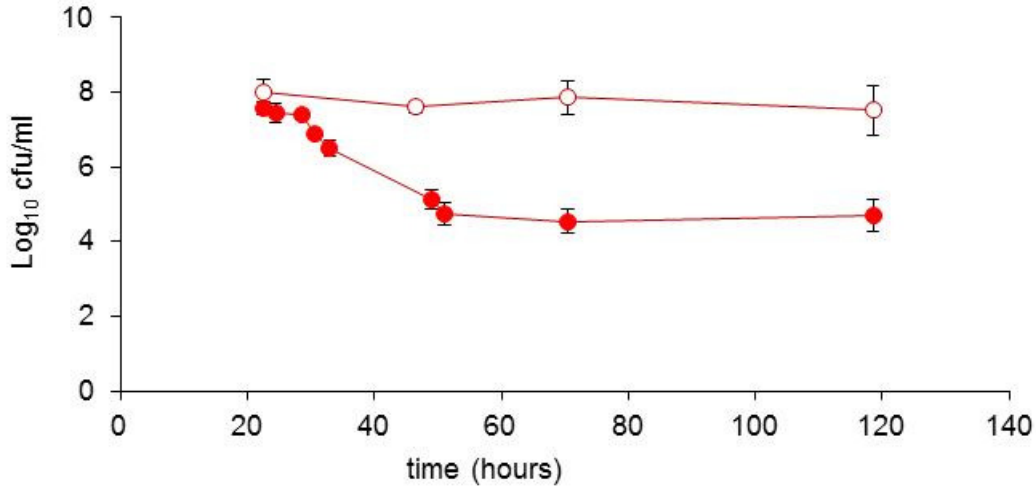


Figure 4.6. *S.* Typhimurium in mixed culture with inactivated bacterial cells. Concentration of *S.* Typhimurium inoculated in medium containing inactivated faecal bacterial cells (○) and in mixed culture with living faecal bacteria (●). The results are the averages of 2 independent experiments set up with different sample donors.

4.5.3 Intercellular structures with different morphology were visualised in cultures of faecal bacteria alone as well as in mixed cultures of faecal bacteria with *S.* Typhimurium

The samples were prepared as explained in 3.4.3.1. The examination of a field of co-cultured cells by electron microscopy revealed visible intercellular tubular formations among the cells of each species, and more importantly, clear protrusions were formed between different species (Figs. 4.7 and 4.9). *S.* Typhimurium could be identified by the presence of flagella in both pure (Fig. 4.8) and mixed cultures (Figs. 4.9 and 4.11) while in faecal samples inoculated alone, flagellated bacteria or flagella-like structures were not observed (Fig. 4.7). Lack of flagella proteins has been also reported in a metaproteomics study of the human distal gut microbiota [249]. In that study, bacterial flagella proteins were not identified and only a very small number of proteins associated to bacterial motility were observed when analysing thousands of proteins

with a non-targeted, shotgun mass spectrometry-based whole community proteomics approach [249].

In faecal cultures, numerous tubular and vesicular structures of different width were observed between cells of apparently different morphology, demonstrating the ubiquitous nature of this phenomenon (Fig. 4.7). In the co-culture of *S*. Typhimurium and the faecal sample, large tubular structures were visualized between flagellated bacteria, which were assumed to be *S*. Typhimurium, and faecal bacteria with varied cell morphology (Figs. 4.9, 4.10 and 4.11). These tubular structures have similar morphology and dimensions to the reported nanotubes for molecular transfer between *B. subtilis*, *S. aureus* and *E. coli* [196].

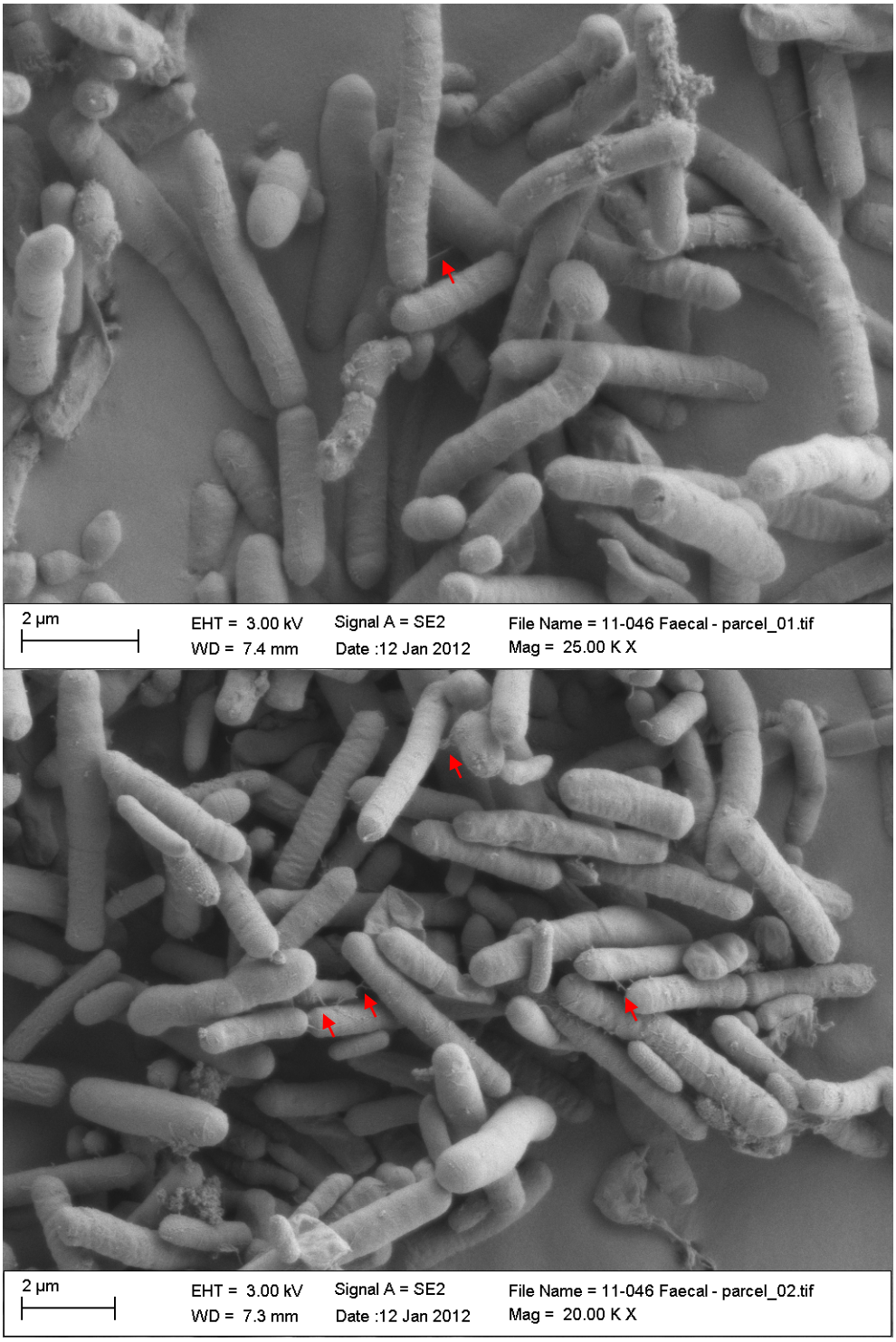


Figure 4.7. Intercellular tubular structures between different species of faecal origin. The cultures were incubated for 24 h after inoculation with faecal bacteria and visualised by HR-SEM-parcel method. Arrows indicate some of the intercellular nanotubes between neighbouring cells with different morphology. Faecal bacteria tend to be aggregated in clusters. Flagellated bacteria were not observed in faecal cultures.

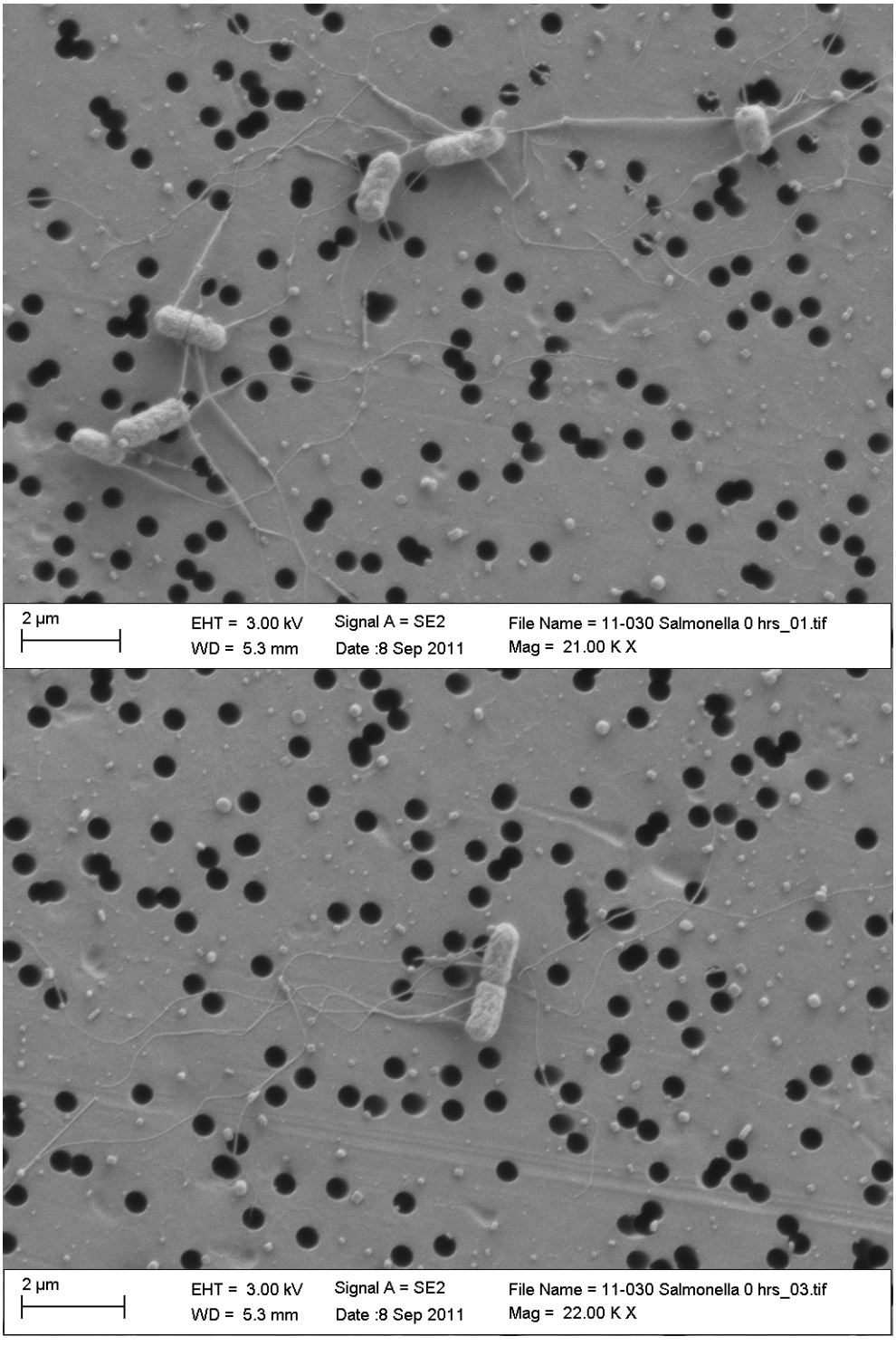


Figure 4.8. *S.* Typhimurium visualised by HR-SEM. *S.* Typhimurium cells in pure culture. Flagella were observed in all cells.

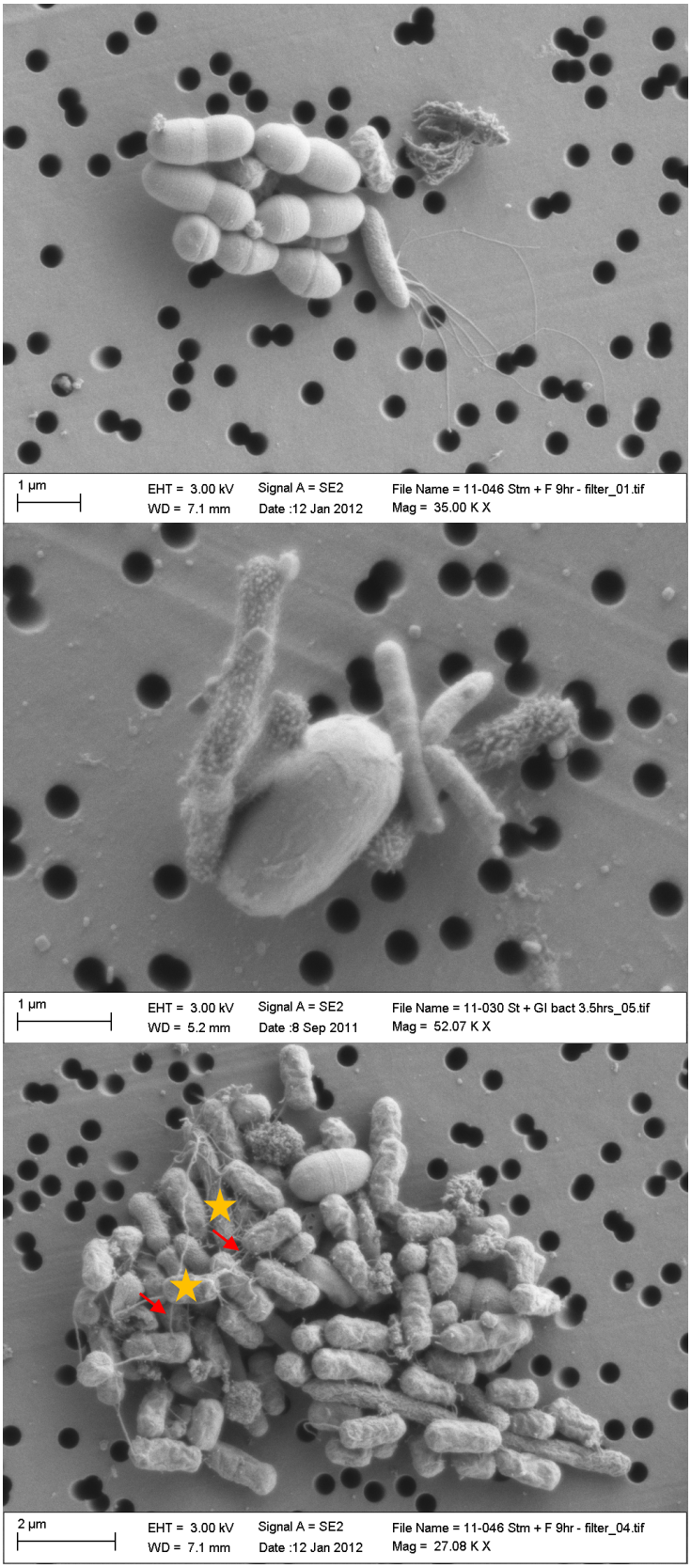
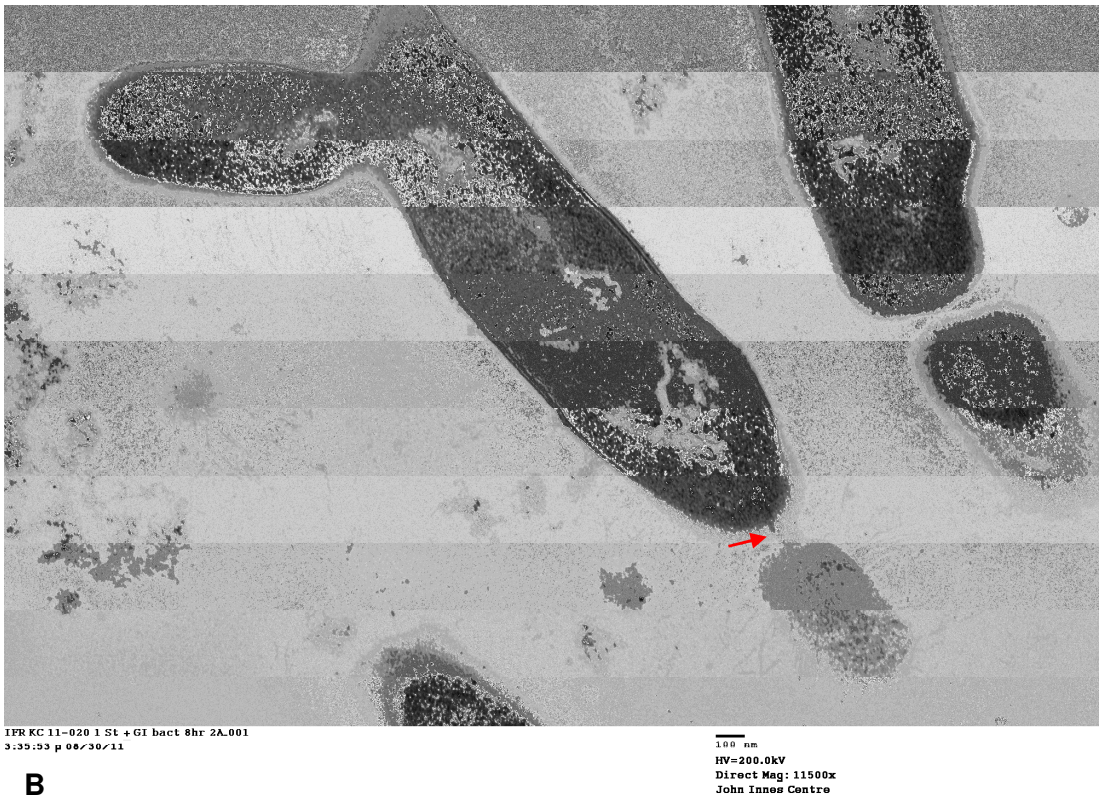


Figure 4.9. Bacterial species of faecal origin in mixed culture with *S.* Typhimurium visualised by HR-SEM. Cultures were incubated for 9 h after inoculation with faecal bacteria and *S.* Typhimurium. Clusters of bacteria were very often detected. Flagellated *S.* Typhimurium cells are indicated by an orange star and arrows indicate nanotubes between *S.* Typhimurium cells and faecal bacteria.

A



B

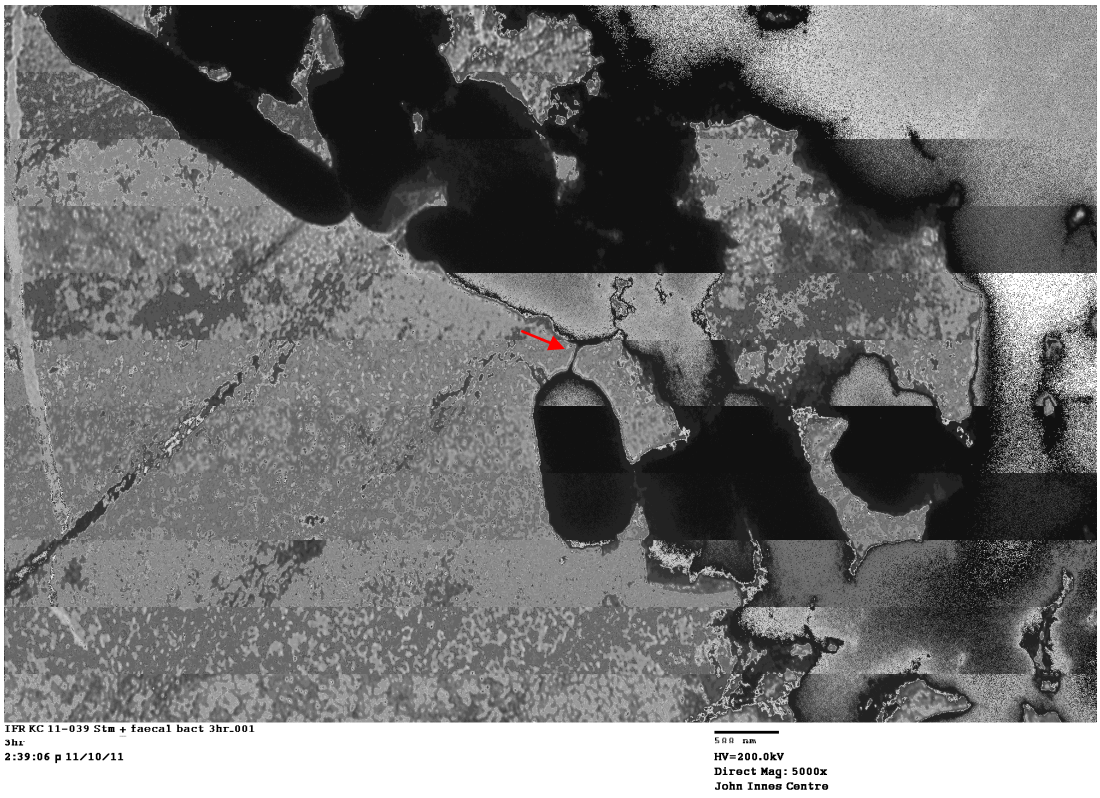
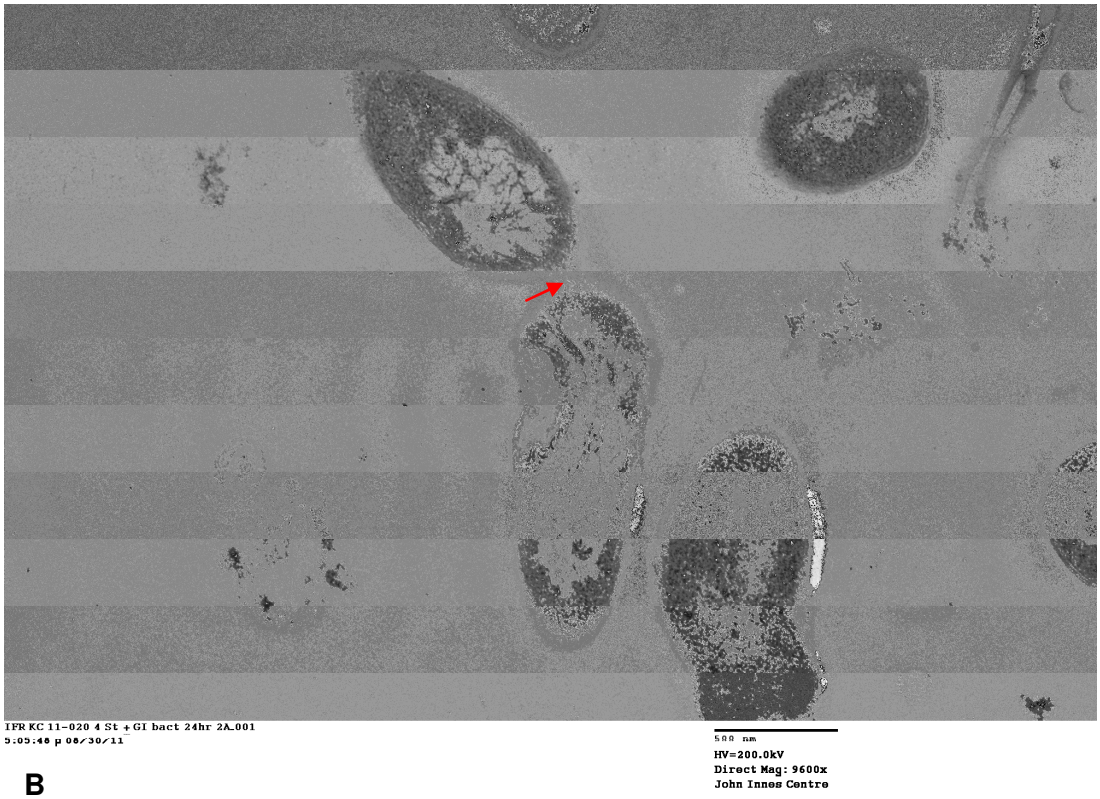


Figure 4.10. Bacterial species of faecal origin in mixed culture with *S.* Typhimurium visualised by negative staining method for TEM. Cultures were incubated for 8 h (A) and 3 h (B) after inoculation with faecal bacteria and *S.* Typhimurium. Clusters of bacteria were very often detected. Arrows indicate nanotubes between neighbouring cells.

A



B

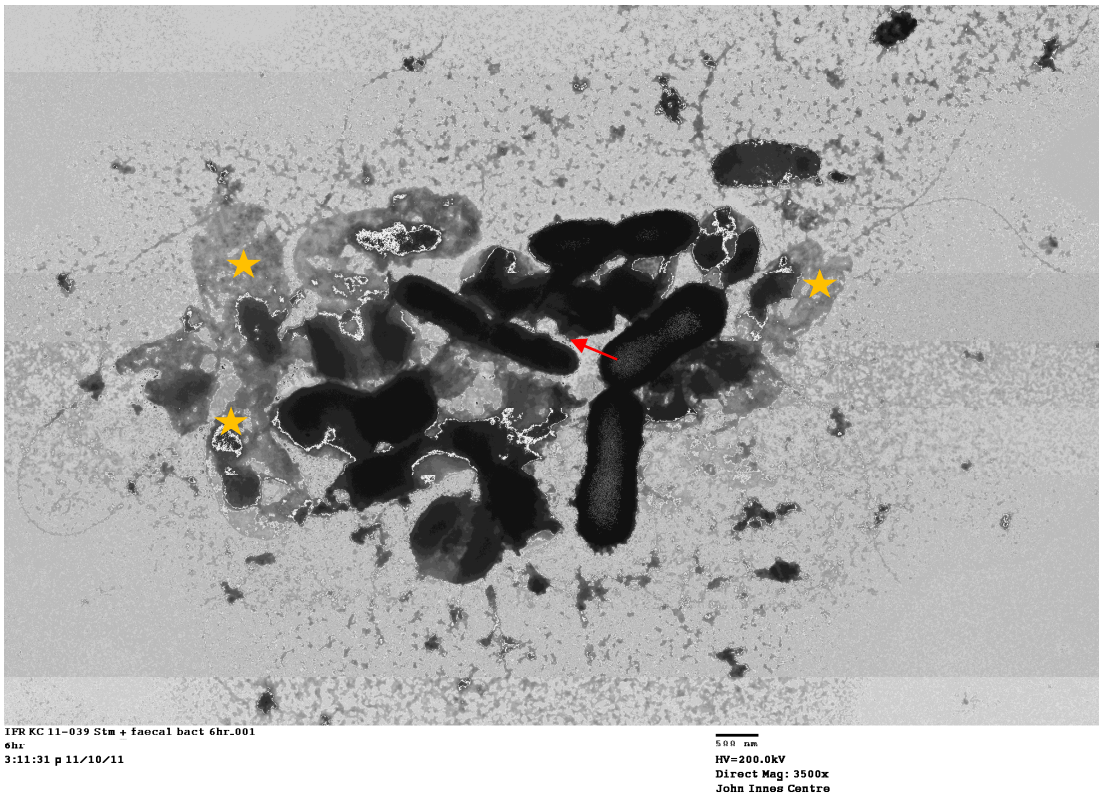


Figure 4.11. Bacterial species of faecal origin in mixed culture with *S.* Typhimurium visualised by negative staining method for TEM. Cultures were incubated for 24 h (A) and 6 h (B) after inoculation with faecal bacteria and *S.* Typhimurium. Clusters of bacteria were very often detected. Flagellated *S.* Typhimurium cells are indicated by an orange star and arrows indicate nanotubes between neighbouring cells.

4.5.4 The loss of culturability of *S.* Typhimurium by local interaction with faecal bacteria is characterised by the probability of inactivation after cell encounter

The hypothesis to be tested was that the probability that an encounter results in loss of culturability, which is quantified by parameter *P* in the model, should be constant under identical experimental conditions. This hypothesis was supported by showing that the fitted value of *P* is the same for all datasets generated with several bacterial concentrations and inoculation protocols. Mixed cultures of faecal bacteria and *S.* Typhimurium were prepared at low, ca. 10^6 cfu/ml (Fig. 4.12A), and high, ca. 10^9 cfu/ml, concentration (Fig. 4.12B). In addition, *S.* Typhimurium was re-inoculated in a mixed culture 72 h after the first inoculation when the initial population of *S.* Typhimurium was already tailing off (Fig. 4.12C). A value of ca. 10^{-5} for the probability of inactivation, *P*, was estimated by fitting the model with the three datasets (Fig. 4.12C). An *F* test showed that this value was not significantly different from those obtained when the model was fitted individually to each dataset (*p* value = 0.63). When the concentration of *S.* Typhimurium (Fig. 4.12B) in the mixed culture with faecal bacteria was high, the predicted and observed initial reduction of the population was larger and at a sharper rate, due to the higher frequency of cell encounters, than when the concentration of *S.* Typhimurium was smaller (Fig. 4.12A). When *S.* Typhimurium was re-inoculated in a mixed culture in which the initial population of *S.* Typhimurium was already tailing off, both predictions and observations indicated that the reduction of the re-inoculated population was smaller than that of the initially inoculated population of *S.* Typhimurium. This was due to a decrease of the frequency of cell encounters because of the relative slight decay of the anaerobic faecal bacteria during course of the experiment. The faecal population decreased from ca.10^9 cfu/ml to ca. $5x10^8$ cfu/ml during the first 72 h preceding the re-inoculation of *S.* Typhimurium. After the first inoculation with 10^9 cfu/ml faecal bacteria, a reduction of two decimal logarithmic units was detected in the population of *S.* Typhimurium in the first 24 h. However, only 1.2 decimal logarithmic units reduction was detected after re-inoculation (Fig. 4.12C). Had not the faecal population decayed, a ca. two-logarithmic-unit reduction would have been also expected after re-inoculation (Fig. 4.12C).

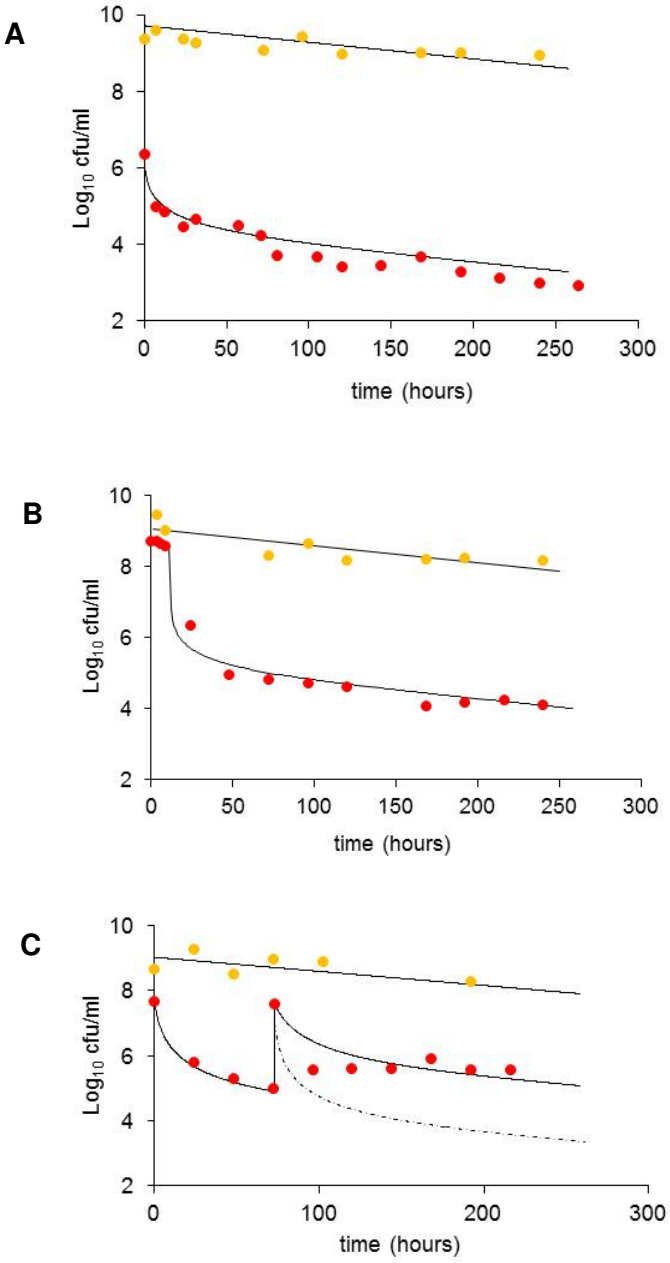


Figure 4.12. Faecal bacteria and different concentrations of *S.* Typhimurium. Model fittings (lines) obtained with the observed concentration of *S.* Typhimurium (●) and anaerobic faecal bacteria (●) generated with various protocols of inoculation: **A)** Faecal inoculum added first to the vessel and incubated for 24 h prior to the inoculation of a relatively low inoculum, ca. 10^6 cfu/ml, of *S.* Typhimurium; **B)** *S.* Typhimurium inoculated first into the vessels and incubated for 24 h to reach a maximum density of ca. 10^9 cfu/ml prior to the addition of the faecal inoculum; **C)** *S.* Typhimurium re-inoculated in a mixed culture with faecal bacteria 72 h after the first inoculation when the initial population of *S.* Typhimurium was already tailing off. Continuous lines represent predictions when the faecal population decays with a rate of $-0.01h^{-1}$. The dashed line shows the predicted concentration of *S.* Typhimurium assuming that that the concentration of the faecal population does not change during the first 72 h of incubation.

4.5.5 The loss of culturability of *S.* Typhimurium by cell-cell contact with faecal bacteria is predicted as a function of the concentration of both populations

As indicated in equation 3, the frequency of cell encounters and thus the rate of loss of culturability were dependent on the concentration of both *S.* Typhimurium and faecal bacteria (Fig. 4.13A).

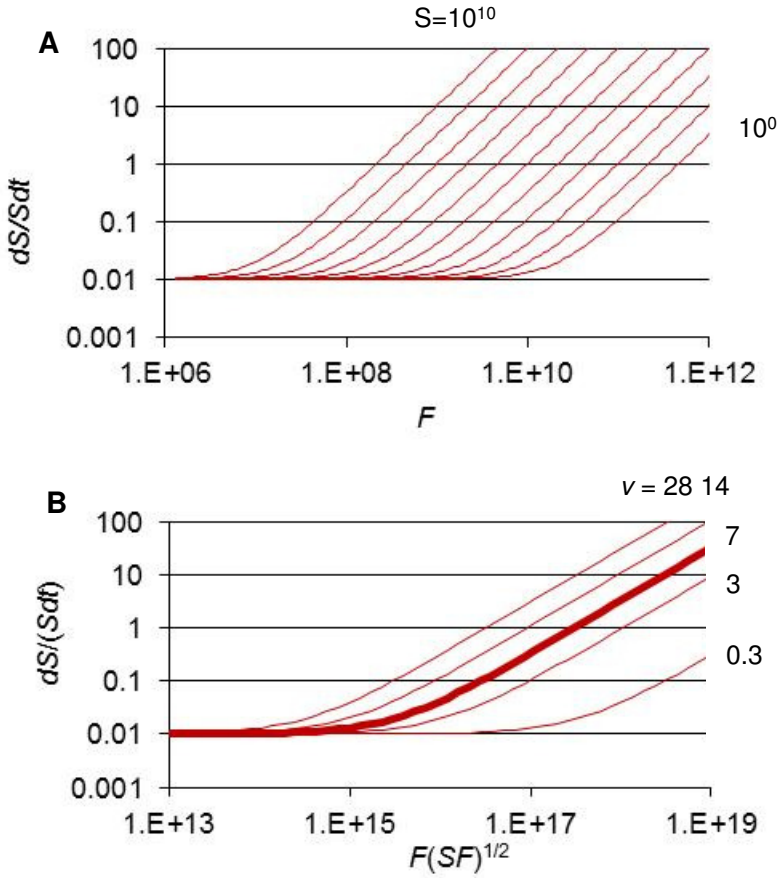


Figure 4.13. Bacterial concentration, cell velocity and prediction of *S.* Typhimurium loss of culturability. **A)** Predicted loss of culturability rate of *S.* Typhimurium as a function of the concentration of faecal bacteria, *F* (cfu/ml), at several constant concentrations of *S.* Typhimurium, *S*, from 1 to 10^{10} cfu/ml; **B)** Predicted relationship between the specific rate of loss of culturability of *S.* Typhimurium, *dS*/(*Sdt*) (h^{-1}), and the concentration of *S.* Typhimurium, *S* (cfu/ml), and anaerobic faecal bacteria, *F* (cfu/ml), assuming several values for the average cell velocity, *v*, from ca. 0.3 to 30 µm/s.

For a concentration of faecal bacteria smaller than $6x10^6$ cfu/ml, the model described in this chapter predicts that the loss of culturability rate of *S.* Typhimurium is practically equal to the decay rate of the population in stationary phase, $r = 0.01$ h^{-1}. When the concentration of *S.* Typhimurium is equal to 1 cfu/ml, more than ca. 10^{10} cfu/ml faecal bacteria are required to observe a loss of culturability rate significantly greater than

0.01 h^{-1}. Fig. 4.13B describes the dependence of the rate of loss of culturability with the concentration of both populations. To predict loss of culturability of *S.* Typhimurium by local interaction with faecal bacteria, i.e. rate significantly greater than the population decay rate in stationary phase, the product $F(S{\cdot}F)^{1/2}$, where *F* is the concentration of faecal bacteria and *S* of *S.* Typhimurium, must be greater than ca. 10^{15} $(cfu/ml)^2$ (Fig. 4.13B). This value is predicted assuming that the average bacterial cell velocity is equal to 7 μm/s. Bacterial cell velocity affects the frequency of encounters between the populations and thus the rate of loss of culturability of *S.* Typhimurium. The required concentrations to observed inactivation by cell-cell contact increase when cells move slower and decrease for faster cell velocities. The required minimum value of $F(S{\cdot}F)^{1/2}$ is 10^{17} $(cfu/ml)^2$ if cell velocity is equal to 0.3 μm/s and 10^{14} cfu/ml when equal to 30 μm/s (Fig. 4.13B). These values define a range of velocities similar to those estimated experimentally by a three dimensional tracking bacterial technique [248].

4.5.6 Metabolomic analysis

Prior to *S.* Typhimurium inoculation in the two-compartment culture system separated by a 0.45 μm membrane (Fig. 4.1), the system was filled up with medium and one compartment was inoculated with faecal bacteria and incubated for 24 h. *S.* Typhimurium was then inoculated into both compartments, which were incubated for 24 h. Loss of culturability of *S.* Typhimurium was detected only in the compartment where *S.* Typhimurium was in contact with faecal bacteria. The NMR profiles of the samples obtained from the two compartments of the culture system were practically identical with the exception of the signal identified potentially as uracil which was not detected in the compartment containing *S.* Typhimurium and faecal bacteria, and the signal for ethanol. The peak for ethanol was slightly higher in the compartment containing *S.* Typhimurium and faecal bacteria. This can be observed in Fig. 4.14, which shows the scatter plot of the NMR intensities in each sample; in this figure, the dots located on the diagonal of the scatter plot represent peaks with very similar NMR intensity in both samples, whereas deviations from the diagonal indicate different amounts of metabolite in the samples. The remaining 124 metabolites were very similar in both samples. Fig. 4.15 shows the entire metabolic profile for each sample.

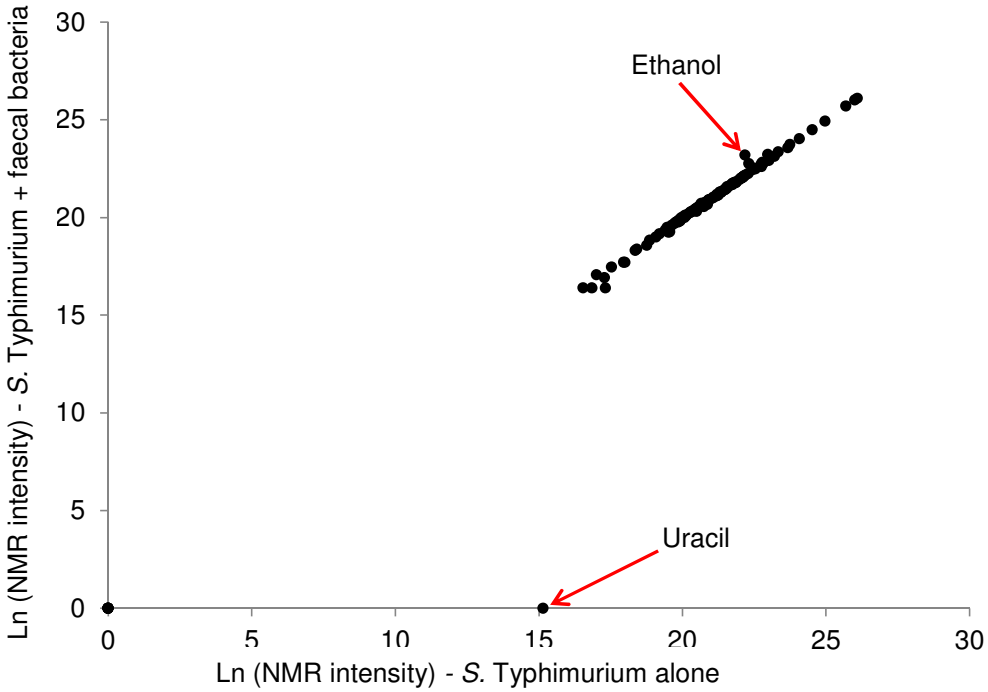


Figure 4.14. NMR intensities for samples containing *S.* Typhimurium and faecal bacteria *vs* samples with only *S.* Typhimurium, obtained from the two-compartment system. The red arrows indicate the metabolites ethanol (slightly higher in the mixed samples) and uracil (absent in the mixed samples).

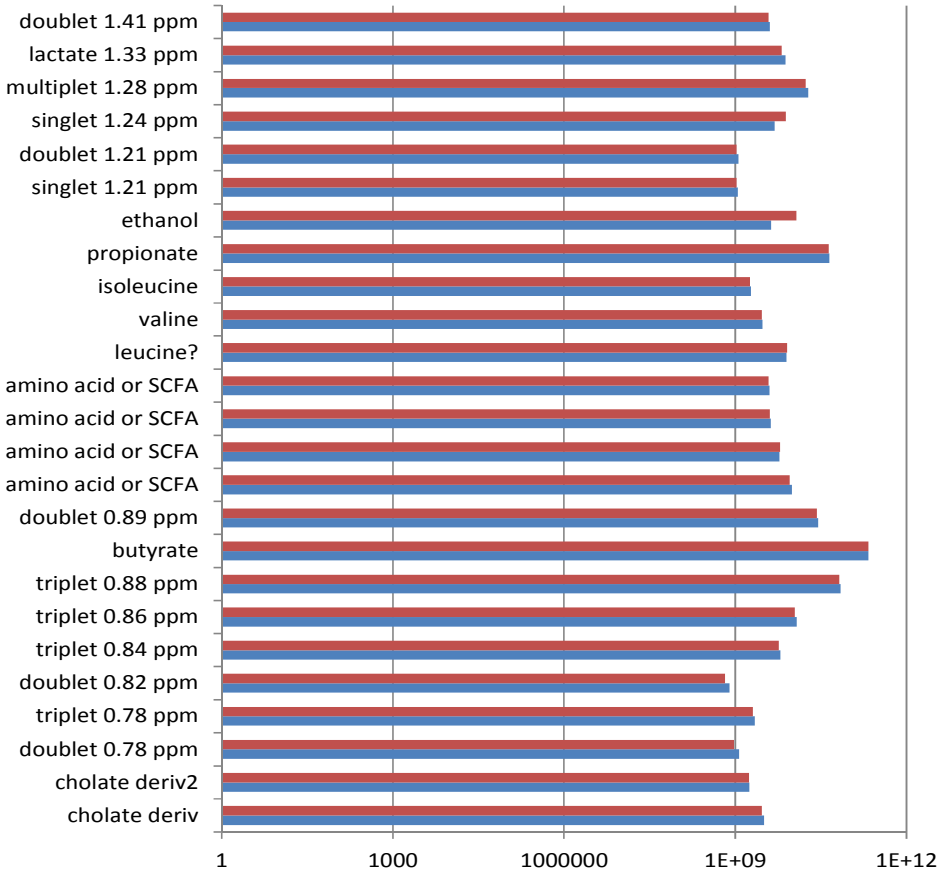


Figure 4.15

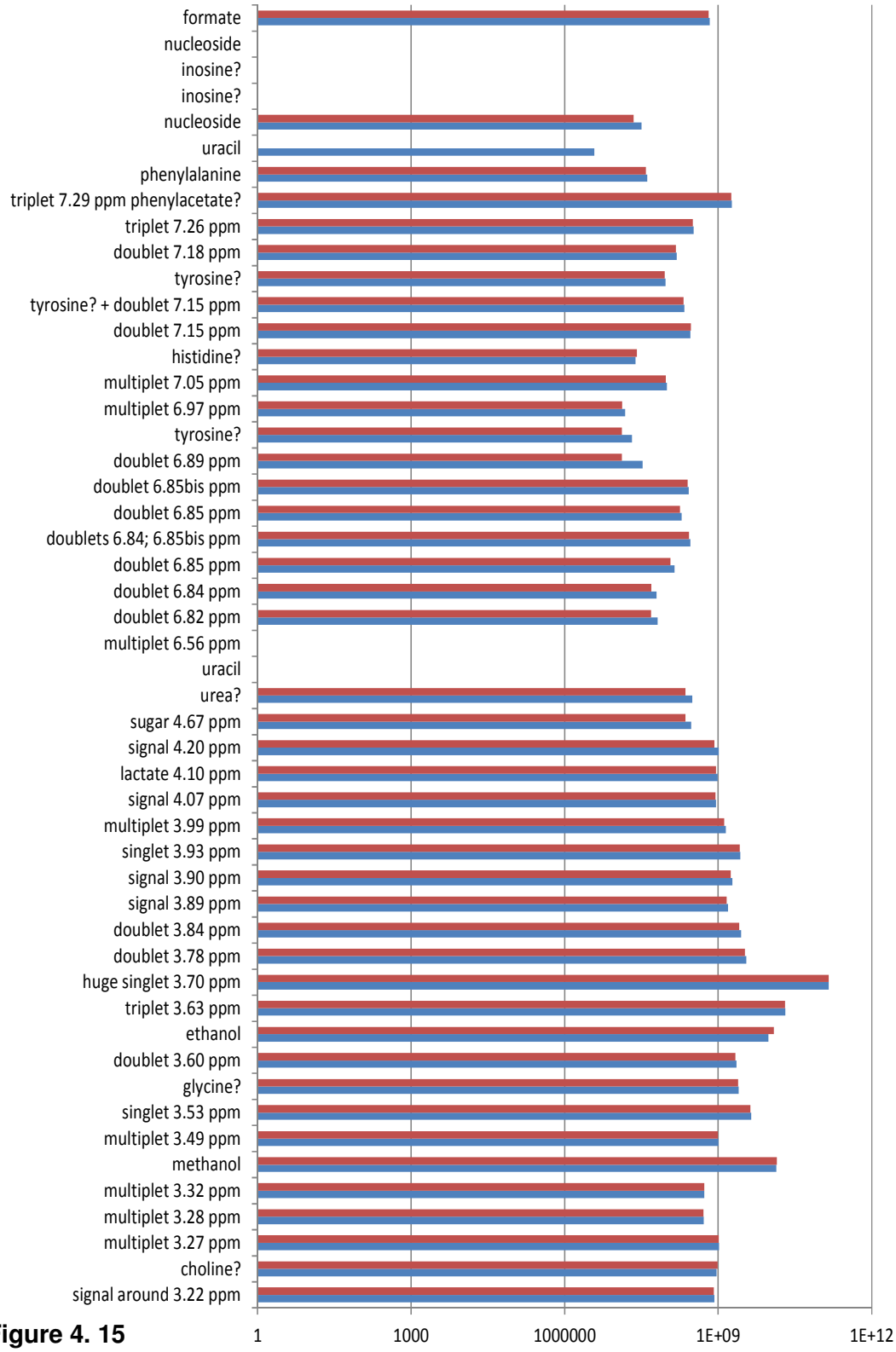

formate
nucleoside
inosine?
inosine?
nucleoside
uracil
phenylalanine
triplet 7.29 ppm phenylacetate?
triplet 7.26 ppm
doublet 7.18 ppm
tyrosine?
tyrosine? + doublet 7.15 ppm
doublet 7.15 ppm
histidine?
multiplet 7.05 ppm
multiplet 6.97 ppm
tyrosine?
doublet 6.89 ppm
doublet 6.85bis ppm
doublet 6.85 ppm
doublets 6.84; 6.85bis ppm
doublet 6.85 ppm
doublet 6.84 ppm
doublet 6.82 ppm
multiplet 6.56 ppm
uracil
urea?
sugar 4.67 ppm
signal 4.20 ppm
lactate 4.10 ppm
signal 4.07 ppm
multiplet 3.99 ppm
singlet 3.93 ppm
signal 3.90 ppm
signal 3.89 ppm
doublet 3.84 ppm
doublet 3.78 ppm
huge singlet 3.70 ppm
triplet 3.63 ppm
ethanol
doublet 3.60 ppm
glycine?
singlet 3.53 ppm
multiplet 3.49 ppm
methanol
multiplet 3.32 ppm
multiplet 3.28 ppm
multiplet 3.27 ppm
choline?
signal around 3.22 ppm
1
1000
1000000
1E+09
1E+12

Figure 4. 15

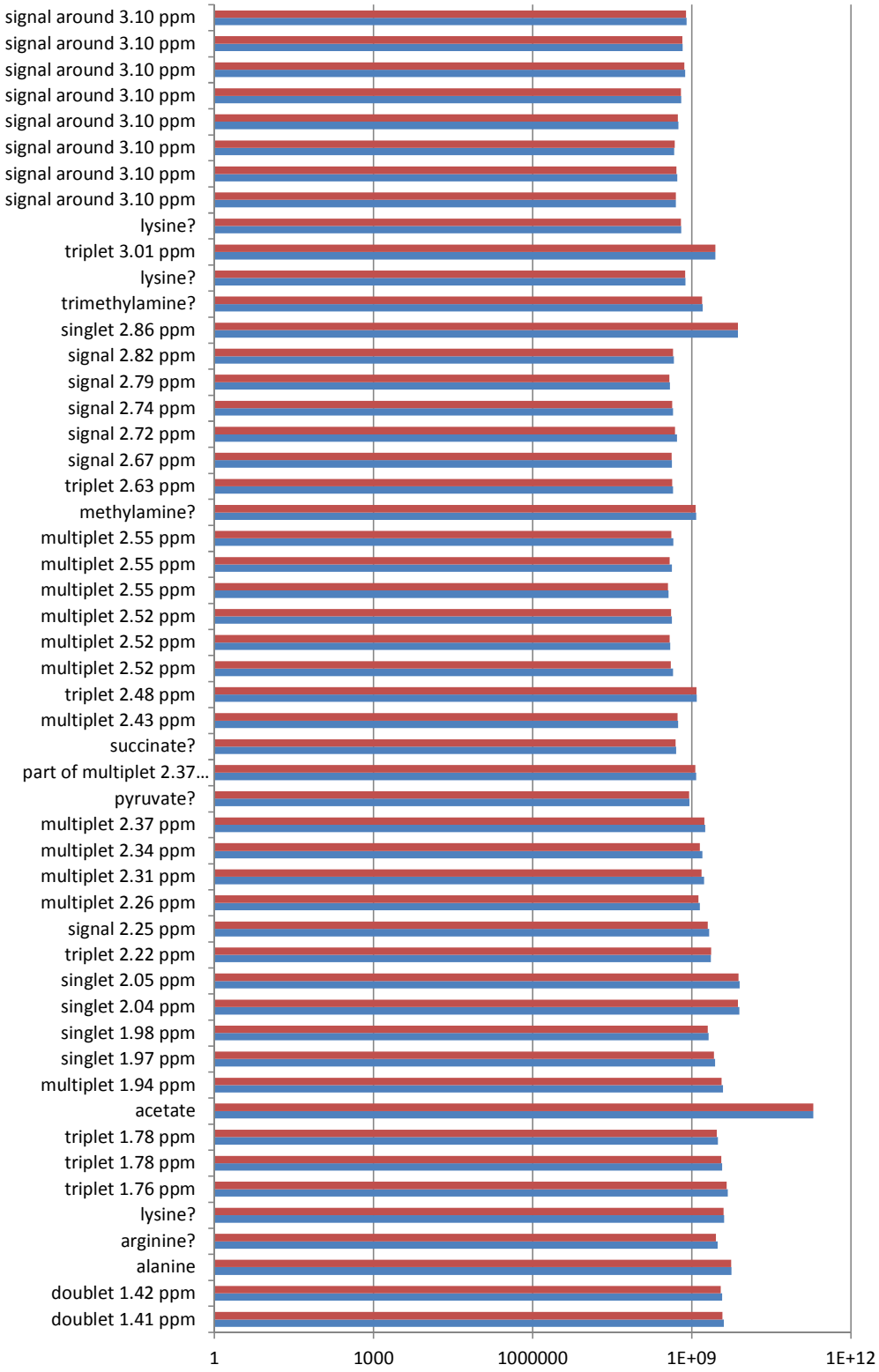


Figure 4.15. Metabolite profile for samples of *S.* Typhimurium alone and co-culture of *S.* Typhimurium and faecal bacteria. S. Typhimurium alone (red); co-culture (blue).

Further comparison of the metabolic profiles of these samples was carried out by principal component analysis. In order to have some reference to understand the magnitude of the distance between metabolic profiles, the samples obtained at 10, 24 and 48 h of incubation from three faecal cultures initiated with faeces from different donors were included in this analysis. The first two principal components (PC 1 and 2) account for practically 70% of the total variability which is mainly due to the origin of samples from different donors (Fig. 4.16). The variability of the metabolic profile between faecal cultures from different human donors or sampling times was much greater than that between the samples of the two compartment culture system, which were located very close to each other. Therefore, the metabolic profile of the compartment with *S.* Typhimurium alone is very similar to that of the compartment where contact killing of *S.* Typhimurium by faecal bacteria takes place.

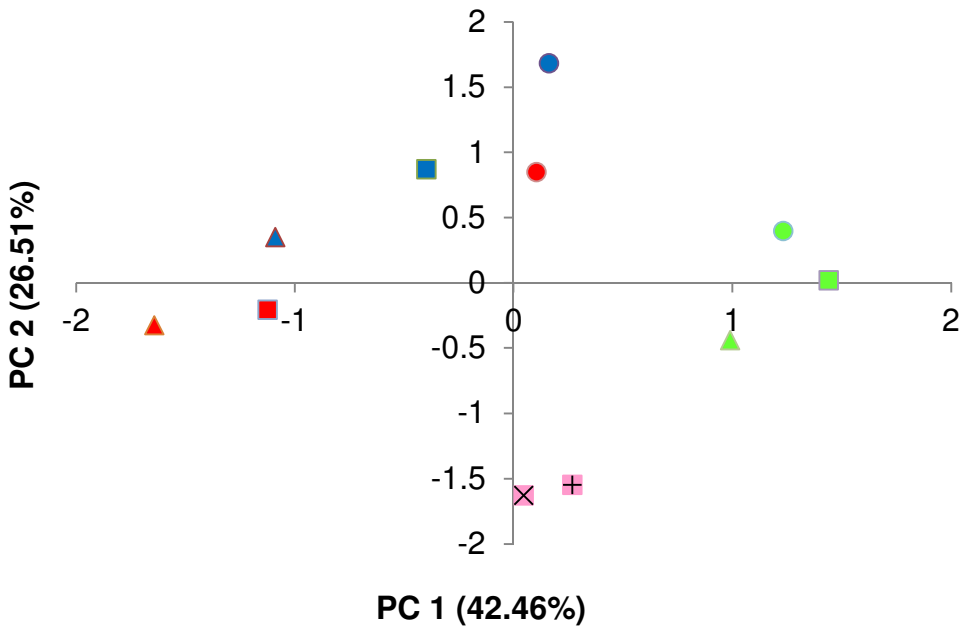


Figure 4.16. Principle component analysis of the metabolite concentrations. Cell free medium collected from faecal cultures incubated for 10 h (circle), 24 h (square) and 48 h (triangle). Samples were obtained from three donors identified by blue, green or red colour. Pink symbols represent the samples obtained from a two-compartment culture system separated by a membrane of pore size equal to 0.45µm. The symbol with a central cross represents the sample from the compartment where *S.* Typhimurium was inoculated alone while the symbol with a central plus represents the sample of the compartment where *Salmonella* was in contact with faecal bacteria.

4.6 Discussion

The work presented in this chapter demonstrates the existence of a novel way of interaction between the gut microbiota and *S.* Typhimurium that requires local interaction such as cell contact and leads to growth inhibition or loss of culturability of *S*. Typhimurium. The model presented explains the observed kinetics of the loss of culturability of *S.* Typhimurium as a function of the frequency of encounters between the two populations and the probability of inactivation after an encounter.

The preferential infection of the small intestine by *Salmonella* spp. is in part explained by the production of SCFA by the gut microbiota. Formate is present at higher concentrations in the small intestine than in the colon and leads to the up regulation of invasion genes in *Salmonella* spp. [250]. By contrast, butyrate, which is present at higher concentrations in the colon than in the small intestine, leads to the down regulation of invasion genes [162].

The experiments presented in this chapter suggest the existence of an additional way of inactivation involving local interaction with the gut microbiota to prevent the colonisation of the large intestine by *S.* Typhimurium. Contact-dependent bacterial interaction has been identified in strains of uropathogenic *E. coli* enabling them to inhibit the growth of other microbes in mixed populations [194]. This phenomenon was mediated by the CdiA/CdiB two partner secretion proteins that bind to the outer membrane protein BamA in target cells [194, 251]. This inhibitory process was dependent on the growth stage; exponential growing bacteria but not stationary phase cells were inhibitory while target cells were inhibited regardless of growth stage [194]. In the experiments described in this chapter, only stationary phase cells of faecal origin were inhibitory for *S.* Typhimurium. When faecal bacteria were inoculated 24 h prior to the inoculation of *S.* Typhimurium, the reduction of the culturable population of *S.* Typhimurium was detected immediately after its inoculation (Fig. 4.2A). However, when the faecal sample was inoculated either simultaneously, or after *S.* Typhimurium, a ~12 h delay was required to observe the reduction of the culturability of *S.* Typhimurium (Figs. 4.2B, 4.2C and 4.12B). The reason for this delay could not be an insufficient concentration of bacteria, because the product $F(S{\cdot}F)^{1/2}$ was greater than ca. 10^{15} $(cfu/ml)^2$, which is the threshold value predicted by the model described above to observe loss of culturability of *S*. Typhimurium by local interaction. It has been reported that *E. coli* evolved in serial passage experiments and became able to kill or inhibit the growth of its own ancestors by cell contact only after reaching stationary phase [244]. A distinct killing or growth inhibitory process requiring cell-cell

contact is described in the later work for non-growing cells of *E. coli* K-12, which lacks both *cdiA* and *cdiB* genes responsible for the inhibition mechanism reported by Aoki *et al*. [194, 251]. *E. coli* K-12, growth inhibition by cell contact was suggested to be associated with mutations in the *glgC* gene and overproduction of glycogen [244]. Similarly, the initial delay observed in the loss of culturability of *S.* Typhimurium may be related to the time required by faecal bacteria to acquire those features that enable them to inhibit bacterial growth. An initial time delay following inoculation can also be observed in the inactivation of *E. coli* by cell contact with *Vibrio cholerae* mediated by the T6SS [245]. The T6SS of *P. aeruginosa* has also been reported to deliver by cell-cell contact two effector proteins, Tse1 and Tse3, in the periplasm of *E. coli* and *Pseudomonas putida* causing cell lysis [247]. An additional strategy suggested as possible mechanisms to deliver toxic molecules to neighbouring cells is the formation of tubular extensions bridging cells or nanotubes [196]. Therefore, cell-cell contact is essential for all these interactions, although the described molecular mechanism leading to loss of culturability, growth inhibition or cell lysis varied between species.

Similar inactivation kinetics to that observed in *S.* Typhimurium was sometimes observed in the population of total facultative aerobes as well as in the population counted on MacConkey agar for *Enterobacteriaceae* (Fig. 3.5) described in the previous chapter. The similarity in the inactivation kinetics indicates that the loss of culturability of these bacterial groups could also require cell contact as demonstrated for *S.* Typhimurium. This implies that loss of culturability upon cell-cell contact with faecal bacteria is not specific for *Salmonella* spp. but it may also affect other species. Simple growth assays on selective media were carried out to follow the population dynamics of the main culturable faecal bacterial groups. For a thorough identification of microorganisms in faecal cultures, recent developed sequencing technology and metagenomic and bioinformatics methods are required [252, 253].

The loss of culturability kinetics observed in these experiments, as well as in other studies of local interactions leading to growth inhibition and/or inactivation [194, 244, 245], is characterised by a rapid reduction of several decimal logarithmic units of the culturable population followed by a gradual tailing off pattern. In the model presented, the rate of loss of culturability is dependent on the product of the frequency of encounters and the probability that after local interaction bacterial growth is inhibited. A significant loss of culturability is detected only if the value of $F\,(S{\cdot}F)^{1/2}$ is above a threshold that depends on the velocity of the cells. That threshold means that, for example, at an average cell velocity of 7µm/s, when the population of *S.* Typhimurium is between 1 and 10^{10} cfu/ml, the required concentration of faecal bacteria is between

10^{10} and 10^{6} cfu/ml, respectively, in order to obtain a frequency of cell encounters sufficient for the inactivation to take place. On the other hand, the hypothesis that the probability of loss of culturability after encounter is related to the mechanism of growth inhibition and therefore should maintain a constant value if estimated for the same local interaction process is supported by the results of these experiments. Indeed, a constant value for the probability of loss of culturability after cell encounter has been estimated for all experimental datasets generated with several concentrations of faecal bacteria and *S.* Typhimurium and inoculation protocols. Thus, the probability of loss of culturability*, P*, could be the parameter characterising the variety of mechanisms of growth inhibition and/or inactivation following cell encounter suggested in other studies [194, 244, 245, 247].

Results presented in this chapter showed for the first time the inactivation of *S.* Typhimurium by local interaction with human faecal bacteria. In the following chapter the transcriptional response of *S.* Typhimurium in mixed cultures with faecal bacteria will be analysed for hypotheses generation on the mechanism of this interaction.

5 Chapter 5 – Studying molecular changes during contact killing of S. Typhimurium by faecal bacteria

5.1 Abstract

As previously reported, the population of *S.* Typhimurium decreased by 10^3-10^4 cfu/ml in 24 h in batch anaerobic mixed cultures with faecal samples from different human donors. Close proximity with faecal bacteria was necessary as this effect was not observed when *S.* Typhimurium was separated from the faecal bacteria by a 0.45 µm pore size membrane. This chapter aims to investigate this phenomenon at the transcriptomic level in order to develop hypotheses on the molecular mechanisms involved in contact inactivation of *S.* Typhimurium by faecal bacteria. Simultaneously, possible changes on the faecal bacterial groups during contact killing of *S.* Typhimurium have been investigated by denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene amplicons.

The microarray slides used for the transcriptional studies were constructed for analysing expression of genes of the genome and plasmids of *S.* Typhimurium strain SL1344. However, DNA genomic hybridisation demonstrated that a high percent of the array features (up to 60%) cross-hybridised, giving positive signals for genomic samples of faecal cultures free from *S.* Typhimurium. Therefore the use of these slides to study gene expression of *S.* Typhimurium in mixed cultures with faecal bacteria is not straightforward. This difficulty was addressed by maximising the concentration of *S.* Typhimurium in the mixed culture with faecal bacteria so that the concentration of transcripts generated by *S.* Typhimurium was equal to or greater than the concentration of transcripts produced by faecal bacteria cross-hybridising onto the slide. In this way, two populations of transcripts were distinguishable in the first 9 h of incubation prior to *S.* Typhimurium inactivation: one population corresponded to genes transcribed by *S.* Typhimurium and the other to faecal bacteria cross-hybridising transcripts. After 9 h, due to the reduction of the population of *S.* Typhimurium in contact with faecal bacteria, *S.* Typhimurium transcripts could not be differentiated from faecal bacteria transcripts cross-hybridising with the microarray features. Many functions associated with the genes expressed in *S.* Typhimurium in co-culture with faecal bacteria were related to stress response.

With regards to changes on the faecal bacterial groups, the molecular profiling of faecal bacteria measured by DGGE did not show any change specifically associated to *S.* Typhimurium inactivation.

Exploring the possible mechanisms of interactions between gut commensals and *S.* Typhimurium at molecular level is relevant for hypotheses development and may be essential for finding new methods for disease prevention and treatment.

5.2 Introduction

The human gut harbours a diverse microbiota that exerts a protective effect against *Salmonella* and other enteropathogens [127, 254]. To inhibit the growth of intestinal pathogens, the commensal bacteria have developed various mechanisms that include the production of bacteriocins, metabolites and SCFA. Other strategies used by the microbiota to outcompete the pathogens consist of consuming common limited resources and altering local pH or ambient oxygen tension [90]. In order to survive these sources of stress, *S.* Typhimurium needs to be competent to sense, react and adapt to changing environments [255]. Like other intestinal pathogens, *S.* Typhimurium is equipped with adaptation systems, mainly transcriptional stress regulators, to cope with these hostile conditions [31].

It has been reported that competition between bacteria can be regulated by the composition of the background community. In other words, the microbial species that define the environment can modify the interaction between competing strains, which implies that the colonisation of a pathogen such as *S.* Typhimurium is dependent on the composition of the gut microbiota [230]. The previous chapters describe the impact of selected GI bacteria and faecal bacteria on the survival of *S.* Typhimurium in mixed cultures, as well as investigate interactions between the commensals and the pathogen by cell-cell contact and mediated by soluble components secreted to the medium. It was observed that *S.* Typhimurium was inactivated in mixed cultures with faecal bacteria and that the inactivation process required local interaction, i.e. cell contact or local proximity between cells.

The aim of this chapter was to study gene expression of *S.* Typhimurium during inactivation by cell contact in mixed cultures with faecal bacteria, using microarrays, as well as possible changes on the faecal population which may take place during this process by DGGE. DGGE studies have previously been carried out for microbial analysis in complex bacterial communities [256, 257]. This technique consists of the separation of DNA fragments of the same size but different base pair sequence based on the electrophoretic mobility of partially denatured DNA in a polyacrylamide gel [258]. On the other hand, the development of the microarray technology has allowed the detection of the expression of vast numbers of genes and the study of how this expression varies as a function of the time and environmental conditions [259]. Microarrays are commonly used to observe gene expression of cells subjected to different environmental conditions, identify mutations in DNA sequences and characterise bacteria in samples containing multiple microbial species such as

environmental samples of soil or human/animal samples of faecal material [260, 261]. With regards to *S.* Typhimurium the publication of the complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2 in 2001 [60] and the later sequence of other strains such as SL1344 by the Sanger Institute (www.sanger.ac.uk/Projects/Salmonella) have prompted transcriptomic studies of this pathogen.

In the present chapter, RNA was isolated after 3.5, 6, 9 and 24 h of incubation from *S.* Typhimurium and faecal bacteria mixed cultures and from pure cultures of *S.* Typhimurium to investigate gene transcription in this pathogen immediately preceding contact killing by faecal bacteria. Since there are many genes involved in the adaptation or survival of *S.* Typhimurium to different stressful conditions [262], a functionality analysis of the genes that were expressed has been performed as they provide valuable signatures to observe the physiological activity of *S.* Typhimurium prior to its loss of culturability in mixed cultures with faecal bacteria.

5.3 Objectives

- Studying gene expression in *S.* Typhimurium immediately prior to its inactivation in mixed cultures with faecal bacteria.
- Studying possible changes on the composition of the faecal bacterial population in mixed cultures with *S.* Typhimurium during the process of inactivation of the pathogen by analysing DGGE profiles.

5.4 Methods

5.4.1 Microarray

5.4.1.1 Array design

Hybridisations were carried out using Agilent microarray slides constructed for the genome and plasmids of *S.* Typhimurium strain SL1344. The arrays were designed using the Agilent eArray programme (8x15k expression format), using the following genomic information: http://www.xbase.ac.uk/genome/salmonella-enterica-subsp-enterica-serovar-typhimurium-sl1344-nctc-13347/XB000024/features?page=2

Agilent microarray design constructed for *S.* Typhimurium strain SL1344 is deposited with GEO database ref. number: GLP15227. Full details of probe sequences can be obtained in the following link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL15227. The design included two 60-oligonucleotide probes for each gene, replicated twice.

5.4.1.2 DNA hybridisations

5.4.1.2.1 Genomic DNA samples for microarray specificity testing

To test the specificity and sensitivity of the microarray slide, genomic DNA isolated from mixed and separated cultures of faecal samples and *S.* Typhimurium were hybridised on the microarray. These hybridisations were non-competitive assays, carried out as one-colour microarrays, where only one DNA genomic sample, labelled with either Cy 3 or Cy 5, was hybridised.

Genomic DNA was extracted from the following samples:

- Sample 1: Culture of faecal bacteria; faeces obtained from a male donor.
- Sample 2: Mixed culture of faecal bacteria and *S*. Typhimurium; faeces obtained from a female donor.
- Sample 3: Mixed culture of faecal bacteria and *S*. Typhimurium; faeces obtained from a male donor.
- Sample 4: Culture of faecal bacteria; faeces obtained from a female donor.
- Sample 5: *S*. Typhimurium pure culture.
- Sample 6: *S*. Typhimurium pure culture.

Two water-jacketed fermenter vessels (Soham Scientific, UK) were prepared as explained in chapter 2; samples containing only faecal bacteria (1 and 4) were taken after inoculating the vessels with 10% (w/v) of faecal sample and incubating them for 24 h. Once the faecal samples had been taken, these vessels were inoculated with 10 ml of a 24 h *S.* Typhimurium culture and samples containing both faecal bacteria and *Salmonella* (2 and 3) were collected, having concentrations of *S.* Typhimurium between 10^7-10^8 cfu/ml and ca. 10^9 cfu/ml of total anaerobes. Samples 5 and 6 were obtained after inoculating a vessel with 10 ml of a 24 h *S.* Typhimurium culture.

5.4.1.2.2 Genomic DNA extraction (Qiagen Genomic DNA Handbook Protocol)

The method was carried out according to the manufacturer's instructions and it is described below. The products used were supplied by Qiagen, catalogue number 13323. Qiagen buffers: Buffer B1: 50 mM Tris·Cl, pH 8.0; 50 mM EDTA, pH 8.0; 0.5% Tween®-20; 0.5% Triton X-100; Buffer B2: 3 M guanidine HCl; 20% Tween-20; Buffer QBT: 750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol, 0.15% Triton X-100;

Buffer QC: 1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol; Buffer QF: 1.25 M NaCl; 50 mM Tris·Cl, pH 8.5; 15% isopropanol.

A sample of 16 ml bacterial culture (24 h incubation) was collected and centrifuged at 3000 x *g* for 20 min. Each bacterial pellet was resuspended in 1 ml of a preparation consisting of 2 µl RNase A solution, (100 mg/ml) and 1 ml Buffer B1 (a bacterial lysis buffer) by vortexing. Then, 20 µl lysozyme stock solution (100 mg/ml) and 45 µl Proteinase K were added to the sample. This was followed by incubation at 37℃ for 30 min. 350 µl Buffer B2 was added and mixed by vortexing for a few seconds before incubation at 50℃ for 30 min. The sample was vortexed for 10 s and applied to a Qiagen Genomic-tip 20/G (an affinity binding column), which had been previously equilibrated by adding 2 ml Buffer QBT and allowing to empty by gravity flow. The column was washed 3 times with 1 ml Buffer QC and then the DNA was eluted by applying 1 ml Buffer QF twice. The DNA was precipitated by adding 1.4 ml isopropanol (Sigma, UK) at room temperature. Centrifugation at 5000 x *g* for 15 min at 4℃ followed and the supernatant was discarded. The DNA was washed with 1 ml cold 70% ethanol (Sigma, UK), vortex-mixed briefly and centrifuged at 5000 x *g* for 10 min at 4℃. After the supernatant was discarded, the DNA was air dried for 10 min. The DNA was dissolved in 50 µl TE buffer and frozen at -20℃. To make Tris EDTA (TE) buffer, a solution of 0.1 M NaCl, 10 mM Tris-HCl (pH 8) and 1 mM EDTA (pH 8) was made in deionised water, autoclaved and stored at ambient temperature.

5.4.1.2.3 Downstream purification of DNA

This was carried out according to [263] and as follows. All the reagents used were from Sigma (UK) unless specified otherwise. DNA preparations that were too viscous for the Genomic-tip 20/G column were diluted further to a final volume of 500 µl TE buffer and transferred into a MaxTract tube (Qiagen, UK), used to enhance the separation between aqueous and organic phases. 500 µl of phenol/chloroform/IAA (50:48:2) was added, vortex-mixed briefly and centrifuged at 18,000 x *g* for 5 min. The aqueous phase, in the top of the tube, was added to 500 µl chloroform in a fresh MaxTract tube, to remove carbohydrates and excess phenol, vortex-mixed briefly and centrifuged at 18,000 x *g* for 5 min. The supernatant was mixed with 20 µl 3 M sodium acetate and 1 ml 100% ethanol. After centrifuging at 18,000 x *g* for 5 min, the supernatant was discarded. Then 500 µl 70% ethanol was added to the pellet to remove the sodium acetate. Following centrifugation as above, the pellet was air dried, dissolved in 50 µl TE buffer and frozen at -20℃.

5.4.1.2.4 DNA digestion

The DNA was digested with *Sau*3AI (Promega, catalogue number R6191), following the manufacturer's protocol, to reduce its average size to fragments of ca. 400 bp. Small pieces of DNA label and hybridise better. An amount of 30 µg DNA per sample, which was considered to be in excess of the amount needed for the entire series of experiments, was digested by addition of 3 µl *Sau*3AI (10U/µl) enzyme, 12 µl 10 x Buffer B (Promega) and H_2O to a total volume of 120 µl as shown in table 5.1 below. The mix was incubated overnight at 37°C.

Samples	**1**	**2**	**3**	**4**	**5**	**6**	
H_2O	63	80	10	90	73	13	(µl)
10 x Buffer B	12	12	12	12	12	12	(µl)
DNA (30µg)	42	25	95	15	32	92	(µl)
Sau 3AI	3	3	3	3	3	3	(µl)

Table 5.1. Amounts of DNA and enzyme used for DNA digestion of each sample

The 120 µl reaction mix was transferred to a MaxTract tube (Qiagen, UK), that had been previously centrifuged for 30 s to pellet the phase separation matrix, and the same volume of phenol/chloroform/IAA (50:48:2) added. After vortex mixing briefly, tubes were centrifuged at 18,000 x *g* for 5 min. The supernatant was transferred to a fresh MaxTract tube, to which 120 µl of chloroform/isoamyl alcohol (24:1) were added. The mix was vortex mixed briefly and centrifuged at 18,000 x *g* for 5 min. The supernatant was transferred to a fresh 0.5 ml tube and 10 µl of 3 M sodium acetate and 300 µl 100% ethanol were added. The mix was vortex mixed briefly and centrifuged at 18,000 x *g* for 5 min to obtain the DNA pellet. It was then washed with 70% ethanol, air dried, dissolved in 50 µl of TE buffer and frozen at -20°C as described above.

5.4.1.2.5 Labelling genomic DNA with fluorescent dye

Precautions: cross-contamination of the samples was carefully avoided; only pipette filter tips were used. All reagents, particularly the enzymes, were kept on ice continuously. The tubes were briefly spun before use to retrieve fluids on the reaction tube wall. After adding fluorophores, the solutions were protected from the light to prevent bleaching, especially from UV light, which could lower signal intensity.

The technique was performed using the BioPrime® Array CGH Genomic Labeling System (Life Technologies) in conjunction with Cy3-nucleotides from Amersham

(Fisher Scientific, UK). Approximately 2 µg *Sau*3A1-digested genomic DNA per sample was added to a 0.5 ml tube and the volume was adjusted to 21 µl with sterile water. After adding 20 µl 2.5 x random hexamer primers the tube was boiled for 5 min, having previously pierced the lid. The tube was then placed on ice and 5 µl 10 x dUTP mix, 3 µl Cy-3 dUTP and 1 µl Exo-Klenow enzyme added. After 3 h incubation at 37°C, the reaction was stopped by adding 5 µl 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 8). EDTA is a chelating agent that binds divalent metal ions such as calcium and magnesium, and so inhibits the function of enzymes such as Klenow polymerase which use these divalent cations. The DNA was purified using a DyeEx 2.0 spin column (Qiagen, UK) and the incorporation of Cy dye was checked with a NanoDrop 1000 Spectrophotometer. After that, the labelled DNA was dried and stored at -20°C.

5.4.1.2.6 Microarray hybridisation of labelled genomic DNA

A volume containing 2 µg fluorescently labelled genomic DNA was air dried in the 'Speed vac', dissolved in 20 µl of sterile water and transferred to a 0.2 ml tube; 25 µl 2 x Agilent Hybridisation Buffer and 5 µl 10 x Agilent Block Solution were added and the samples were boiled for 3 min to denature probe DNAs and then incubated at 37°C for 30 min.

The gasket slide was placed in the hybridisation chamber (Agilent, UK) base and 45 µl of the sample carefully pipetted in the centre of the gasket slide, taking care not to make any bubbles. Then the microarray slide was gently lowered onto the gasket slide. After positioning the hybridisation chamber top, the clamp was tightened and the chamber was rotated to ensure that the samples were moving freely and contained no bubbles. The slides were placed in the Agilent rotary hybridisation oven and incubated at 64°C for 16 h.

5.4.1.2.7 Post hybridisation washing

After incubation, the hybridisation chamber clamp and top were removed. Using forceps and gloves the gasket and microarray slide, still joined together, were placed in a tray containing Wash 1 Solution at 40°C. Ensuring that the slides were fully submerged, the microarray slide was separated from the gasket using forceps. The gasket slide was discarded and the microarray slide was placed in a 50 ml plastic conical (Falcon) centrifuge tube containing Wash 1 solution at 37°C. The tube was rotated for 5 min at room temperature on the roller apparatus (Stuart Scientific roller mixer). Using plastic forceps and gripping the bar code only, the microarray slide was transferred to another 50 ml tube containing Wash 2 solution and was rotated for 5

min at room temperature in the roller apparatus. The slide was then transferred to a third tube containing acetonitrile to remove most residual water and rotated for 1 min at room temperature. The dry slide was slowly removed from the acetonitrile and placed into a slide holder box to protect it from the light.

5.4.1.2.7.1 Microarray wash solutions

Wash 1: Containing 6 x SSPE (Sigma S1027 saline sodium phosphate EDTA buffer), 0.005% NLS (N-Lauryl sarcosine). Prepared by adding 300 ml 20 x SSPE, 0.5 ml 10% NLS and H_2O to 1 litre; **Wash 2**: Containing 0.06 x SSPE and 0.18% PEG200 (Polyethylene glycol 200). Prepared by adding 3 ml 20 x SSPE, 1.8 ml PEG200 and H_2O to 1 litre.

5.4.1.2.8 Microarray scanning

For a pre-scan the slide was introduced into the scan tray of the microarray scanner according to the manufacturer's instructions. The Cy3 green laser light channel and the Cy5 red laser light channel were selected for labelled DNA. In general, the Photomultiplier (PMT) gain of both channels was set to 600. Positive control spots (*ttrC* probe) were used for defining optimal signal intensity. This pre-scan allowed manipulation of the PMT gain to achieve settings which avoided too many pixels possessing intensities outside the dynamic range of the machine. The array field was defined and a full scan was performed. The hybridised slides were scanned with an Axon GenePix 4000B laser scanner (Axon, Foster City, CA) using a resolution of 5 µm per pixel and the feature intensities were quantified using GenePix Pro 6.1 Software (Axon, Foster City, CA).

GenePix Pro includes an integrated Array List Generator which generates GenePix Array List (GAL) files from plain text files. GAL files describe the size and position of blocks, the layout of feature-indicators in them, and the names and identifiers of the printed substances associated with each feature-indicator.

5.4.1.2.9 Genomic DNA hybridisation data analysis

One-colour arrays were used to analyse the sensibility and specificity of the microarray slides. Histograms constructed with the $\log_2$ of the fluorescent intensities detected in the samples with *S*. Typhimurium were used to estimate cut-off intensities or minimum fluorescent intensities detected in hybridised *S*. Typhimurium genes. These cut-off values were applied to find out if cross-hybridising genes showed fluorescence measurements statistically similar to those of *S*. Typhimurium genes. A

t test was carried out to find out if average intensities of features were greater ($p<0.05$) than the cut off.

5.4.1.3 cDNA hybridisations

5.4.1.3.1 RNA sample preparation from mixed cultures of *S.* Typhimurium and faecal bacteria

Studies of *S.* Typhimurium transcription were carried out using mixed cultures of *S.* Typhimurium and faecal bacteria in which the concentration of *S.* Typhimurium was equal to or greater than that of faecal bacteria. To do this, the vessels were inoculated with 10 ml of a 24 h *S.* Typhimurium culture and incubated for 24 h reaching concentrations of ca.10^9 cfu/ml, before being inoculated with a volume of faecal inoculum equal to 10% of the vessel content (Fig. 5.1A). Thus, the initial concentration of *S.* Typhimurium and the total faecal bacteria counts were both ca. 10^9 cfu/ml. Samples were taken and processed for genomic analysis of transcripts 3.5, 6, 9 and 24 h after the addition of the faecal inocula. The concentration of *S.* Typhimurium and faecal bacteria was measured in these samples; the concentration of *S.* Typhimurium was not significantly affected within the initial 9 h of incubation in mixed cultures with faecal bacteria, which allowed *Salmonella* transcripts to be detected because they were not masked by faecal bacteria RNA during this period. The inactivation of *S.* Typhimurium population was initiated after the first 9 h of incubation. After 24 h of incubation of the mixed culture of *S.* Typhimurium and faecal bacteria, the population of *S.* Typhimurium was 10^5-10^6 cfu/ml, whereas the total faecal anaerobes maintained counts of ca. 10^9 cfu/ml throughout the experiment (Fig. 5.2).

The experimental design to set up hybridisation assays is described in Fig. 5.1B. Cultures were prepared by inoculating one vessel containing a 24 h culture of *S.* Typhimurium and two vessels containing sterile medium with faecal inocula. For competitive hybridisation assays, samples were obtained at 3.5, 6, 9 and 24 h after the addition of the faecal inoculum. At each sampling time, samples from the mixed culture, of faecal bacteria and *S.* Typhimurium, had RNA prepared either with Cy3 or Cy5, and mixed with samples labelled with Cy5 or Cy3, respectively, obtained from each of the vessels containing only faecal bacterial; the mixture was hybridised onto the microarray slide. Furthermore, at each sampling time, control hybridisation experiments were set up by differentially labelling a sample from each of the two vessels containing faecal bacteria cultures free from *Salmonella;* the labelled samples were mixed and hybridised onto the slide (Fig. 5.1B).

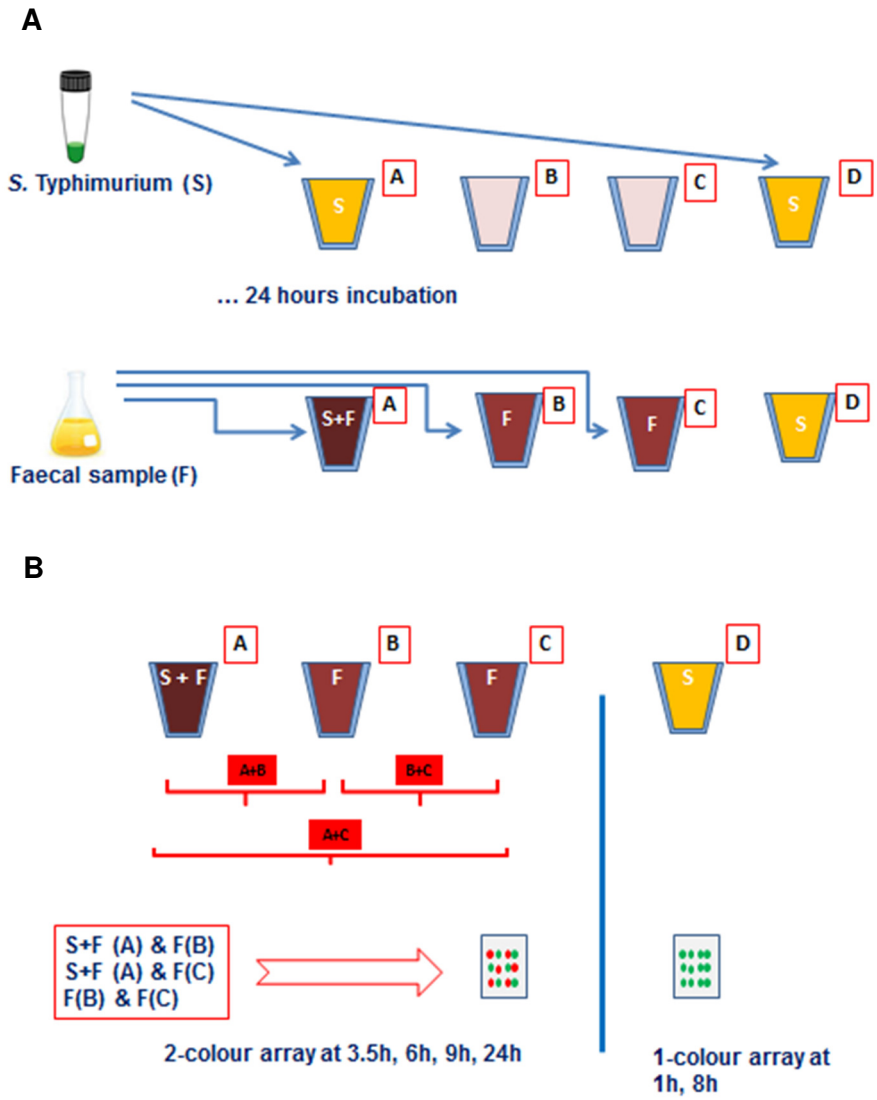


Figure 5.1. RNA sample preparation from mixed cultures of *S.* Typhimurium and faecal bacteria. **(A)** Inoculation with faecal slurry of two vessels containing fresh medium (B and C) and one vessel containing a 24 h *S.* Typhimurium culture (A). **(B)** Samples were collected 3.5, 6, 9 and 24 h after adding the faecal inoculum. Samples from the control *S.* Typhimurium culture (D) were taken 1 and 8 h after inoculating the other vessels with faecal material. For two-colour hybridisations, samples from the mixed culture of *S.* Typhimurium and faecal bacteria, were labelled with either Cy3 or Cy5, and hybridised with labelled samples with either Cy5 or Cy3, respectively, from each one of the vessels with faecal bacteria free from *S.* Typhimurium (A+B and A+C). Control hybridisations were carried out for faecal bacteria by hybridising, after differentially labelling, samples from the two vessels containing faecal bacteria only (B+C). For *S.* Typhimurium control, one-colour hybridisations were carried out with samples from the vessel containing a pure culture of *S.* Typhimurium.

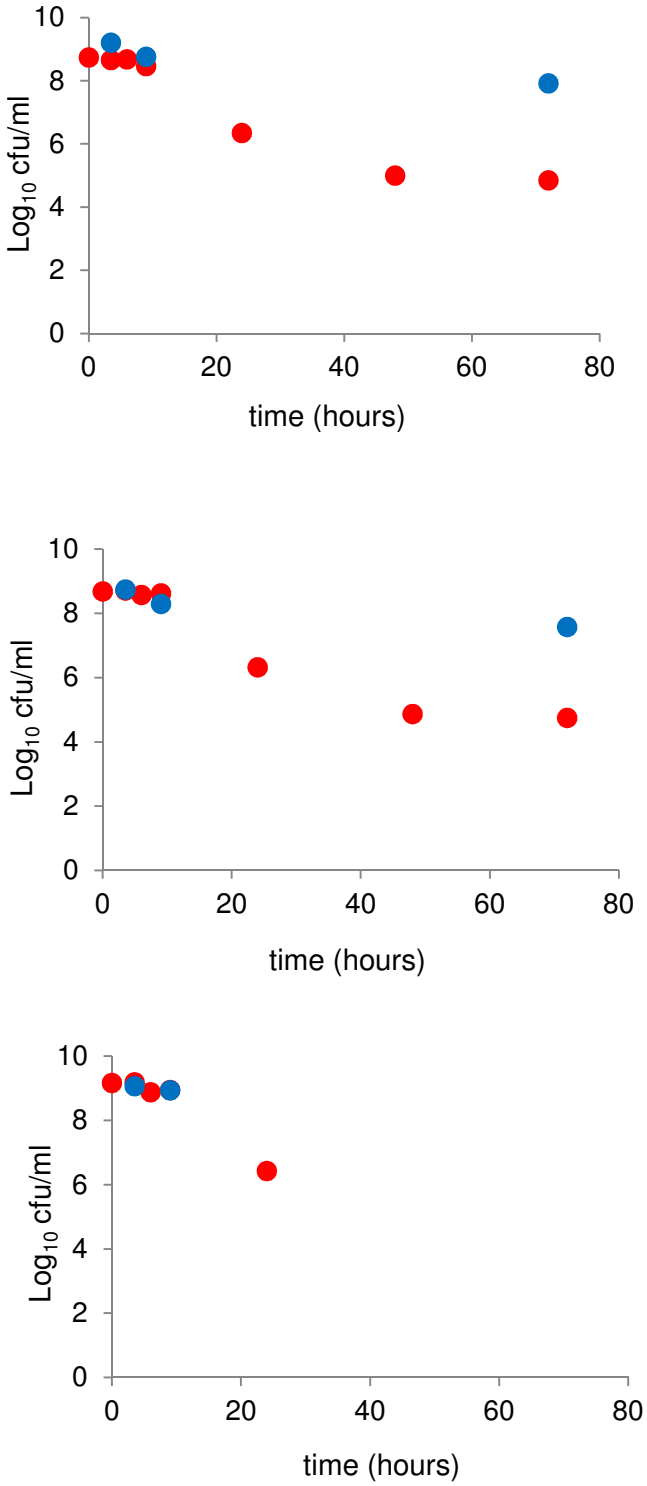


Figure 5.2. Concentration of *S.* Typhimurium and total anaerobes during RNA sample collection. Concentration of *S.* Typhimurium (●) and total anaerobic bacteria (●) in the mixed cultures used to analyse gene expression of *S.* Typhimurium during its inactivation by contact with faecal bacteria. Each plot corresponds to each of the experiments set up with faecal samples from different donors to study transcription.

An extra vessel containing a 24 h culture of *S.* Typhimurium was used to analyse transcription in pure cultures of this pathogen. Samples from the control *S.*

Typhimurium culture were taken 1 and 8 h after inoculating the other vessels described above with the faecal material (Fig. 5.1B). One-colour hybridisation assays were carried out to analyse cDNA in these samples.

This experiment was repeated 3 times with faecal samples from different donors. These samples were used for RNA extraction and to analyse changes in the population composition by DGGE analysis in both mixed cultures of faecal bacteria and *S.* Typhimurium and cultures of faecal bacteria alone.

5.4.1.3.2 RNA extraction

The extraction of RNA and purification was carried out following [264] and as explained here. The cell pellets were thawed on ice for 10 min and resuspended in 500 µl of ice cold TES buffer. This 500 µl cell suspension was transferred to 2 ml tubes containing 0.5 ml (1.2 g) silica beads (0.1 mm) and phenol (pH 4.3)/chloroform (6:1). Cell disruption was achieved by performing a cycle of 2 min at speed setting 6 in the beadbeater (Savant Fastprep FP120) followed by 2 min at 60°C, repeated a total of 3 times. The tubes were chilled on ice for 5 min and then centrifuged at 3,000 x *g* for 1 min at 4°C. The top phase (ca. 500 µl) was transferred to a 2 ml MaxTract High Density (HD) tube (Qiagen, UK) containing 500 µl phenol (pH 4.3)/chloroform (6:1) which was then vortexed and centrifuged at 20,000 x *g* for 5 min at 4°C. This step was repeated once more; then ca. 350 µl from the top phase was added to a 2 ml MaxTract HD tube containing 600 µl of chloroform. After vortexing the tube was centrifuged at 20,000 x *g* for 5 min at 4°C. The top phase (ca. 200 µl) was added to a tube containing 20 µl 3M Na Acetate, pH 4.8 and 550 µl ice cold 95% ethanol. The tube was vortexed and after allowing precipitation for 20 min at -80°C, it was centrifuged at 20,000 x *g* for 20 min at 4°C. The supernatant was discarded and the pellet washed once with 600 µl of 70% ethanol, which was followed by centrifugation at 20,000 x *g* for 10 min at 4°C. The pellet was air dried and resuspended in 100 µl of 1 mM sodium citrate, pH 6.4 (RNA storage solution Ambion, UK).

5.4.1.3.3 DNase treatment

The following was added to 100 µl re-dissolved RNA: 2 µl RNase inhibitor mix (Promega RNasin Ribonuclease inhibitor, N2511); 10µl DNase 10 x reaction buffer mix (Promega RQ1 RNase free DNase, M6101) and 2 µl RNase free DNase. It was then incubated at 37°C for 30 min.

5.4.1.3.4 Post-DNase treatment purification

It was decided to purify the RNA with phenol rather than risk reducing yield by using the Qiagen columns. For this process, 2 ml MaxTract HD tubes (Qiagen, UK) were used. A volume of 0.5 ml of saturated phenol, pH 4.3: chloroform: isoamylalcohol (IAA) (25:24:1) was pipetted into the MaxTract tube. The RNA sample was then added to the tube and vortexed, followed by centrifugation at 23,000 x *g* for 3 min. Meanwhile, 0.5 ml of chloroform: IAA (24:1) was pipetted into a fresh MaxTract tube. The top layer of the centrifuged sample was transferred into this fresh tube and vortexed. It was then centrifuged again at 23,000 x *g* for 3 min and the supernatant transferred to a 2 ml Eppendorf tube. 50 µl 3 M sodium acetate buffer solution (Sigma, UK) was added followed by 2.5 volumes of 100% ethanol. The tubes were placed at -80℃ for 1 h and then centrifuged at 23,000 x *g* for 20 min at 2℃. The supernatant was removed by pipette, being careful not to lose the RNA pellet. After adding 1 ml of 70% ethanol and inverting the tube gently so that the pellet came loose, the tube was centrifuged at 23,000 x *g* for 5 min. The supernatant was removed and the tube was centrifuged at 23,000 x *g* for 30 s and the rest of the supernatant removed. The pellet was air dried and dissolved in 100 µl of RNA storage solution (Ambion, UK).

5.4.1.3.5 RNA quality control

In addition to the NanoDrop 1000 spectrophotometer (Thermo Scientific, UK), Agilent 2100 Bioanalyzer (Agilent Technologies, USA) was used to assess the quality of the total RNA extracted from mixed and separated cultures of faecal and *S.* Typhimurium. The procedure was carried out using an Agilent RNA 6000 Nano Kit. This bioanalytical tool is based on microcapillary electrophoretic RNA separation to estimate the integrity of RNA samples unequivocally [265]. The quality of bacterial mRNA can easily be determined through visual inspection of the electropherogram of each sample. The Bioanalyzer electropherogram of total RNA shows two clear ribosomal peaks corresponding to 16S and 23S for prokaryotic RNA and a relatively flat baseline between the 5S and 16S ribosomal peaks. The rRNA ratios (16S/23S) indicate RNA quality in terms of degradation; for good quality RNA, the height of the 23S rRNA peak should be at least twice that of the 16 S rRNA peak.

5.4.1.3.6 cDNA synthesis and labelling

All reagents were supplied by Amersham Pharmacia unless specified otherwise. To anneal primers to RNA, 10 µg of each RNA sample was transferred into a 1.5 ml Eppendorf tube and the contents were vacuum dried (SpeedVac Concentrator, Savant). Then 9.7 µl of a preparation of stock primers consisting of 7.7 µl

diethylpyrocarbonate (DEPC)-treated water and 2 µl random nonamers were added to the tube and the RNA was gently resuspended. The sample was incubated at 70°C for 5 min and then cooled in ice for 10 min. To label the sample, 2 µl 10x RT buffer, 2 µl 0.1 M DTT, 0.6 µl 50 x dNTPs, 3 µl of 100 nMol Cy3 or Cy5 dCTP dye and 3 µl of Affinity script reverse transcriptase (Agilent, UK) were added to the tube. The reaction mix was incubated at room temperature for 10 min and then at 42°C overnight.

To degrade the RNA and so remove it from the newly synthesised DNA, 15 µl of freshly made 0.1M NaOH was added and the tube was incubated at 70°C for 10 min. Then 15 µl of 0.1M HCl were added to neutralise the alkali. DyeEx columns were used for probe purification. Unincorporated nucleotides remained on top of the column and cDNA was eluted into 1.5 ml Eppendorf tubes.

Hybridisation of the labelled cDNA was carried out as described for genomic DNA, but the 2 µg of cDNA added to the microarray comprised a mixture of the two cDNA samples differentially labelled. Post hybridisation washing and slide scanning was carried out as described above.

5.4.1.3.7 cDNA hybridisation data analysis

5.4.1.3.7.1 Analysis of the intensities detected in two-colour cDNA hybridisation experiments with cDNA from faecal cultures and mixed cultures of *S.* Typhimurium and faecal bacteria

In standard experimental conditions, the fluorescence intensities of the two cDNA labelled samples hybridised onto the slide are proportional to each other

$$F1_i \propto F2_i \tag{1}$$

where $F1_i$ is the fluorescence intensity from cDNA sample 1 (labelled with either Cy3 or Cy5) and $F2_i$ from cDNA sample 2 (labelled with the other dye, i.e. either Cy3 or Cy5) detected in the *i-th* feature. By taking logarithms in equation (1) the following expression is obtained:

$$\log_2(F1_i) = R_i + \log_2(F2_i) \tag{2}$$

And therefore the logarithm to the base 2 of the ratio between intensities for the *i-th* feature is

$$R_i = \log_2\left(\frac{F1_i}{F2_i}\right) \tag{3}$$

In standard microarray analysis and after normalization, which can be done by using either global or local average intensities or LOWESS non-parametric regression, it is a common approach to consider that when R_i takes values greater than 2 the gene corresponding to the *i-th* feature on the array is differentially expressed in one of the samples [266]. The analysis here was, however, different from existing approaches as the two populations of intensities shown in Fig. 5.3 were separated so that genes expressed by *Salmonella* can be separated from genes detected in the faecal sample only. One population includes transcripts expressed in *S.* Typhimurium and maybe some of the transcripts of faecal bacteria cross-hybridising on the array; the other population includes only features cross-hybridising with faecal bacteria cDNA.

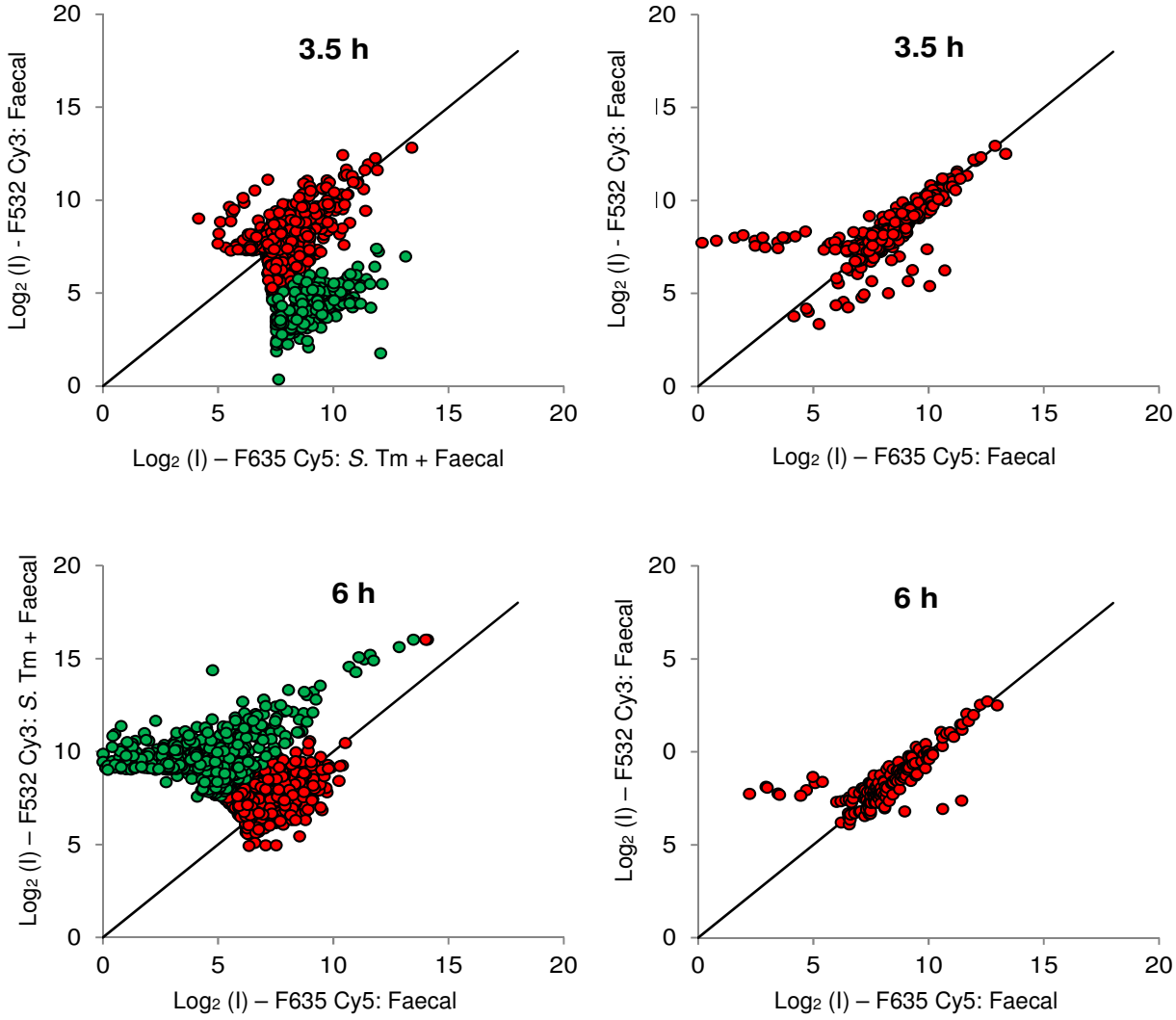


Figure 5.3

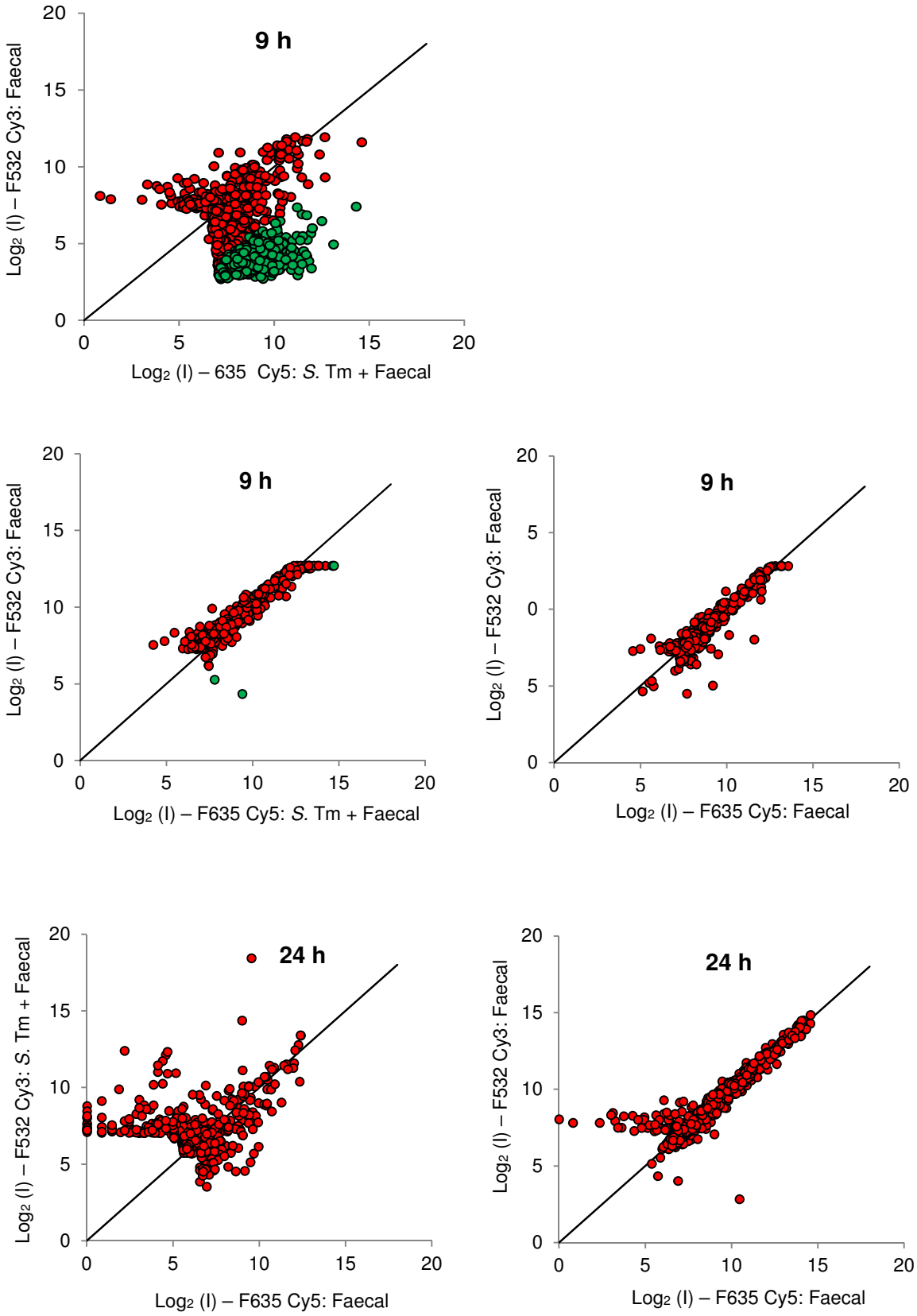


Figure 5.3. Intensities of the transcripts. Figures in the left: Intensities of transcripts in competitive hybridisation experiments of samples from mixed cultures of *S.* Typhimurium, *S.* Tm, and faecal bacteria (Green) *vs* samples from faecal cultures free from *Salmonella* (Red). Figures in the right: Intensities of transcripts in competitive hybridisation experiments between samples from faecal cultures free of *Salmonella* established with the same inoculum (Red).

The approach for data standardisation and selection of features expressed in each population used in this study is based on that developed to evaluate transcription based on microarray hybridisations [267] and it is as follows. Data standardisation was based on a set of 100 features randomly selected from the group of features visually identified as expressed in the faecal sample only. These were used as reference intensities for standardisation. These features, in which only transcripts from faecal bacteria were hybridised, should have similar fluorescent signals for both samples, with and without *Salmonella*. These values could therefore be used as a reference to standardise the whole hybridisation dataset. In order to do this, the average of the logarithm of the ratio between intensities measured in the reference features was estimated and denoted as R_r. The logarithms of the ratio between intensities measured from all features in the array, including reference features, were standardised by subtracting R_r. Therefore the standardised logarithm of the ratio between intensities for the *i-th* feature is calculated $R_i' = R_i - R_r$

In order to determine if the intensities for both channels were significantly different, it was assumed that the logarithm of the ratio between intensities has a Gumbel distribution with density function

$$g_R(r) = \frac{1}{b} e^{\frac{A-r}{b}} e^{-e^{\frac{A-r}{b}}} \qquad -\infty < r < \infty \tag{4}$$

where A and b are the location and scale parameters, respectively. The expected value of R is $E(R) = A + 0.5772\, b$ while the variance is $V(R) = (\pi b)^2/6$. The parameter b is invariant and estimated from the reference features. The location parameter, A, is a linear transformation of the ratios and therefore its use to evaluate the value of the ratio between intensities of a feature is straightforward. When the scale parameter, b, is known, the Gumbel distribution belongs to the exponential family and a conjugate family of distributions exists for the parameter A, i.e. the prior and posterior distributions of A differ only in the value of a finite parameter vector. A conjugate family of distributions for the parameter A of the Gumbel distribution is given by:

$$f_A(a) = \frac{1}{b} \frac{\beta^\alpha}{\Gamma(\alpha)} e^{\frac{a}{b}\alpha} e^{-\beta e^{\frac{a}{b}}} \qquad -\infty < a < \infty \tag{5}$$

where $\Gamma(\alpha) = \int_0^\infty t^{\alpha-1} e^{-t} dt$ is the gamma function. It is worth to notice that $X = e^{\frac{A}{b}}$ is

distributed according to a gamma distribution with shape parameter *α* and scale parameter 1/*β*.

It can be demonstrated that a priori, the expected value of *A* is

$$\mathrm{E}[A] = b\left(\psi(\alpha) - \ln\beta\right) \tag{6}$$

where $\psi(\alpha) = \frac{\Gamma'(\alpha)}{\Gamma(\alpha)}$ is the digamma function and its variance is

$$\mathrm{V}[A] = b^2\left(\frac{1}{\alpha} + \frac{1}{2\alpha^2}\right) \tag{7}$$

Bayes theorem gives a posterior distribution for *A* as:

$$f_A(a|r_1..r_n) = \frac{g(r_1..r_n|a)f(a)}{\int_{-\infty}^{\infty} g(r_1..r_n|a)f(a)da} \quad -\infty < a < \infty \tag{8}$$

where $g(r_1..r_n|a) = \frac{1}{b^n} e^{\frac{na}{b}} e^{-\sum_{i=1}^{n}\frac{r_i}{b}} e^{-\tilde{k}e^{\frac{a}{b}}}$ is the density function of the sample of measurements conditioned by *A* = *a*; *n* is the number of independent measurements for the gene in study and $\tilde{k}$ is a sample statistic: $\tilde{k}(r_1..r_n) = n \cdot e^{|\bar{r}/b|}$ where $\bar{r}$ is the mean value of the ratios measured for the gene in study. $\tilde{k}$ is a function of the absolute value of the average of the ratios so that the sign of the difference between the log intensities do not affect the result. $\tilde{k}$ takes values in the interval [1, ∞).

Therefore, substituting

$$f_A(a|r_1..r_n) = \frac{e^{\frac{na}{b}} e^{-\tilde{k}e^{\frac{a}{b}}} e^{\frac{a}{b}\alpha} e^{-\beta e^{\frac{a}{b}}}}{\int_{-\infty}^{\infty} e^{\frac{na}{b}} e^{-\tilde{k}e^{\frac{a}{b}}} e^{\frac{a}{b}\alpha} e^{-\beta e^{\frac{a}{b}}} da} \quad -\infty < a < \infty \tag{9}$$

The denominator integrates to $b\frac{\Gamma(\alpha+n)}{\left(\beta+\tilde{k}\right)^{\alpha+n}}$ and thus the posterior distribution of *A* is equal to

$$f_A(a|r_1..r_n) = \frac{1}{b}\frac{\left(\beta+\tilde{k}\right)^{\alpha+n}}{\Gamma(\alpha+n)} e^{\frac{a}{b}(\alpha+n)} e^{-(\beta+k)e^{\frac{a}{b}}} \quad -\infty < a < \infty \tag{10}$$

which belongs to the same family as the prior distribution with parameters $\alpha' = \alpha + n$ and $\beta' = \beta + k$. *A posteriori*, the expected value of *A* is $\mathrm{E}[A] = b\left(\psi(\alpha+n) - \ln(\beta+k)\right)$ and its variance is

$$\mathrm{V}[A] = b^2\left(\frac{1}{\alpha+n} + \frac{1}{2(\alpha+n)^2}\right). \quad (11)$$

Thus, the probability that a feature is expressed in *Salmonella* is estimated as

$$P(A \geq a_0 \,|\, r_1..r_n) = \int_{a_0}^{\infty} f_A(a \,|\, r_1..r_n)\, da \quad (12)$$

For features expressed only in faecal samples, or features similarly expressed in both samples, the value of the location parameter, *A*, is centred on a value a_0. To avoid an arbitrary asymmetry in the results, the hypothesis test is made by using only one tail of the posterior distribution of *A*.

The estimation of the parameters was carried out with the ratios measured from the selected reference features expressed only in the faecal sample.

The parameter *b* of the Gumbel distribution was estimated by the method of moments as $\hat{b} = \frac{\sqrt{6}}{\pi} sd_r$ where sd_r represents the standard deviation of the ratios measured from the reference features.

After data standardization, the ratios of genes expressed only in faecal samples are expected to be centred on 0. Thus, the centred value, a_0, of the parameter *A* is estimated by the method of moments as $\hat{a}_0 = 0 - 0.5772 \cdot \hat{b}$

To estimate the parameters α and β in the prior distribution of A, the transformation $X = \exp(A/b)$ was use; *X* has a gamma distribution with shape parameter α and scale parameter $1/\beta$ which were estimated by the maximum likelihood method as described by [268]. For the gamma function, the approximation derived by [269] was used. The digamma function was approximated by using the formula 6.3.16 p.259 of [270].

The analysis was carried out by using the in-house developed Excel macro freely available online http://www.ifr.ac.uk/safety/ArrayLeaRNA/.

5.4.1.3.7.2 Analysis of the intensities detected in one-colour hybridisation experiments with cDNA samples from pure cultures of *Salmonella*

The $\log_2$ intensity was assumed to have a Gumbel distribution. The density function of the Gumbel distribution was fitted to the empirical histogram in order to estimate the percentile 0.5% or value of the $\log_2$ intensity smaller than 99.5% of all measurements. All features with greater intensities than this value were considered expressed in *S.* Typhimurium.

5.4.1.3.7.3 Cluster analysis of transcriptional profiles

The transcriptional profile of each sample was coded by assigning a value of 1 to expressed genes and 0 to the others. Cluster analysis of the sample transcriptional profiles was carried out by using the Nei coefficient of association, CA, [271] as distance measurement. This was estimated as CA= $2a/(2a+b+c)$, where *a* is the number of genes expressed in both samples and *b* and *c* are the number of bands expressed only in one of the samples.

The matrix of dissimilarity distances estimated as 1-CA was subjected to the UPGMA (unweighted pair-group method with arithmetic average) hierarchical clustering method using the SAS 9.3 software.

The number of clusters was determined by plotting Eigen values *vs.* number of clusters and visually assessing the number of clusters until which the associated Eigen value changed significantly.

5.4.1.3.7.4 Correspondence analysis of transcriptional profiles

It was run on the transcriptional profiles of the samples, coded by assigning a value of 1 to expressed genes and 0 to the others, using the SAS 9.3 software.

5.4.1.3.7.5 Functionality analysis of transcriptional profiles

To carry out the analysis of cellular functions and metabolic pathways associated with expressed genes, a previously constructed genome network for *S*. Typhimurium was applied [272]. This bipartite genome scale network includes all genes in the genome and plasmids of *S*. Typhimurium SL1344. The network is bipartite because network edges connect two sets of nodes. All genes described in the Genome Project NCBI database for *S*. Typhimurium SL1344 constitute one of these sets of nodes. The other set of nodes includes metabolic pathways and cellular functions, according to the KEGG database, the CMR-TIGR database [89][22][22] and the COGs (Clusters of Orthologous Groups of proteins) functional categories obtained from the Genome

Project NCBI database [273][23][23]. The number of nodes is 5153, from which 4717 are genes and the remaining 436 nodes represent metabolic pathways and cellular functions. There are 11626 edges between these two sets of nodes. The use of this network for functional analysis of transcriptional profiles has been demonstrated for *S*. Typhimurium exposed to environmental stresses [274].

Previous multivariate analysis were carried out using samples profiles obtained in replicated conditions in order to explore similarities and consistence between replicated samples under the same conditions. For functional analysis, profiles were constructed for each assay condition, i.e. culture condition and sampling time. The criterion used to decide whether genes were expressed in a given condition was as follows: only genes detected in more than half the independent replicates at a given sampling time and culture condition and/or at two consecutive sampling times under the same culture conditions were considered expressed for that culture conditions and sampling time. In this way, expression profiles were prepared for each assay condition. The genes forming each profile for each condition were used to extract from the genome network the sub network containing all cellular functions and metabolic pathways associated to that condition. This means that the genome network was applied to translate expressed genes in each condition into cellular functions and metabolic pathways. To consider a metabolic pathway or cellular function expressed in a given condition the number of genes belonging to that function or pathway and expressed under that condition had to be significant. Significance was determined with the following test.

Statistical test on the significance of the changes in expression of metabolic pathways and cell functional categories

For each condition, the statistical evaluation of the up-regulation of a particular metabolic pathway or functional category was carried out as follows:

Let X denotes the number of expressed genes belonging to a metabolic pathway or cell functional category. If X follows the commonly assumed hypergeometric distribution, then

$$P(X=k)=\frac{\binom{T-M}{n-k}\binom{M}{k}}{\binom{T}{n}} \qquad (k=1\ldots n) \qquad (13)$$

where:

T = total number of genes evaluated in the genome and plasmids;

M = number of genes in the total genome and plasmids known to belong to that metabolic pathway or cell functional category;

n = total number of expressed genes in the condition.

The probability that the number of genes associated to that metabolic pathway or cell functional category is equal to or greater than k, under the hypothesis that no differential expression took place (i.e. the p-value associated to an observed k number) can be calculated as

$$p(k) = P(X \geq k) = \sum_{i=k}^{\min\{n,M\}} P(X = i) \qquad (14)$$

When the p-value was smaller than 0.05, the $X=k$ event being unlikely to have happened purely by chance, the metabolic pathway, cell functional category or operon was considered to be significantly differentially expressed.

5.4.2 Denaturing Gradient Gel Electrophoresis

5.4.2.1 DNA extraction (FastDNA spin kit for soil (Qbiogene)-A VTT modified protocol)

Samples of 1.5 ml were obtained from the cultures in the fermenter vessels and were frozen at -80°C prior to DNA extraction using the methods described below.

The method was used according to [275] and as explained here: After thawing the samples in ice, 200 µl were dispensed into a 10 ml sterile tube followed by 978 µl sodium phosphate buffer and 122 µl MT buffer; the tube was vortex mixed thoroughly until completely homogenised. The tube was then kept at 4°C for 1 h. Samples (1ml) were transferred to a Lysing Matrix E Tube. Using FastPrep Instrument (Bio 101 Savant, Holbrook, NY) samples were lysed three times for 60 s at a speed of 6.5 m/s, cooling them in ice (1 min) between lysing steps. The Lysing Matrix E Tube was then centrifuged for 1 min at full speed. The supernatant was transferred to a clean Eppendorf tube, 250 µl PPS reagent added and mixed by shaking the tube by hand 10 times. The tube was centrifuged for 5 min at 14,000 x *g*; the supernatant was transferred to a sterile 15 ml tube and 1 ml binding mix suspension added. The tube was inverted by hand for 2 min and after letting it stand in a rack for 3 min to allow the settling of the silica matrix, 1 ml supernatant was removed and discarded. The

binding matrix was resuspended in the remaining supernatant. Approximately 600 µl of the mixture were transferred to a spin filter tube and centrifuged for 1 min at 14,000 x *g*. This was followed by the addition of 500 µl SEWS-M (wash buffer) into the spin filter tube before centrifugation for 1 min at 14,000 x *g*. The wash was repeated twice more and then centrifuged for 2 min at 14,000 x *g* to remove from the matrix any residual SEWS-M wash solution. The spin filter was removed and placed in a fresh catch collection tube where it was air dried for 5 min at room temperature. DNase/Pyrogen Free Water (DES) (ca. 100 µl) was added and the matrix was gently stirred on the filter membrane with a pipette tip for efficient elution of DNA. It was then centrifuged at 14,000 x *g* for 1 min. The resulting eluted DNA was stored at -80℃.

5.4.2.2 Polymerase chain reaction (PCR)

The DNA was used as a template to amplify the variable regions V6–V8 of the bacterial 16S rRNA genes with primers U968-GC-f (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and L1401-r (5'-CGG TGT GTA CAA GAC CC-3') [276]. A GC clamp is included at the 5'end of the forward primer. Each PCR reaction mixture of 50 µl contained 0.4 µl of 25 mM dNTPs (Thermo scientific, UK), 5 µl of 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl 10 x buffer, 1.5 µl of 2U/µl Dynazyme II (Thermo scientific, UK), 40.1 µl of deionised water, 1 µl of each primer (10 pmol/ µl) and 1 µl DNA solution. PCR was performed using the Biometra TProfessional Basic (Thistle scientific, UK) thermal cycler as follows: initial denaturation at 94℃ for 5 min, denaturation at 94℃ for 30 s, primer annealing at 50℃ for 20 s and primer extension at 72℃ for 40 s. The PCR amplification was run for a total of 35 cycles followed by a final extension for 7min at 72℃ and cooling at 4℃. The presence of the product was confirmed by visualising on a 0.8% agarose gel. Primers were obtained from Sigma-Genosys (UK) and annealing temperatures were calculated using the website http://www.basic.northwestern.edu/biotools/olig℃alc.html [277].

5.4.2.3 PCR product clean-up

PCR products (greater than 200 bp) were cleaned following Cycle-Pure Spin Protocol (E.Z.N.A.) to remove short primers, unincorporated dNTPs, enzymes, short (failed) PCR products, and salts from PCR reactions. The PCR product was transferred to a 1.5 ml microcentrifuge tube and 5 volumes of CP buffer added. The samples were vortex mixed for 30 s and the tube was briefly spun to collect any drops from the inside of the lid. A HiBind DNA Mini Column was placed in a 2 ml collection tube and the mix was added. The tube was centrifuged for 1 min at 13,000 x *g* at room

temperature. The flow-through liquid was discarded and the HiBind DNA Mini Column was placed back into the same collection tube. A volume of 700 µl DNA wash buffer was added and the column was centrifuged at 13,000 x *g* for 1 min. The flow-through liquid was discarded and the HiBind DNA Mini Column was placed back into the same collection tube. A volume of 500 µl of DNA wash buffer was added and the column was centrifuged at 13,000 x *g* for 1 min. The flow-through liquid was discarded and the HiBind DNA Mini Column was placed back into the same collection tube. The empty HiBind DNA Mini Column was centrifuged at 13,000 x *g* for 2 min to dry the column matrix, which is critical for the removal of ethanol from the HiBind DNA column. It was then placed into a clean 1.5 ml microcentrifuge tube and 50 µl elution buffer (10 mM Tris, pH 8.5) added directly onto the centre of the column matrix. After incubation at room temperature for 2 min, the tube was centrifuged for 1 min at 13,000 x *g* to elute the DNA.

5.4.2.4 Gel preparation and running conditions

Approximately 150 ng of PCR product from the samples containing faecal samples and 100 ng of PCR product from the *S*. Typhimurium pure cultures were analysed by DGGE with the BioRad D Code Universal Mutation Detection System. PCR samples and DNA ladders (made from pooled amplified PCR products from a faecal sample) were applied to 8% (wt/v) polyacrylamide gels and TAE buffer (20 mM Tris, 10 mM acetic acid, 5 mM EDTA, pH 8.3), with 40-60% gradient of formamide that increased in the direction of the electrophoresis, plus 0.08% (wt/v) ammonium persulphate (Sigma, UK) and 0.14% NNN'N'-tetramethylethylenediamide (Fisher Scientific, UK) to make the gel polymerise. The denaturing gel was overlaid by a stacking gel which lacked denaturing agents. Electrophoresis was carried out in 0.5 x TBE buffer (Fisher Scientific, UK) at 60℃ at a constant voltage of 85 V. The DNA molecules are immobilised at differing points in the acrylamide gel depending on the electrophoretic mobility of a partially melted DNA molecule in it, as partial separation of the DNA strands (denaturisation) prevents further migration. After 16 h the gels were stained using SYBR Green (Invitrogen, UK) for 45 min, rinsed with distilled water and visualised using the BioRad Pharos FX Plus Molecular Imager with an external laser at 65% voltage. Images were captured using Quantity One software and exported to Phoretix 1D Pro for band profile analysis.

5.4.2.5 Cluster analysis of DGGE

Cluster analysis of DGGE profiles was carried out as described for transcriptional profiles, but in this case, sample profiles were generated by assigning a value of 1 to

bands present in the gel and 0 if not detected at that position. Cluster analysis of the DGGE profiles was carried out by using the Nei coefficient of association, CA, [271] as distance measurement. The distance between each pair of samples was estimated as CA= $2a/(2a+b+c)$, where *a* is the number of bands in common in both samples and *b* and *c* are the number of bands observed only in one the samples. As above, the matrix of dissimilarity distances estimated as 1-CA was subjected to the UPGMA (unweighted pair-group method with arithmetic average) hierarchical clustering method using the SAS 9.3 software.

5.4.3 Quantification of nucleic acid

Nucleic acids were regularly monitored during processing using the Nanodrop instrument and software (Thermo Scientific, UK) following the manufacturer's instructions. After selecting the nucleic acid (DNA or RNA) to be quantified, the instrument was cleaned with lint-free tissue moistened with ultra-pure water. The instrument was calibrated by taking a blank measurement with 1 µl ultra-pure water and dried with tissue. The measurements were carried out using 1 µl of sample and cleaning with tissue between samples. Nucleic acid peaks were normally at 260 nm. Peaks at 280 nm were an indication of protein contamination and samples with high absorption at 280 nm were re-extracted. An absorbance ratio (260/280) of between 1.7 and 2 denoted a pure extraction of good quality.

The approximate quantity of nucleic acid could also be estimated by running an agarose gel and comparing the intensity of the bands obtained with the known quantities of the Hyper I DNA ladder (Bioline Ltd). Agarose gels had a concentration of 0.8% (Melford Laboratories Ltd) and were run in 0.5 x Tris borate EDTA (TBE) gel electrophoresis buffer (Fisher Scientific, UK). A positive and a negative control were tested on each gel and the sample volume was typically 5-10 µl. To apply a loading dye, 1 µl spots of loading buffer (0.015% bromophenol blue (Sigma, UK), 10% glycerol (Sigma, UK) in 0.5 x TBE buffer) were dispensed onto parafilm (M Laboratory) and mixed with samples prior to loading into the gel. The gels were stained in 1 mg/l ethidium bromide for 30 min, briefly rinsed in deionised water and visualised with transilluminating UV light using the AlphaImager software (ProteinSimple).

5.5 Results

5.5.1 Analysis of the specificity and sensibility of the microarrays for *S.* Typhimurium by genomic DNA hybridisations

With the aim of analysing the sensitivity and specificity of the microarray slides, DNA from mixed and separated cultures of faecal bacteria and *S.* Typhimurium were extracted and hybridised onto the arrays. One-colour arrays were used for this purpose. Samples containing *S.* Typhimurium had higher intensity signals than faecal samples with no *S.* Typhimurium. To estimate a cross hybridisation indicator in faecal samples free from *Salmonella*, a cut-off intensity level was estimated from the hybridisation of genomic DNA obtained from the sample containing *S.* Typhimurium and faecal bacteria. Cut-off intensities were estimated as the 0.5 percentile of the empirical histogram i.e. the intensity value smaller than 99.5% of all these measurements (Fig. 5.4). Cut-off $\log_2$ intensities were equal to 8.7 for both the female and male faecal samples. A *t* test was carried out to find out how many features printed on the array cross-hybridised with the DNA from the faecal sample free from *Salmonella* and therefore had average intensities greater ($p<0.05$) than the cut off. The percent of features in the array cross-hybridising with faecal bacterial DNA was 28% for the male faecal sample and 63% for the female sample (Table 5.2). Other methods of estimation and/or donors could result in different values for the percentage of cross-hybridisation, which would very likely be high and question the use of this array in mixed cultures of *S.* Typhimurium with faecal bacteria. Therefore, the use of these array slides to measure gene expression in *S.* Typhimurium when in mixed cultures with faecal bacteria is not straightforward.

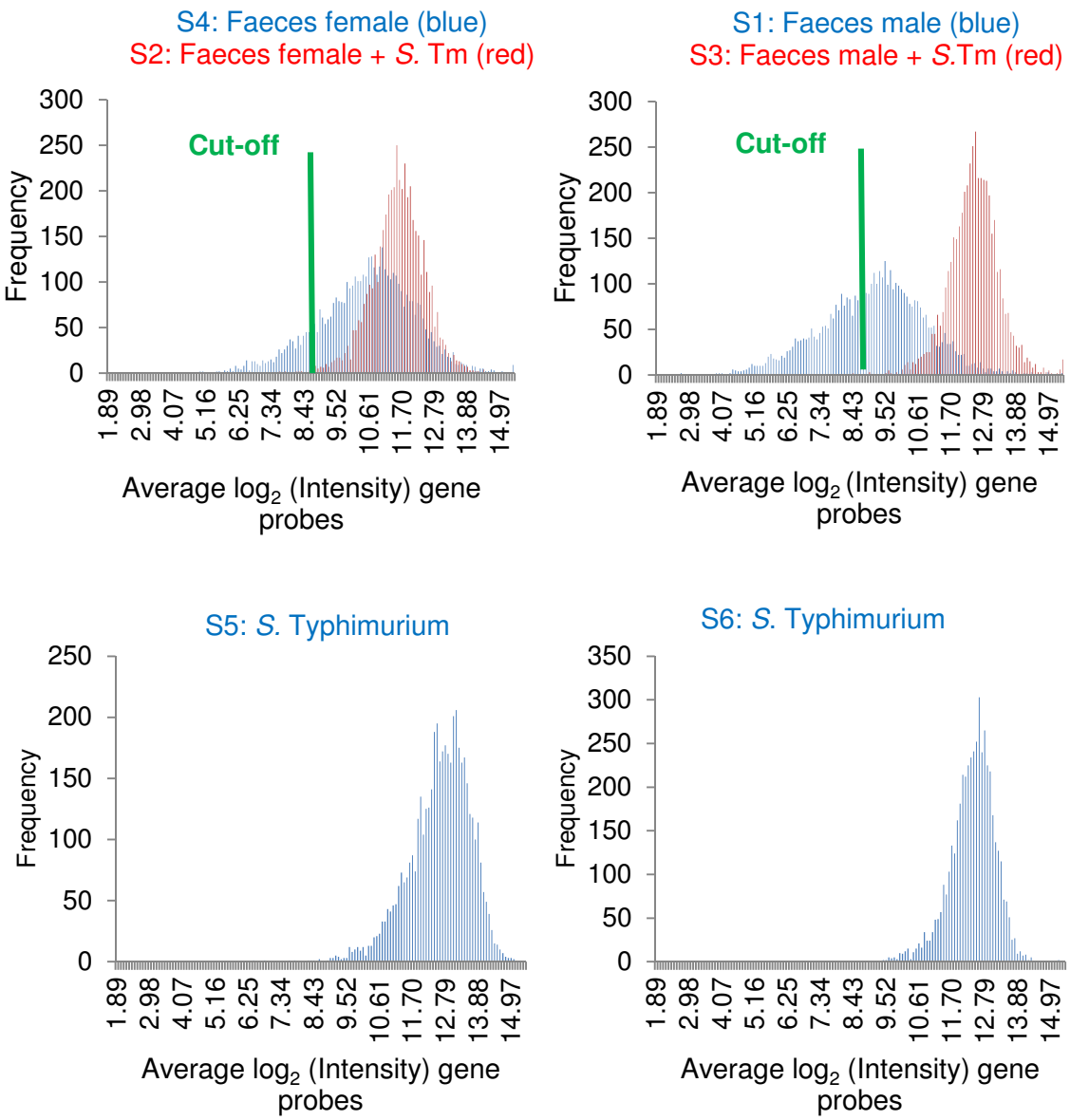


Figure 5.4. DNA genomic hybridisations. Log_2 of intensities from DNA genomic hybridisations (one colour arrays) of samples extracted from cultures of faecal bacteria and/or *S.* Typhimurium (*S.* Tm).

Sample	DNA extracted from	Number of genes	Mean	Standard deviation	Median	* Percentile 0.5 (Cut-off)	** Genes with intensity >cut-off	*** %
S1	Faeces (male)	4744	9.07	1.74	9.17	8.70	1323	28
S3	Faeces (male) + *S.* Typhimurium	4744	12.42	0.84	12.43			
S4	Faeces (female)	4744	10.48	1.59	10.60	8.70	3009	63
S2	Faeces (female) + *S.* Typhimurium	4744	11.51	0.90	11.53			
S5	*S.* Typhimurium	4744	12.55	1.01	12.67			
S6	*S.* Typhimurium	4744	12.33	0.74	12.41			

*Percentiles were estimated from histograms constructed from measurements

**Cut-off =percentile 0.5 (error equal to 5/1000) of the same faecal sample with *S.* Typhimurium, i.e. number genes cross-hybridising with DNA from S1 were calculated using percentile 0.5 of the histogram constructed with measurements from sample S3 as cut-off. Similarly cross hybridisations in S4 were estimated using percentile 0.5 from histogram S2 as cut off

*** proportion of genes cross-hybridising in the faecal sample

Table 5.2. Intensities (Log_2) from DNA genomic hybridisations (one-colour arrays) of samples extracted from cultures of faecal bacteria and/or *S.* Typhimurium cultures. In S1 and S4 a t test was used to decide whether the intensity was greater than the cut-off ($p<0.05$).

5.5.2 Analysis of the intensities detected in the two-colour hybridisation experiments to quantify gene expression in *S.* Typhimurium prior to its inactivation in mixed cultures with faecal bacteria

Samples from mixed cultures of *S.* Typhimurium and faecal bacteria in competitive hybridisation with samples from cultures of faecal bacteria

Two groups of intensities could be observed after competitive hybridisation of these samples. The group with a higher intensity in one of the channels included genes expressed by *S.* Typhimurium. The other group, showing intensities with similar values in both channels, belonged to transcripts produced only by faecal bacteria that cross-hybridised on the slide (Fig. 5.3) as explain below:

1) The group of intensities with higher values in one of the two fluorescent channels is identified with features where cDNA corresponding to transcripts produced by *S.* Typhimurium hybridised. Features with hybridised cDNA corresponding to genes expressed only by *S.* Typhimurium had a detectable intensity signal in the fluorescent channel corresponding to the label of the mixed culture sample, whereas no signal was detected in the other channel. Array features with hybridised transcripts produced by both *S.* Typhimurium and faecal bacteria had detectable signals in both channels; however, the signal in the fluorescent channel corresponding to the mixed culture label was higher because there was a greater amount of cDNA in that sample corresponding to transcripts from faecal bacteria, that cross-hybridised in the array, plus transcripts of *S.* Typhimurium, as described in Fig. 5.5; the signal in the channel corresponding to the faecal culture was lower because the sample was free from *Salmonella* and contained transcripts from faecal bacteria only. This group of intensities with higher values might include transcripts from genes up-regulated in faecal bacteria in response to the presence of *S.* Typhimurium in the culture. However, after 9 and 24 h of incubation of the mixed culture and coincidentally with the inactivation of *S.* Typhimurium, this entire group of genes is not detected anymore. The lack of detection could be due to *Salmonella* inactivation itself, because this implies the removal of the factor that allegedly triggered up-regulation of these genes in faecal bacteria. However, 'memory' or 'hysteresis' has been reported at the transcriptional level, meaning that although gene up-regulation is triggered

very quickly in response to environmental stimuli, transcription repression does not take place immediately after stimuli are removed [274].

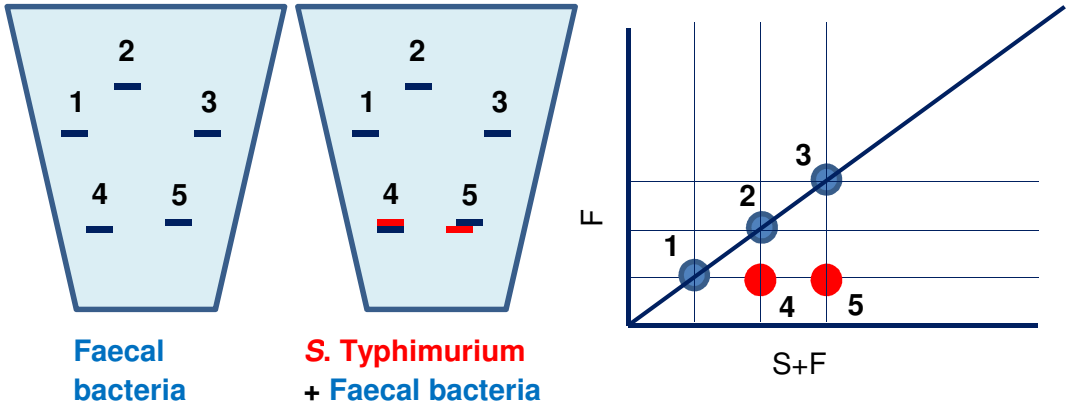


Figure 5.5. Diagram illustrating how competitive cDNA hybridisations between samples from mixed cultures of *S.* Typhimurium and faecal bacteria and samples from faecal bacteria only give rise to two groups of intensities. Transcripts able to hybridise on 5 features of the array are represented by bars in red for *S.* Typhimurium, S, and blue for faecal bacteria, F, in the left drawing and plotted according to the intensities from each sample in the right graph. Transcripts hybridising on features 1, 2 and 3 have similar concentrations in both samples; this results in the detection of similar intensities in both channels and their position on the diagonal of the scatter plot. Transcripts hybridising on features 4 and 5 have higher concentrations in the mixed culture which displace them from the diagonal of the scatter plot.

2) The group of intensities with similar values for both fluorescent channels were associated to genes with equal amount of transcripts in both samples and therefore those are genes expressed only by faecal bacteria present in both samples that cross-hybridised on the array.

These two groups of intensities were observed in hybridisations with samples taken at 3.5 and 6 h, while the initial concentration of viable *S.* Typhimurium was still high. However, genes belonging to *S.* Typhimurium were not always detected in samples after 9 h of incubation (Fig. 5.3); in these samples, the detection of *S.* Typhimurium transcripts was variable. In two of the three replicated experiments only one group of transcripts was detected, and these datasets exhibited similar patterns to those observed in control hybridisations of faecal samples without *S.* Typhimurium (Fig. 5.3). This may be due to the fact that the inactivation of *S.* Typhimurium could have already been initiated. The slight decrease in the concentration of *S.* Typhimurium observed after 9 h of incubation in the various replicates (Fig. 5.2) should be sufficient

to prevent the detection of its transcripts because they would be outnumbered by faecal bacteria transcripts in the mixed culture. Only the group of genes expressed by faecal bacteria would be detected in this case, resulting in patterns similar to those observed in control hybridisations of faecal samples without *S.* Typhimurium (Fig. 5.3). In other words, in the competitive hybridisations of cDNA from mixed cultures of *S.* Typhimurium and faecal bacteria with cDNA from cultures with only faecal bacteria, genes expressed by *S.* Typhimurium could be measured only during the first 9 h of incubation. In samples taken after that time, the decrease of viable *S.* Typhimurium did not allow the detection of its transcripts. This experimental design allowed the study of genes expressed by *S.* Typhimurium prior to its inactivation in mixed cultures with faecal bacteria, however, changes in the level of expression of genes in *S.* Typhimurium could not be inferred.

Control competitive hybridisations between samples from cultures of faecal bacteria established with the same inoculum

To detect genes expressed in cultures containing only faecal bacteria (without *S.* Typhimurium) and cross-hybridising on the array, control competitive hybridisation experiments were carried out by labelling cDNA from faecal samples collected at the same time from two independent faecal cultures that had been inoculated with faeces from the same donor without *Salmonella*. These hybridisations resulted in very neat datasets with most of the genes equally expressed in both faecal samples (Fig. 5.3). This reinforces the principle behind the experimental design and proves that independent cultures established with faecal samples from the same donor have similar concentrations of transcripts or similar intensities in both fluorescent channels, as they are located around the diagonal in the scatter plot (Fig. 5.3); when one of the independent cultures is inoculated with *S.* Typhimurium, two groups of intensities are detected in the scatter plot (Fig. 5.3).

The group of transcripts obtained from faecal bacteria that hybridised in the array did not represent the totality of faecal bacteria genes that were being expressed; the array was designed specifically for *S.* Typhimurium and will not include all the necessary features for a complete analysis of gene expression of faecal bacteria.

Table 5.3 lists all the hybridisation experiments carried out, showing in the comment column which of those were successful and which ones were not.

Array	Sampling time (hours)	F635_Cy5 Green	F532_Cy3 Red	*replicate or faeces donor	**hybridization	***dye	****Comments
Slide 5_Array 1	3.5	Faeces + S. Tm	Faeces	1	3.5h 1A x 1B	Cy-5 x Cy-3	OK
Slide 5_Array 2	3.5	Faeces + S. Tm	Faeces	1	3.5h 1A x 1C	Cy-5 x Cy-3	OK
Slide 5_Array 3	3.5	Faeces	Faeces	1	3.5h 1B x 1C	Cy-5 x Cy-3	OK
Slide 5_Array 4	3.5	Faeces + S. Tm	Faeces	2	3.5h 2A x 2B	Cy-5 x Cy-3	unclear separation of populations
Slide 5_Array 5	3.5	Faeces + S. Tm	Faeces	2	3.5h 2A x 2C	Cy-5 x Cy-3	unclear separation of populations
Slide 5_Array 6	3.5	Faeces	Faeces	2	3.5h 2B x 2C	Cy-5 x Cy-3	OK
Slide 5_Array 7	3.5	Faeces + S. Tm	Faeces	3	3.5h 3A x 3B	Cy-5 x Cy-3	OK
Slide 5_Array 8	3.5	Faeces + S. Tm	Faeces	3	3.5h 3A x 3C	Cy-5 x Cy-3	unclear separation of populations
Slide 4_Array 1	3.5	Faeces	Faeces	3	3.5h 3B x 3C	Cy-3 x Cy-5	very low intensity

Table 5.3

Slide 4_Array 2	6	Faeces	Faeces + S. Tm	1	6h 1A x 1B	Cy-3 x Cy-5	very low intensity
Slide 4_Array 3	6	Faeces	Faeces + S. Tm	1	6h 1A x 1C	Cy-3 x Cy-5	OK
Slide 4_Array 4	6	Faeces	Faeces	1	6h 1B x 1C	Cy-3 x Cy-5	OK
Slide 4_Array 5	6	Faeces	Faeces + S. Tm	2	6h 2A x 2B	Cy-3 x Cy-5	OK
Slide 4_Array 6	6	Faeces	Faeces + S. Tm	2	6h 2A x 2C	Cy-3 x Cy-5	OK
Slide 4_Array 7	6	Faeces	Faeces	2	6h 2B x 2C	Cy-3 x Cy-5	very low intensity
Slide 4_Array 8	6	Faeces	Faeces + S. Tm	3	6h 3A x 3B	Cy-3 x Cy-5	very low intensity
Slide 7_Array 1	6	Faeces	Faeces + S. Tm	3	6h 3A x 3C	Cy-5 x Cy-3	very low intensity
Slide 7_Array 2	6	Faeces	Faeces	3	6h 3B x 3C	Cy-5 x Cy-3	very low intensity
Slide 7_Array 3	9	Faeces + S. Tm	Faeces	1	9h 1A x 1B	Cy-5 x Cy-3	low intensity
Slide 7_Array 4	9	Faeces + S. Tm	Faeces	1	9h 1A x 1C	Cy-5 x Cy-3	looks like a control experiment

Table 5.3

Slide 7_Array 5	9	Faeces	Faeces	1	9h 1B x 1C	Cy-5 x Cy-3	low intensity
Slide 8_Array 1	9	Faeces	Faeces + S. Tm	2	9h 2A x 2B	Cy-3 x Cy-5	unclear separation of populations
Slide 8_Array 2	9	Faeces	Faeces + S. Tm	2	9h 2A x 2C	Cy-3 x Cy-5	unclear separation of populations
Slide 8_Array 3	9	Faeces	Faeces	2	9h 2B x 2C	Cy-3 x Cy-5	OK
Slide 8_Array 4	9	Faeces	Faeces + S. Tm	3	9h 3A x 3B	Cy-3 x Cy-5	OK
Slide 8_Array 5	9	Faeces	Faeces + S. Tm	3	9h 3A x 3C	Cy-3 x Cy-5	OK
Slide 8_Array 6	9	Faeces	Faeces	3	9h 3B x 3C	Cy-3 x Cy-5	OK
Slide 9_Array 5	24	Faeces + S. Tm	Faeces	1	24h 1A x 1B	Cy-5 x Cy-3	OK
Slide 9_Array 6	24	Faeces + S. Tm	Faeces	1	24h 1A x 1C	Cy-5 x Cy-3	low intensity
Slide 9_Array 7	24	Faeces	Faeces	1	24h 1B x 1C	Cy-5 x Cy-3	OK
Slide 9_Array 8	24	Faeces + S. Tm	Faeces	2	24h 2A x 2B	Cy-5 x Cy-3	low intensity

Table 5.3

Slide 10_Array 1	24	Faeces	Faeces + S. Tm	2	24h 2A x 2C	Cy-3 x Cy-5	OK
Slide 10_Array 2	24	Faeces	Faeces	2	24h 2B x 2C	Cy-3 x Cy-5	OK
Slide 10_Array 3	24	Faeces	Faeces + S. Tm	3	24h 3A x 3B	Cy-3 x Cy-5	OK
Slide 10_Array 4	24	Faeces	Faeces + S. Tm	3	24h 3A x 3C	Cy-3 x Cy-5	OK
Slide 10_Array 5	24	Faeces	Faeces	3	24h 3B x 3C	Cy-3 x Cy-5	OK
Slide 7_Array 6	1	S. Tm			1h S. Tm (1)	Cy-5	OK
Slide 7_Array 7	1	S. Tm			1h S. Tm (2)	Cy-5	OK
Slide 7_Array 8	1	S. Tm			1h S. Tm (3)	Cy-5	OK
Slide 8_Array 7	8		S. Tm		8h S. Tm (1)	Cy-3	OK
Slide 8_Array 8	8		S. Tm		8h S. Tm (2)	Cy-3	OK

Table 5.3

Slide 9_Array 1	8	S. Tm	8h S. Tm (3)	Cy-5	OK

*Replicas: 1, 2 and 3. Faeces from person 1, 2 and 3.

**Vessel A was inoculated with *S.* Typhimurium after 24 h with a faecal sample from the same donor as B and C.

***Cy3: Signal is read in F532 channel. Cy5: Signal is read in F635 channel.

****Only samples with OK results were used.

(S. Tm = *S.* Typhimurium)

Table 5.3. List of competitive hybridisations carried out in microarrays.

From the 24 (three donors x four sampling times x two faecal cultures free of *S*. Typhimurium established from same donor) competitive hybridisations set up between samples from mixed cultures of *S*. Typhimurium and faecal bacteria and samples from faecal cultures free from *S*. Typhimurium, only 12 were successful. Successful hybridisations resulted in two groups of genes: genes expressed by *Salmonella* and genes expressed by faecal bacteria that cross-hybridised with features in the microarray.

From the 12 (three donors x four sampling times) competitive hybridisations between independent faecal cultures free from *S*. Typhimurium established from the same donor, eight were successful.

The six (three replicates x two sampling times) one-colour non-competitive hybridisations established from pure cultures of *S*. Typhimurium were successful.

The reasons for the failures were either that the two groups of intensities belonging to *S*. Typhimurium and faecal bacteria could not be distinguished or the intensity measurements were very low.

5.5.3 Cluster analysis of the transcription profiles of samples collected from individual cultures as well as co-cultures of *S.* Typhimurium and faecal bacteria

Gene expression similarities between samples were explored by cluster analysis. All sample profiles were used in this analysis. Thus, these profiles included:

1) Genes expressed by *S.* Typhimurium in co-cultures of *S.* Typhimurium and faecal bacteria at 3.5, 6, 9 and 24 h after inoculation.

2) Genes detected as expressed (cross-hybridising) by faecal bacteria in co-cultures of *S.* Typhimurium and faecal bacteria at 3.5, 6, 9 and 24 h after inoculation.

3) Genes detected as expressed (cross-hybridising) by faecal bacteria in cultures of faecal bacteria free from *S.* Typhimurium at 3.5, 6, 9 and 24 h after inoculation.

4) Genes expressed by *S.* Typhimurium in pure cultures at 1 and 8 h after inoculation.

Distance-based methods of hierarchical clustering were used to analyse gene expression data with the purpose of identifying similarities between expression patterns in the different mixed and separated cultures of *S*. Typhimurium and faecal

bacteria. Cluster analysis was run on the matrix of dissimilarities constructed using all sample profiles (Fig. 5.6A), gene expression profiles of only *S.* Typhimurium (Fig. 5.6 B) and profiles of only faecal bacteria (Fig. 5.6C).

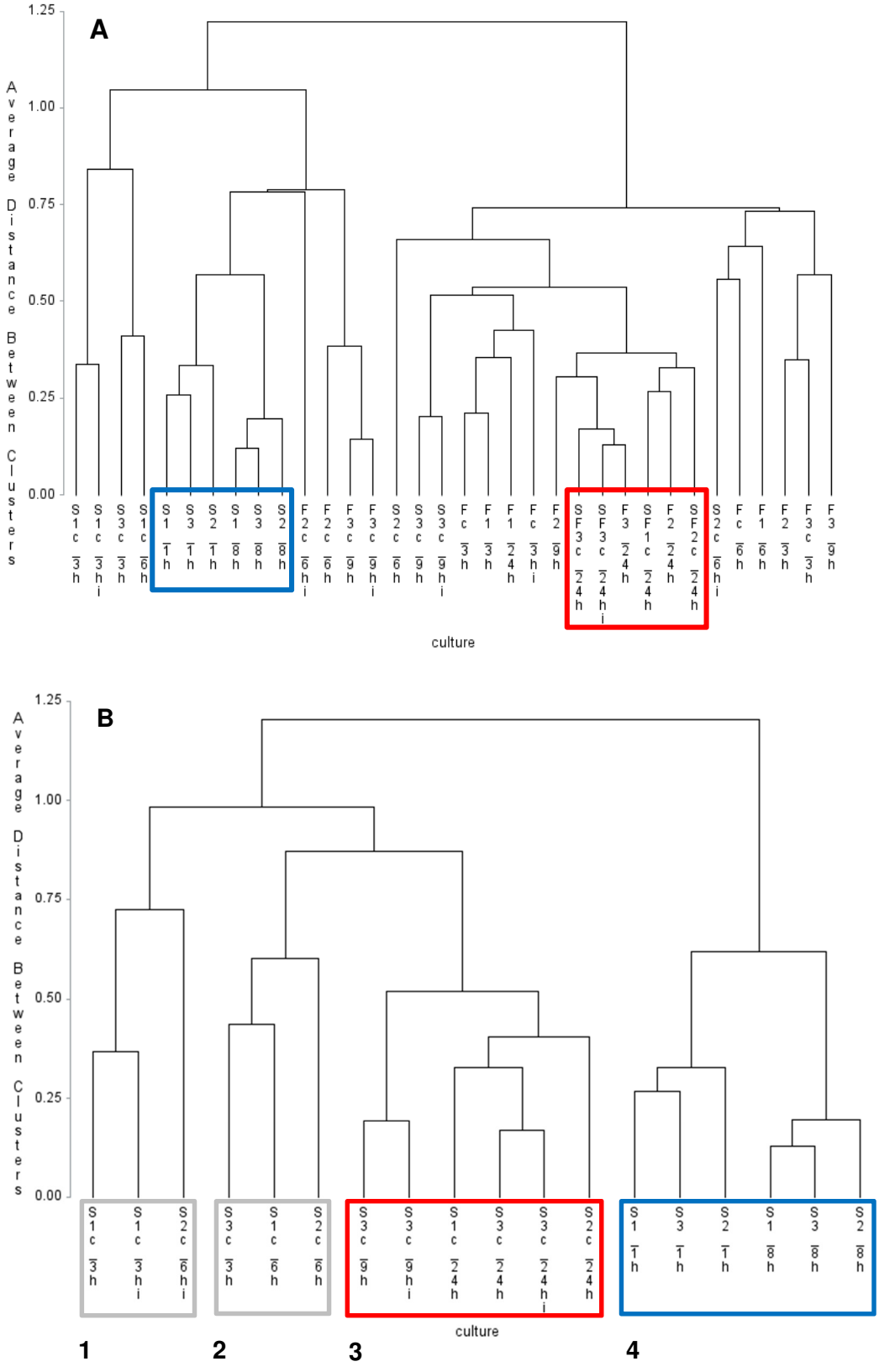


Figure 5.6

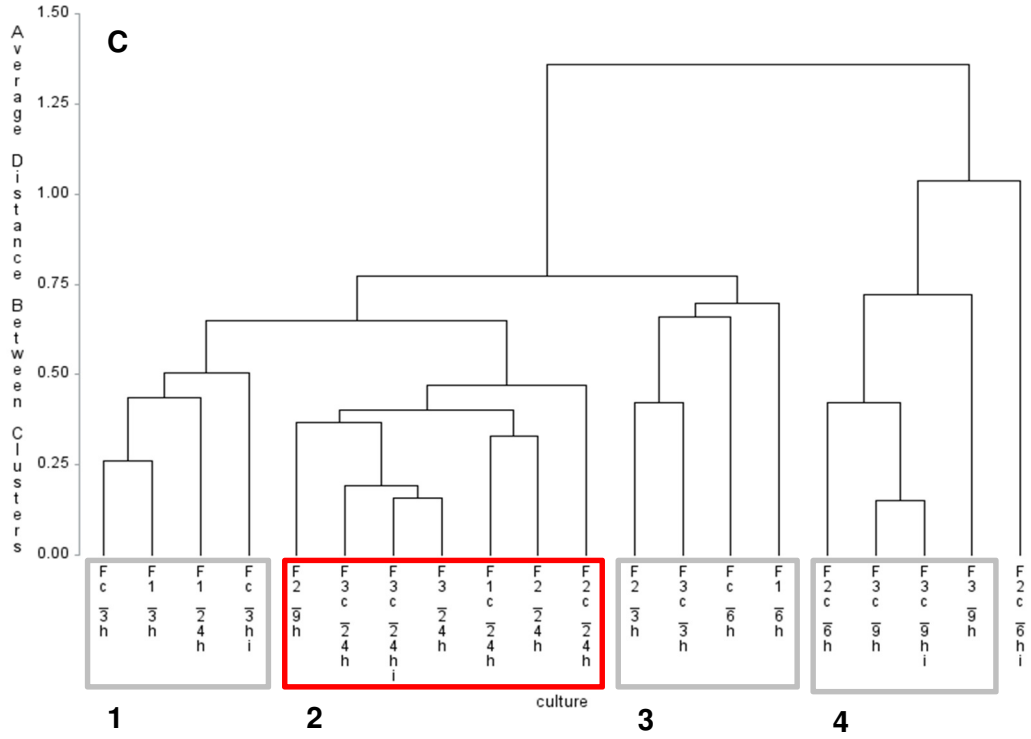


Figure 5.6. Cluster trees of the transcription profiles of samples collected from individual cultures as well as co-cultures of *S.* Typhimurium and faecal bacteria. Cluster trees of all samples (A); samples of *S.* Typhimurium (B) and samples of faecal bacteria (C). The coding of the samples is:

1) "S" or "F" denoting genes detected in *S.* Typhimurium or in faecal bacteria, respectively.
2) "1", "2" or "3" for each of the replicated independent experiments and/or donors of faecal samples.
3) "c" indicates co-culture of *S.* Typhimurium and faecal bacteria.
4) "3h" (representing '3.5h'), "6h", "9h" or "24h" indicates the sampling time.
5) "i" distinguishes the two competitive hybridisations established between samples from co-cultures of *S.* Typhimurium and faecal bacteria and each of the faecal bacteria cultures inoculated with samples from the same donor.

The dendrogram resulting from clustering all samples of *S.* Typhimurium and faecal bacteria showed two main clusters of similar samples: a group including all *S.* Typhimurium expression profiles obtained in pure cultures both at 1 and 8 h of incubation and a second group with all but one of the faecal bacteria cultures after 24 h of incubation that had initially been inoculated either alone or in co-culture with *S.* Typhimurium (Fig 5.6A). The explanation for the clustering of samples from co-cultures of *S.* Typhimurium and faecal bacteria with samples from cultures of faecal bacteria is that after 24 h, the concentration of viable *S.* Typhimurium had decreased

to 10^6 cfu/ml (Fig. 5.2); therefore, only the group of transcripts corresponding to faecal bacteria and cross-hybridising onto the array was detectable.

To explore in more detail similarities between profiles of samples of *S.* Typhimurium in pure culture and co-culture, a dendrogram was constructed using only samples with *S.* Typhimurium. These sample profiles clustered into four major groups in the dendrogram (Fig. 5.6B). Groups 1 and 2 had similar composition and both included samples after 3 and 6 h of incubation. Group 3 contained samples at 9 and 24 h of incubation. As shown in Fig. 5.2 and explained in chapter 3, a reduction of ca. 3-4 logarithmic units in *S.* Typhimurium concentration was detected in the presence of faecal bacteria within 24 h of incubation (Fig. 3.5, Table 3.4). Samples collected at 9 h of incubation were closer to samples obtained after 24 h than to samples obtained after 3.5 or 6 h of incubation. This indicates that transcripts of *S.* Typhimurium were not detectable after 9 h of incubation possibly due to a reduction of its concentration as explained above. The fourth cluster included all samples of *S.* Typhimurium in pure culture.

When samples of faecal bacteria were represented alone, four main clusters were observed. Cluster 1 and 3 included mainly samples obtained after 3.5 and 6 h of incubation. Cluster 4 included mainly samples obtained after 9 h of incubation. Cluster 3 included all but one of the observations at 24 h. All clusters included indistinctively samples from co-cultures with *S.* Typhimurium and samples from faecal cultures alone (Fig. 5.6C).

5.5.4 Correspondence analysis of the transcription profiles of samples collected from individual cultures as well as co-cultures of *S.* Typhimurium and faecal bacteria

Correspondence analysis was carried out to further explore patterns in the expression of genes of *S.* Typhimurium in separated or mixed cultures with faecal bacteria. Correspondence analysis is conceptually similar to principal component analysis, but applies to categorical data rather than to continuous data.

When carrying out the analysis of DNA microarray data, the genes, the experimental conditions or both can be considered as variables. In this work, as the genes were taken as variables, the analysis of the data produces a series of new 'dimensions', which are the combinations of the original variables that best describe the variability of the experimental responses. This is a very common data exploratory technique applied to generate new variables which allow observations to be plotted in a reduced number of meaningful dimensions and patterns visualized. The analysed dataset

consisted of 1401 genes which were detected at least in one of the samples of either *S.* Typhimurium or faecal bacteria. Correspondence analysis reduced the 1401 original variables to four principal dimensions which retained 14, 12, 11 and 8% of the total variability, respectively. Fig. 5.7 represents the expression profiles of *S.* Typhimurium and faecal bacteria plotted in the new dimensions.

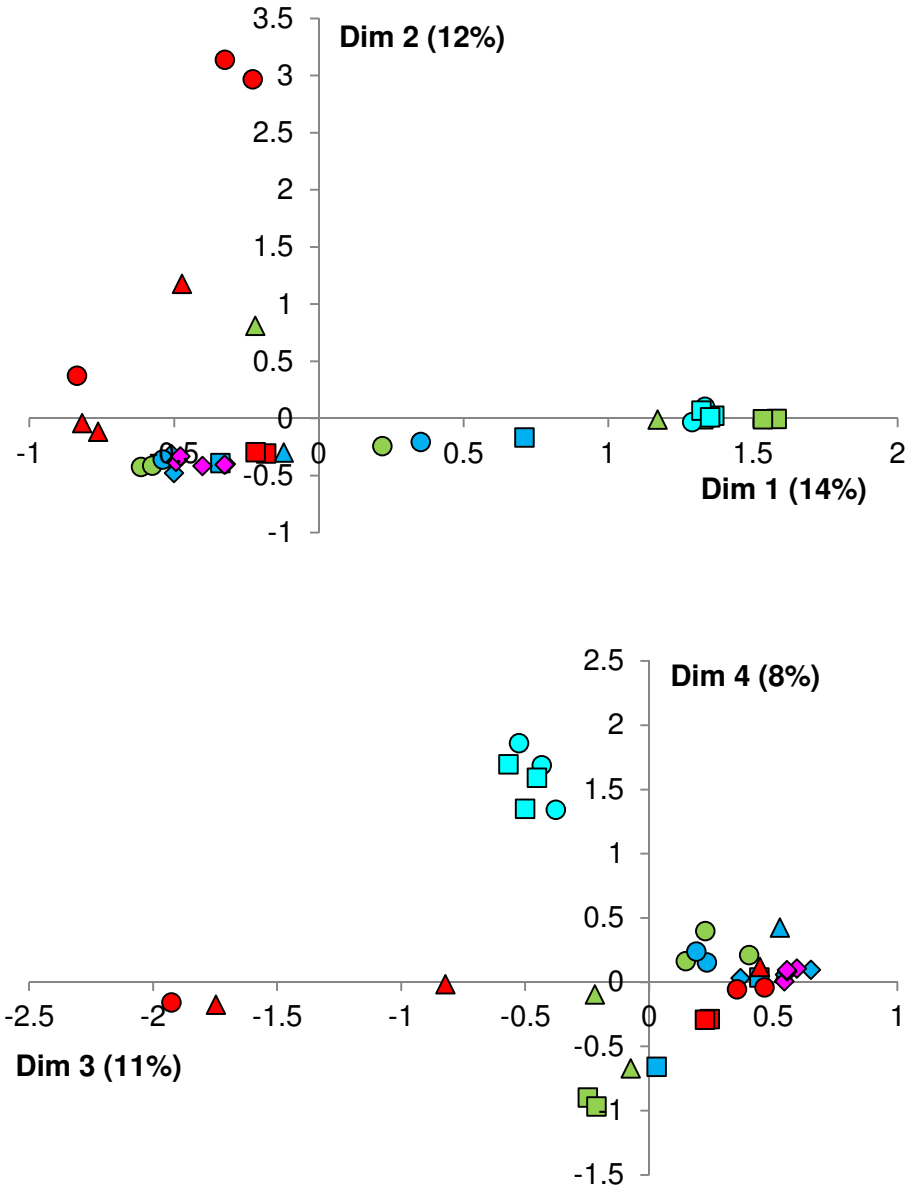


Figure 5.7. Correspondence analysis of the transcriptional profiles of *S.* Typhimurium and faecal bacteria in mixed or separated cultures. Samples were taken at 3.5 (circles), 6 (triangles), 9 (squares) and 24 (diamonds) h. Faecal bacteria alone ,*S.* Typhimurium alone , faecal bacteria in mixed culture , *S.* Typhimurium in mixed culture and mixed culture of faecal bacteria and *S.* Typhimurium after 24 h .

As observed in cluster analysis, two groups of samples can be observed indicating greater similarities between the profiles within each group than between the rest of transcriptional profiles. One group consists of samples of *S*. Typhimurium in pure cultures while the other group comprises samples after 24 h of incubation from both mixed cultures of *S*. Typhimurium and faecal bacteria and cultures of faecal bacteria alone. In addition, transcriptional profiles of faecal bacteria, from mixed cultures with *S.* Typhimurium as well as cultures without the pathogen, were mainly associated with dimension 1 which shows the variability associated with sampling times. The transcriptional profiles of *S*. Typhimurium in mixed cultures with faecal bacteria were associated with Dimension 2. Dimension 3 does not have a straightforward interpretation while dimension 4 mainly accounts for the variability between profiles of *S.* Typhimurium in pure cultures and in other conditions.

5.5.5 Quantification of the number of genes expressed in each culture condition and sampling time

Previous multivariate analysis showed that the transcriptional profiles of replicated samples under the same conditions were consistent. For further analysis of transcription, profiles for each assay condition, i.e. culture condition and sampling time, rather than for each replicated sample were obtained. The criterion used to decide whether genes were expressed in a given condition was as follows: only genes detected in more than half the independent replicates at a given sampling time and culture condition and/or at two consecutive sampling times under the same culture conditions were considered expressed for that culture conditions and sampling time. Gene expression profiles were prepared by coding 1 if the gene was expressed or 0 otherwise for each assay condition. These profiles can be found in Appendix 2. The assay conditions and transcription profiles that were further analysed included:

- Transcription profiles of *S*. Typhimurium in co-culture with faecal bacteria after 3.5, 6 and 9 h of incubation.
- Transcription profiles of *S*. Typhimurium in pure culture after 1 and 8 h of incubation.
- Transcription (cross-hybridisation) profiles of faecal bacteria in cultures free from *Salmonella* after 3.5, 6 and 9 h of incubation.
- Transcription (cross-hybridisations) profiles of faecal bacteria after 24 h of incubation in mixed cultures with *S.* Typhimurium and in faecal cultures free from *Salmonella*. Samples from mixed cultures of *S*. Typhimurium and faecal bacteria after 24 h of incubation were grouped together with samples from

faecal bacteria alone because transcripts of *S.* Typhimurium were not detected due to its inactivation.

Cluster and correspondence analysis indicate that the transcriptional profiles in mixed cultures of faecal bacteria and *S.* Typhimurium and faecal cultures alone are very similar after 24 h of incubation (Fig. 5.6 and Fig. 5.7).

Transcriptional profiles of faecal bacteria (cross-hybridisation) in co-cultures with *S.* Typhimurium were not analysed. This set of genes was not considered because it is very likely to be incomplete. Genes expressed in both *S.* Typhimurium and faecal bacteria in mixed cultures would be detected as expressed in *S.* Typhimurium and missing from the set of genes associated with faecal bacteria. Table 5.4 shows the number of genes expressed in each of these conditions as well as in each pair of conditions.

	S+F 3.5h	S+F 6h	S+F 9h	S 1h	S 8h	F 3.5h	F 6h	F 9h	F 24h
S+F 3.5h	473	310	120	26	32	92	14	98	75
S+F 6h	310	600	272	30	37	192	60	203	167
S+F 9h	120	272	465	50	45	288	96	313	259
S 1h	26	30	50	163	106	116	34	117	31
S 8h	32	37	45	106	169	108	34	115	33
F 3.5h	92	192	288	116	108	649	133	509	318
F 6h	14	60	96	34	34	133	146	129	114
F 9h	98	203	313	117	115	509	129	658	370
F 24h	75	167	259	31	33	318	114	370	427

Genes expressed by *S.* Typhimurium in mixed cultures with faecal bacteria (S+F)
Genes expressed by *S.* Typhimurium in pure cultures (S)
Genes expressed by faecal bacteria in cultures with no *S.* Typhimurium (F)
F 24h includes also mixed cultures of faecal bacteria and *S.* Typhimurium

Table 5.4. Coincidental number of genes expressed in the assay conditions

To achieve a more meaningful analysis of the number of genes expressed in each condition, the Nei coefficient of association [271] was estimated for every pair of conditions as CA= $2a/(2a+b+c)$ where a is the number of genes expressed in both conditions and b and c are the number of genes expressed only in one of the conditions (Table 5.5). This coefficient takes into account the coincidental expression between any pair of conditions relative to the total number of the genes expressed in each sample. The values of CA range between 0, if no common genes are expressed, and 1, when all genes are expressed in both conditions. A value of 0.5 means that 50% of the total genes expressed in two conditions is coincidental.

	S+F 3.5h	S+F 6h	S+F 9h	S 1h	S 8h	F 3.5h	F 6h	F 9h	F 24h
S+F 3.5h	1.00	0.58	0.26	0.08	0.10	0.16	0.05	0.17	0.17
S+F 6h	0.58	1.00	0.51	0.08	0.10	0.31	0.16	0.32	0.33
S+F 9h	0.26	0.51	1.00	0.16	0.14	0.52	0.31	0.56	0.58
S 1h	0.08	0.08	0.16	1.00	0.64	0.29	0.22	0.29	0.11
S 8h	0.10	0.10	0.14	0.64	1.00	0.26	0.22	0.28	0.11
F 3.5h	0.16	0.31	0.52	0.29	0.26	1.00	0.33	0.78	0.59
F 6h	0.05	0.16	0.31	0.22	0.22	0.33	1.00	0.32	0.40
F 9h	0.17	0.32	0.56	0.29	0.28	0.78	0.32	1.00	0.68
F 24h	0.17	0.33	0.58	0.11	0.11	0.59	0.40	0.68	1.00

Genes expressed by *S.* Typhimurium in mixed cultures with faecal bacteria (S+F)

Genes expressed by *S.* Typhimurium in pure cultures (S)

Genes expressed by faecal bacteria in cultures with no *S.* Typhimurium (F)

F 24h includes also mixed cultures of faecal bacteria and *S.* Typhimurium

Table 5.5. Nei coefficient of association of the coincidental number of genes expressed in the assay conditions.

According to the CA, there are 58% of common genes expressed in *S.* Typhimurium at 3.5 and at 6 h of incubation in mixed cultures with faecal bacteria, however, when comparing the profiles obtained at 3.5 and 9 h of incubation, the percentage of common genes decreases to 26%. This means that similarity between transcription profiles decreases as the time of incubation progresses (Table 5.4 and 5.5). The CA between genes expressed by *S.* Typhimurium after 9 h of incubation in mixed cultures with faecal bacteria and genes detected in faecal cultures alone at 3.5, 9 and 24 h of incubation was between 0.5-0.6. This relatively high number of coincidences indicates that after 9 h *S.* Typhimurium genes are not detected in mixed cultures with faecal bacteria. A high CA value was also observed for *S.* Typhimurium in pure culture at 1 h and 8 h of incubation. Faecal cultures alone have high values of CA at different sampling times; however no tendency in time was detected (Table 5.5).

Particularly low CA values, 0.08, were observed between gene expression of *S.* Typhimurium in pure cultures and in mixed cultures with faecal bacteria. Additionally, low CA values were detected between gene expression of faecal cultures free from *S.* Typhimurium and gene expression of *S.* Typhimurium after 3.5 and 6 h of incubation with faecal bacteria (Table 5.5).

5.5.6 Network analysis of the cellular functions and metabolic pathways associated with the transcriptional profiles detected in *S.* Typhimurium in mixed cultures with faecal bacteria

In chapter 3, it was described that the concentration of *S.* Typhimurium decreased sharply when in contact with faecal bacteria; a reduction of ca. 3-4 decimal logarithmic units of *S.* Typhimurium concentration was detected within 24 h (Fig. 3.5, Table 3.4).

In addition, it was also observed that in *Salmonella*-free faecal cultures, *Enterobacteriaceae,* as well as facultative aerobic bacteria, were inactivated with kinetics comparable to that of *S.* Typhimurium (Fig. 3.5, Table 3.4). The aim here was to analyse cell functions and metabolic pathways expressed by *S.* Typhimurium during inactivation by contact with faecal bacteria. To do that, cellular functions and metabolic pathways were associated to the transcriptional profiles derived from the replicated samples for each of the conditions described in the previous section. The association between transcription profiles and cell functions and metabolic pathways was carried out by network analysis techniques. A previously developed genomic network connecting all genes of the genome and plasmids of *S.* Typhimurium with cellular functions and metabolic pathways was used [272]. Genes forming each transcriptional profile for each condition were used to extract, from the genome network, the sub-networks containing all cellular functions and metabolic pathways associated to each transcriptional profile.

Fig. A. 3. 1 in Appendix 3 compares the cellular functions and metabolic pathways expressed by *S.* Typhimurium in mixed culture with faecal bacteria and those expressed in pure culture. The number of functions/pathways expressed by *S.* Typhimurium in mixed cultures with faecal bacteria was 122. These included two-component signalling systems, ABC transporters, protein turnover systems and chaperones, heat shock proteins, protein folding catalysts, amino-acid biosynthesis and metabolism, carbohydrate and lipid transport and metabolism, DNA replication, recombination and repair. There were 160 functions/pathways found to be expressed in pure cultures of *S.* Typhimurium and from those 95 functions/pathways were not detected in mixed cultures of *S.* Typhimurium with faecal bacteria. This suggests that possibly some genes are down regulated when in culture with gut microbiota with respect to pure cultures.

After excluding functions/pathways detected in pure cultures of *S.* Typhimurium, the number of functions expressed by *S.* Typhimurium in mixed cultures with faecal bacteria was 56 (Appendix 3, Fig. A. 3. 2). Functions/pathways expressed by *S.* Typhimurium in pure cultures are not likely to be specifically involved in the inactivation process of the pathogen when in contact with faecal bacteria.

To further narrow down the number of functions/pathways associated with the inactivation process, functions expressed in either mixed cultures of *S.* Typhimurium and faecal bacteria or in faecal cultures alone after 24 h of incubation were also excluded (Appendix 3, Fig. A. 3. 3). After 24 h of incubation the transcripts produced

by *S*. Typhimurium in mixed cultures with faecal bacteria could no longer be detected because of the pathogen inactivation. As explained in chapter 3, in *Salmonella*-free faecal cultures, *Enterobacteria* and facultative aerobes are inactivated with a very similar kinetics to that observed in *S*. Typhimurium (Fig. 3.5, Table 3.4); therefore, the transcriptional response, due to cross-hybridisation, detected in faecal cultures could have common elements with that of *S.* Typhimurium. However, after 24 h of incubation, transcripts produced by *Enterobacteria* and facultative aerobes should no longer be detected and therefore expression patterns are not likely to be associated with the inactivation process. After these considerations, the total number of expressed functions/pathways associated with the inactivation of *S*. Typhimurium was reduced to 42 (Appendix 3, Fig. A. 3. 3). These 42 functions/pathways were related to open pathways to obtain energy such as the activation of fatty acid phospholipid and pyruvate and glutamate metabolism. Genes encoding biosynthesis functions of amino acids such as lysine, tyrosine and tryptophan as well as flagellar assembly, transcription and elongation factors were also expressed. But remarkably, 40% of the functions were stress related. The genes *htpX* and *grpE*, encoding heat shock proteins, were detected. There was activation of five two-component systems which are responsible for environmental information processing, signal transduction and the activation of invasion response regulators [278]. These included TctE/TctD, NarQ/NarP, BarA/UvrY, phosphotransferase and CreC/CreB two-component systems. Furthermore, three ABC transporter systems, four chaperones, parvulin and detoxification mechanisms were activated. In addition, approximately 40% of the total number of genes included in plasmids 1 and 2, which are 103 and 100 respectively, and 30% of the 60 genes associated to mobile extrachromosomal transposons were expressed in *S*. Typhimurium when in contact with faecal bacteria.

A total of 128 functions were expressed in cultures of faecal bacteria free from *Salmonella* (Appendix 3, Fig. A. 3. 4). Many of these functions were of a similar nature to those expressed by *S.* Typhimurium in mixed cultures with faecal bacteria. As explained above, *Enterobacteria* and facultative aerobes are also inactivated in faecal cultures and thus the transcriptional response observed in faecal bacteria could include functions associated with the inactivation of *S.* Typhimurium. After 24 h of incubation, transcripts of bacteria undergoing the inactivation process can no longer be detected. Appendix 3, Fig. A. 3. 5 shows cellular functions and metabolic pathways, 91 in total, expressed in faecal cultures free from *S.* Typhimurium excluding those detected after 24 h of incubation.

With the aim of developing testable hypotheses on the inactivation of *S*. Typhimurium by contact with faecal bacteria, the 42 functions associated to the inactivation of *S*. Typhimurium (Appendix 3, Fig. A. 3. 3) were compared with the 91 detected in faecal cultures free from *Salmonella* during the inactivation of *Enterobacteria* and facultative aerobic bacteria (Appendix 3, Fig. A. 3. 5). These functions excluded those detected in pure cultures of *S.* Typhimurium, in mixed cultures of *S.* Typhimurium and faecal bacteria and *Salmonella*-free faecal cultures after 24 h of incubation. The reasoning behind this comparison was that the same mechanism of inactivation responsible for the contact killing of *S*. Typhimurium by faecal bacteria could be responsible for the inactivation of *Enterobacteria* and facultative aerobic bacteria in faecal cultures. If that was the case, genes equivalent to those expressed by *S.* Typhimurium could be expressed by *Enterobacteria* and facultative aerobes and could cross hybridise onto the array slide. There were nine coincidental elements between these two groups of cellular functions and/or metabolic pathways (Appendix 3, Fig. A. 3. 6). Six of them were stress related. These included the phosphotransferase and CreC/CreB two-component signalling systems, the HSP100 and HSP24 heat shock proteins, parvulin metabolism and regulatory RNA interactions.

5.5.7 Analysis of changes in the population composition by DGGE during the inactivation of *S.* Typhimurium in mixed cultures with faecal bacteria

Possible changes on the microbial composition of the faecal bacterial population during the inactivation of *S*. Typhimurium in mixed cultures were investigated by DGGE. Analysed samples were taken from mixed cultures of *S.* Typhimurium and faecal bacteria and from faecal cultures free from *Salmonella* (vessels A and B in Fig. 5.1 B), after 3.5, 6, 9, 24 and 48 h of incubation. The experiment was repeated three times with faecal samples from different donors. Fig. 5.8 shows the banding patterns on the DGGE gels for each sample. In mixed cultures of *S*. Typhimurium and faecal bacteria, the intensity of the band located in the position corresponding to *S*. Typhimurium DNA decreased as the incubation time increased, and it was not detected after 48 h of incubation. This reflects the decrease of the population of *S.* Typhimurium in mixed cultures with faecal bacteria. In *Salmonella*-free faecal cultures a band positioned at the level of *S*. Typhimurium is detected in many of the samples; these bands can correspond to multiple bacterial groups which are part of the complex faecal bacterial population. These bands were not detected after 48 h of incubation and could be reflecting the population of faecal facultative aerobic bacteria that follows similar inactivation kinetics to that detected in *S.* Typhimurium.

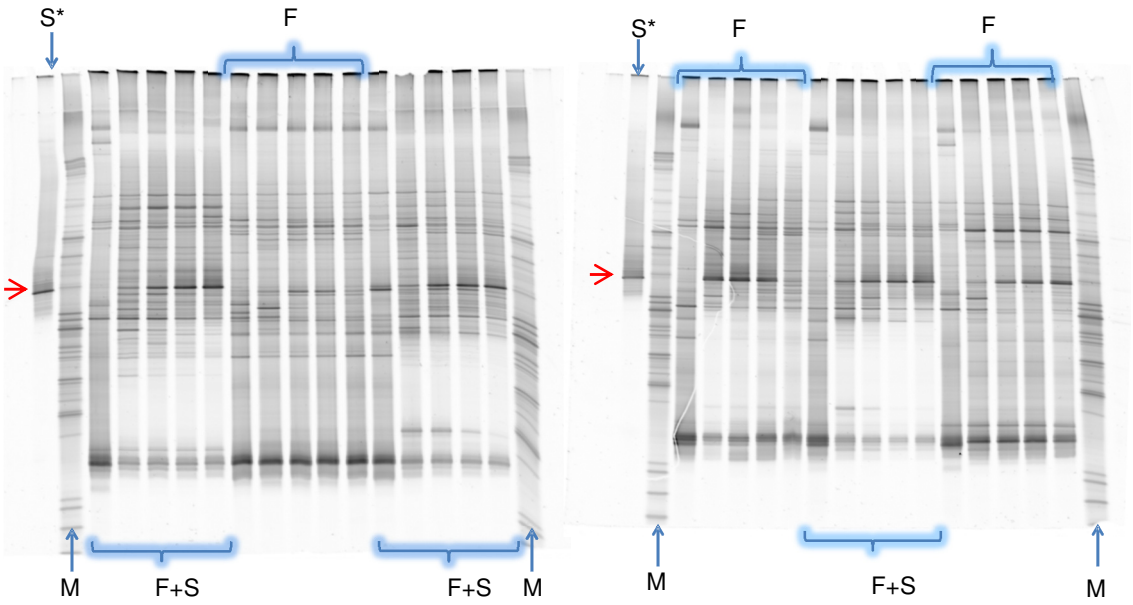


Figure 5.8. DDGE gel images of faecal bacteria culture and mixed culture of faecal bacteria and *S.* Typhimurium taken at 3.5 h, 6 h, 9 h, 24 h and 48 h. Each blue bracket comprises gel tracks that correspond, from right to left, to these hours respectively. The red arrows indicate the band for corresponding to *S.* Typhimurium in pure cultures.
F + S: Mixed cultures of faecal samples and *S.* Typhimurium
S*: *S.* Typhimurium in pure cultures
M: Marker
F: Cultures of faecal samples free from *S.* Typhimurium

Distance-based methods of hierarchical clustering were used to analyse the band profiling generated by DGGE in order to identify changes in the population during the inactivation of *S.* Typhimurium. The dendrogram (Fig. 5.9) shows six main clusters below the cut-off established according to the eigenvalues criteria. Clustering analysis did not indicate a clear pattern of association between samples other than a tendency of the samples from the same donor to group together. Of the six groups, one corresponds to *S.* Typhimurium and another group corresponds to the markers; the other four groups involve mixed cultures of faecal bacteria and *S.* Typhimurium and faecal cultures alone with no clear tendency.

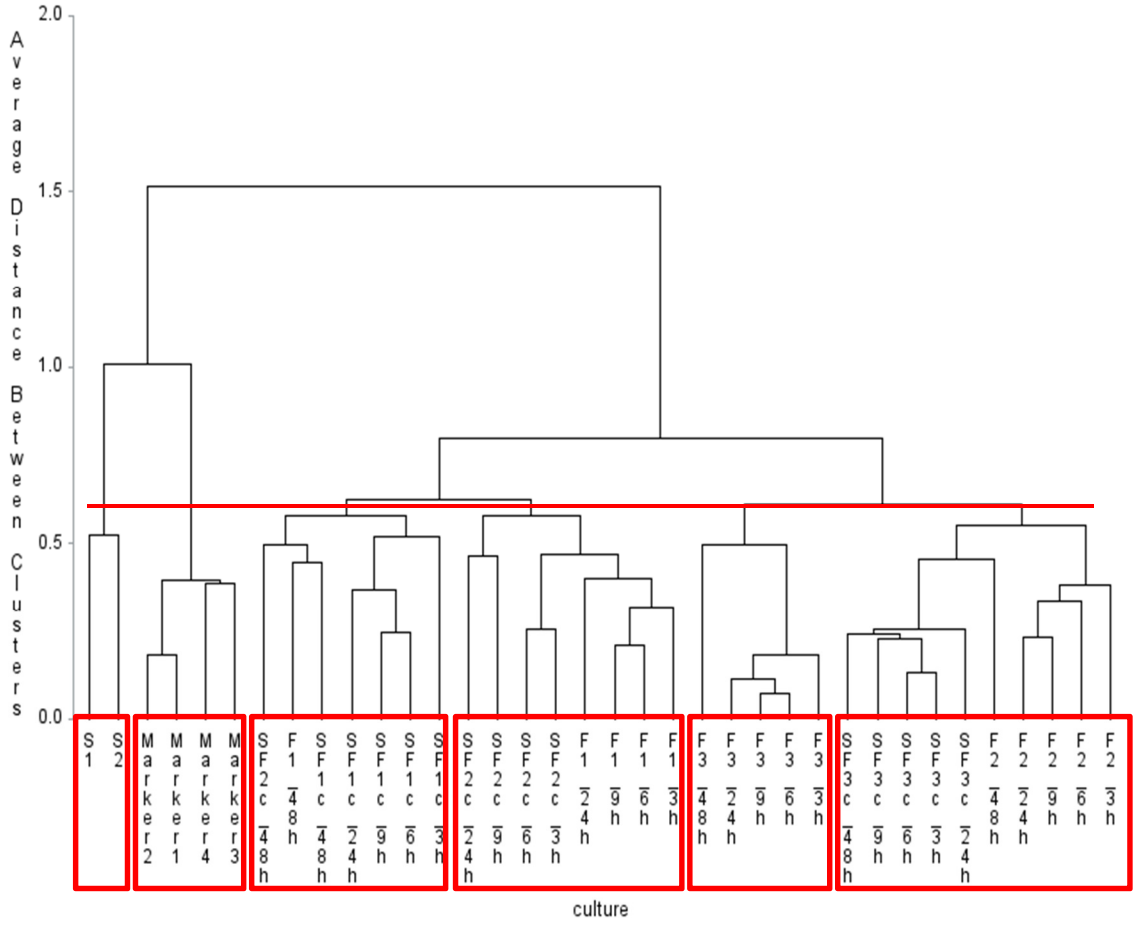


Figure 5.9. Cluster analysis tree of DGGE profiles in mixed cultures of *S.* Typhimurium and faecal bacteria and faecal cultures alone.
The coding of the samples is:

1) "F" or "S" denoting genes detected in *S.* Typhimurium or in faecal bacteria, respectively.
2) "1", "2" or "3" for each of the replicated independent experiment and/or donor of faecal samples.
3) "c" indicates co-culture of *S.* Typhimurium and faecal bacteria.
4) "3h" (representing '3.5h'), "6h", "9h" or "24h" indicates the sampling time.
5) "i" distinguishes the two competitive hybridisations established between samples of co-cultures of *S.* Typhimurium and faecal bacteria and each of the faecal bacteria cultures initiated with samples from the same donor.

Cluster analysis was repeated after deleting bands located at the same position as *S.* Typhimurium genome bands in samples from pure cultures (Fig. 5.10). This was done to evaluate the effect of those bands on clustering results. Cluster number and composition did not vary significanlty when compared with results obtained with

complete profiles including all bands. There was some shifting of samples between clusters but again no pattern of association could be observed.

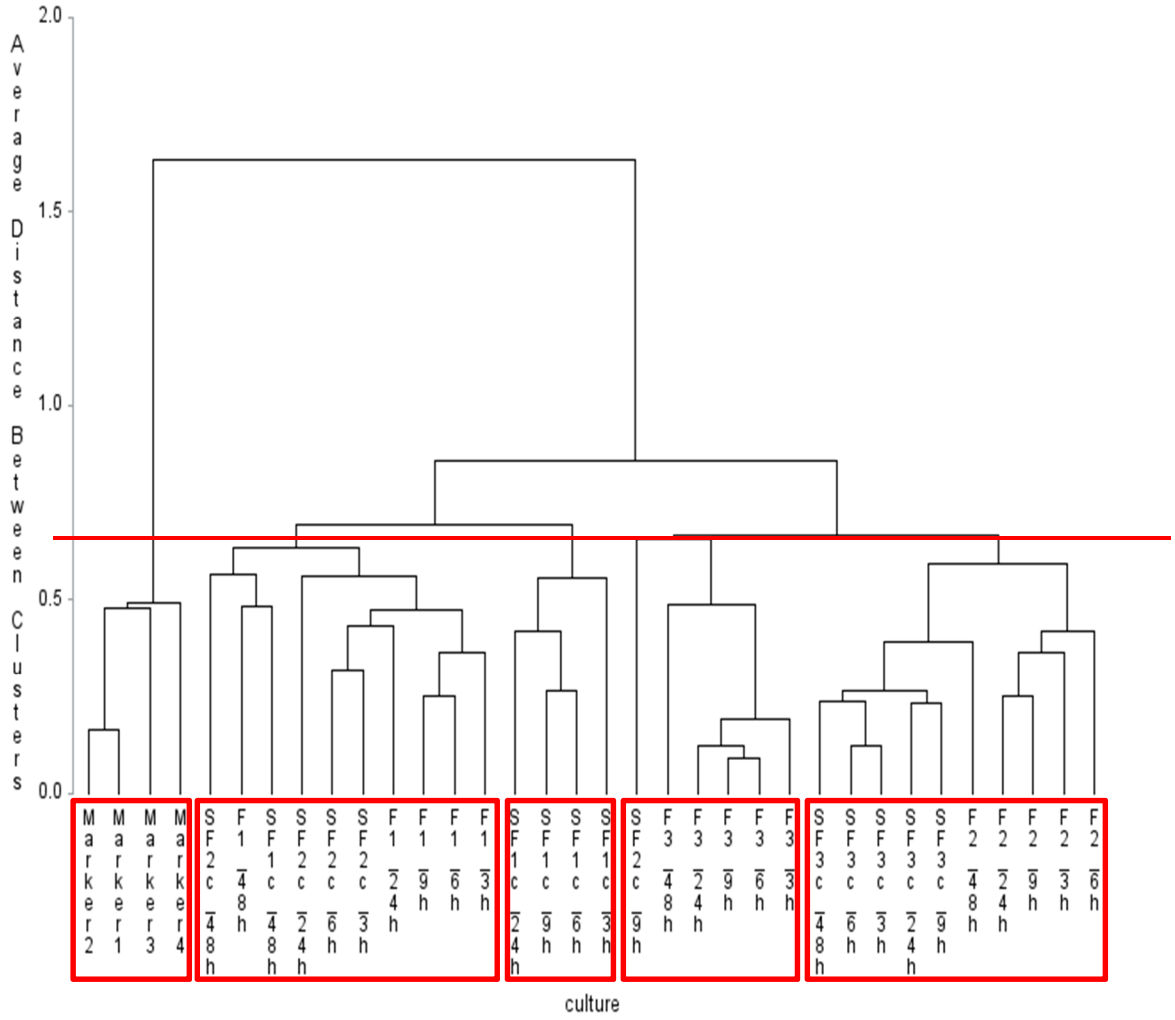


Figure 5.10. Cluster analysis tree of DGGE profiles in mixed cultures of *S.* Typhimurium and faecal bacteria and faecal cultures alone after removing bands in positions corresponding to *S.* Typhimurium genome.
The coding of the samples is:

1) "F" or "S" denoting genes detected in *S.* Typhimurium or in faecal bacteria, respectively.
2) "1", "2" or "3" for each of the replicated independent experiment and/or donor of faecal samples.
3) "c" indicates co-culture of *S.* Typhimurium and faecal bacteria.
4) "3h" (representing '3.5h'), "6h", "9h" or "24h" indicates the sampling time
5) "i" distinguishes the two competitive hybridisations established between samples of co-cultures of *S.* Typhimurium and faecal bacteria and each of the faecal bacteria cultures initiated with samples from the same donor.

5.6 Discussion

5.6.1 Sensitivity and specificity of the microarray

A microarray specific for *S.* Typhimurium SL1344 was used to study the influence of the gut microbiota on the gene expression of this pathogen. Its specificity and sensitivity was tested by hybridising genomic DNA isolated from mixed and separated cultures of faecal bacteria and *S.* Typhimurium. The proportion of probes in the array that cross-hybridised with DNA of faecal bacteria was 0.28 and 0.63 for male and female faecal samples respectively; this meant that the use of these arrays to measure gene expression of *S.* Typhimurium in mixed cultures with faecal bacteria was not straightforward.

As with other studies, the specificity of the microarrays was variable for the detection of bacteria in faecal samples. Specificity levels ranging from 66% to 84% have been reported for bacterial identification in faecal samples depending on the length of the probes that were used in the microarray: 50-mer probes showing specificity of 66% and 40-mer probes showing 75% [279]. Other reports also associate higher specificity to shorter length of the microarray probes, suggesting 19-21-mer probe as the optimal length for specificity [280]. The match/mismatch ratio has been demonstrated to be inversely proportional to the length of the oligonucleotide; the reason being that longer oligonucleotides have more probabilities to energetically accommodate a single nucleotide mismatch at a central position [281]. A fair compromise between specificity and sensibility of microarrays has been suggested to be 30-mer oligonucleotides [281], even 19 to 21-mer probes, as mentioned earlier, are thought to be ideal in terms of specificity. The microarrays used here consisted of 60 oligonucleotide probes, but the possible effect of the extra length on specificity was compensated by printing two different probes for each gene. Thus, the lack of specificity of the array when used with faecal bacteria is not related to the array design but rather to the complexity of the faecal population.

Studies of bacteria detection in natural environments using microarrays describe detection limits of 1 ng of pure genomic DNA and 25 ng of soil community DNA [260], which implies that in mixed cultures the concentration of the target bacteria must be higher. The same work reports a strong linear quantitative relationship (r^2 = 0.89-0.94) between signal intensity and target DNA concentration in the range of 1 to 100 ng for genomic DNA from pure cultures and mixed communities [260].

Regarding the use of microarrays for transcriptional studies, it has been demonstrated that for RNA extracted from mixed cultures, the hybridisation signal intensity is significantly higher than the background signal when the bacterial concentration is greater than 1.3 x 10^7 cfu/ml; for bacteria concentrations of 10^6 cfu/ml, the signal intensity is not different from the background level [261]. In microarray experiments involving dilutions of *Salmonella* into manure and soil to get concentrations from 10^{10} to 10 cfu/g, the highest detection limit for species-specific genes was 10^9 cfu/g [282]. Other studies have reported, on the other hand, that the use of optimised microarray protocols and short-oligonucleotide microarrays can have detection limits of 8.8 x 10^4 cfu/g from a total population of 10^{13} bacteria/g of faecal contents [279].

Here, competitive hybridisations were carried out between cultures of faecal bacteria and mixed cultures of faecal bacteria and *S.* Typhimurium. Two groups of microarray hybridisation signal intensities could be observed in samples obtained from cultures that had incubated for less than 9 h. The group with the highest intensity corresponded to the genes that had been expressed by *S.* Typhimurium and maybe faecal bacteria; on the other hand, the group with lower intensity related to the transcripts expressed by faecal bacteria only (Fig. 5.3). However, when the cultures had incubated for 9 h or longer, only one group of signal intensity was found, similar to that observed in cultures of only faecal bacteria (Fig. 5.3). This can be attributed to the fact that *S.* Typhimurium, in mixed culture with faecal bacteria, became less viable amongst all the microbial population sometime after the first 9 h of incubation, and in particular its population was drastically reduced by contact killing in the first 24 h; this led to *S.* Typhimurium transcripts not being distinguishable from transcripts of bacteria of faecal origin. Therefore, although the experimental design described here was optimised in order to track transcription in *S.* Typhimurium in mixed cultures by microarrays, the study was not straightforward and limited to those cases in which the concentration of *S.* Typhimurium was greater than that of faecal bacteria.

5.6.2 Cellular functions and metabolic pathways expressed by *S.* Typhimurium during inactivation in mixed cultures with faecal bacteria

The analysis of the functions expressed by *S.* Typhimurium in mixed cultures with faecal bacteria (Appendix 3, Fig. A. 4 and A. 5) revealed that many of these functions are associated to a stress response; *S.* Typhimurium is, after all, being inactivated by faecal bacteria (Fig. 5.2). The survival of bacteria in complex and challenging environments is dependent on the interaction between different regulatory pathways which leads to the expression of adaptive responses [283]. The following are some

of the functions that were expressed by *S.* Typhimurium in mixed cultures with faecal bacteria:

5.6.2.1 Two-component system (TCS):

Two-component regulatory or signal-transduction systems take part in bacterial adaptation to various environmental conditions [284]. They act as a stimulus-response mechanism to allow microbes to detect and react to changes in numerous environmental conditions [285]. The system usually involves a membrane-bound histidine protein kinase that detects a specific environmental stimulus and a response regulator protein that controls the cellular response by regulating the expression of target genes [286]. Two-component signalling pathway are usually controlled by phosphotransfer reactions [286]. The TCS expressed in *S*. Typhimurium in mixed cultures with faecal bacteria were:

- BarA-UvrY is used by *E. coli* to cope with oxidative stress [287]. BarA is the sensor-kinase protein and UvrY is its corresponding response regulator [288]. The ortholog of BarA-Uvry expressed in *Salmonella* is BarA-SirA [289].

- CreC/CreB (formerly known as phoM) is a TCS involved in catalytic regulation in response to environmental signals [290]. *E. coli* heat-shock factor, sigma32, induces the expression of the membrane-localised histidine kinases CreC and its cytoplasmic response regulator [291].

- NarQ/NarP is involved in the detection of nitrate and nitrite signals and anaerobic respiration and fermentation. In anoxic conditions *E. coli* can use nitrate or nitrite as electron acceptors. NarQ and its response regulator NarP regulate the genes involve in this process [292]. Ttrs/TtrR TCS, expressed here, is involved in the pathway that enables numerous bacteria, including *S.* Typhimurium, to use tetrathionate as a terminal respiratory electron acceptor, and allowing bacterial growth in minimal media [293].

- The phosphotransferase system used by bacteria for the uptake of carbohydrates and using phosphoenolpyruvate as the source of energy. This TCS is activated in order to optimise the utilisation of carbohydrates and bacterial catabolism in complex environments [294].

- The TctE/TctD TCS, which is involved in the tricarboxilic acid transport is another bacterial shock-sensitive transport system that is repressed by the addition of D-glucose [295].

- The TCS RstB/RstA has been associated with *E. coli* resistance to antibiotics such as troleandomycin and the upregulation expression of *asr* (acid shock RNA) [296, 297]. RstA is involved in acid tolerance and anaerobic respiration [298]. RstB/RstA TCS can be induced by PhoP/PhoQ TCS under low Mg^{2+} growth conditions [299].

- PhoP/PhoQ TCS regulates the adaptation to low Mg^{2+} conditions and the response to other limiting environments by regulating the expression of 1% of the genes in some gram-negative species [300].

5.6.2.2 Heat shock proteins (HSP):

Functions involving HSP and chaperones, particularly HSP100 and HSP24, were expressed by *S.* Typhimurium in mixed culture with faecal bacteria. Cell exposure to adverse environmental conditions, such as high temperatures, arsenite, heavy metals, amino acid analogues and oxidants, lead to acute or chronic stress that results in the expression of HSP [301]. This heat-shock response increases the expression of proteolytic factors, proteases and molecular chaperones to regulate proteolysis [302]. Generally, the signal produced by different stress stimuli is protein damage (denatured or mis-folded proteins); this triggers the expression of HSP and chaperones that leads to either the repair of the damaged proteins and survival of the cell or apoptosis if the stress if too severe [301]. HSP100 are able to neutralise any protein that becomes aggregated after severe stress. They are not essential during normal growth conditions and are generated as a consequence of extreme heat or other severe stresses [303]. HSP24 has been reported to increase resistance of microorganisms to salt, drought and heat stresses [304].

Expression of histone-like heat unstable proteins functions was also observed. These proteins contribute to the looping of DNA, its organisation and regulation [305].

5.6.2.3 Chaperones:

A relatively large number of genes expressed by *S*. Typhimurium in mixed cultures with faecal bacteria were involved in post-translational modification protein turnover and chaperone functions; many of them specifically associated to protein folding stabilisation functions. Their role is similar to that of chaperones in that they assist in the correct folding of proteins and stabilise unfolded polypeptides so they do not aggregate into nonfunctional structures [306]. Many proteins can self-assemble into functional structures while others require additional proteins, molecular chaperones, which do not form part of the final structure but help prevent dysfunctional

configurations. Chaperones are therefore involved in protein biogenesis in physiological conditions but also in mature proteins unfolded and/or misfolded as a consequence of environments stresses [307].

In particular, genes involved in parvulin metabolism were expressed by *S.* Typhimurium in mixed cultures with faecal bacteria. Parvulins are enzymes (propyl isomerases) that have chaperone-like activity and assist protein folding by catalysing the *cis-trans* isomerisation of propyl peptide bonds in polypeptides [308, 309]. *Salmonella* spp. is equipped with regulatory systems intended for detecting and adapting to harsh conditions by regulating the expression of target genes [308]. One of these regulatory systems, sigma RpoE, regulates genes that express proteins and enzymes involved in the correct folding and assembly of outer membrane proteins [308, 309].

5.6.2.4 DNA repair and RNA interactions:

The DNA is exposed to environmental genotoxic agents and metabolomic intermediates; the expression of DNA repair functions are SOS responses that are essential for the survival of the cells [310]. Bile salts exposure causes oxidative DNA damage in *Salmonella enterica* and induces the expression of DNA repair functions [311]. DNA repair functions are required for *S. enterica* virulence [312]. DNA repair is critical for the accurate repair of the damaged incurred to DNA double-strand breaks [313]. Also single-strand breaks repair functions such as base or nucleotide excision single-strand base repair have been expressed by *S.* Typhimurium in mixed cultures. Small regulatory RNAs are involved in the regulation of a wide variety of adaptive responses in bacteria [314].

5.6.2.5 Transporters:

- Function Multidrug ABC2 transporter. The upregulation of the gene *ABC2* has been associated to the treatment with various toxicants, including antibiotics, in organisms such as *Magnaporthe grisea* [315]. ABC2 is one of the multidrug transporters, a vast protein superfamily that can be found in all living organisms and that are involved in the transport of chemical substances from the inside of the cell to the outside [316]. The upregulation of ABC transporters has been observed in *Candida krusei*, when exposed to antifungal treatments; these transporters recognise the drugs and lead to drug-resistance of microorganisms [317]. The function of the ABC transporters to interact with drugs and to expel them across the outer membrane has also been observed in gram-negative bacteria such as *E. coli* and *S.* Typhimurium [318, 319].

- The Major Facilitator Superfamily: This is a large group of transporters that are responsible for the transport across membranes of numerous compounds such as ions, sugar phosphates, drugs, neurotransmitters, nucleosides, amino acids and peptides. These proteins have nutrient uptake and drug-efflux pump functions and are involved in bacterial antibiotic resistance according to the National Center for Biotechnology Information www.ncbi.nlm.nih.gov.
- Nitrate/nitrite transporters: Proteins that transport these molecules across the membrane; nitrates are essential for nitrogen metabolism and it is reduced to nitrites; however the accumulation of cellular nitrites is toxic for the cell [320].

5.6.2.6 Transcription factors Helix-turn-helix MerR family:

Bacteria are equipped with specific transcription factors that are appropriate for their growth and/or stress response depending of their environmental conditions. The MER family of transcription factors consists of proteins that contain a sensing domain that identifies the environmental signal to which a response is necessary. These proteins include the regulator of mercuric-ion resistance, from which it receives its name; however, they involve other metal ion-sensing regulators (copper, lead, zinc, etc.) and regulators with the ability to detect drugs, oxidative and osmotic stress, starvation and pH and temperature changes [321].

5.6.2.7 Mobile extra-chromosomal element and transposon functions:

It is known that *Salmonella* possess plasmids involved in antimicrobial resistance and virulence [322]. SL1344 genome contains 3 plasmids (pSLTSL1344, pCol1B9SL1344 and pRSF1010SL1344) that encode 212 genes [67]. These mobile genetic elements participate actively in the quick adaptation to environmental pressures and are a tool for transferring information inter and intra bacterial species. Further, these mobile genetic elements can encode virulence factors and confer antibiotic resistance to bacteria [323].

Upon the study of these different adaptive responses, it is remarkable that HSP, RNA interactions, HSP100, TCS and parvulins are five of the nine functions expressed by *S.* Typhimurium in mixed cultures and also in faecal cultures alone during the period of inactivation of *Enterobacteria* and facultative aerobes. (Appendix 1, Fig. A6). This suggests that *S*. Typhimurium, and possible *Enterobacteria* and facultative aerobic bacteria, are under adverse conditions similar to those generated by starvation, low pH, heat/cold and osmolarity shock or oxidative damage.

5.6.3 Exploring changes in bacterial composition during *S.* Typhimurium inactivation in mixed cultures with faecal bacteria

DGGE analysis revealed no evidence of changes in bacterial composition associated with the killing of *S.* Typhimurium in mixed cultures with faecal bacteria. Numerous published works have highlighted the alteration of the gut microbiota caused by *S.* Typhimurium due to its ability to trigger an inflammatory response of the host, being the pathogen unable to overcome the commensal barrier in the absence of inflammation [121-123]. In this study, the effect on the gut microbiota could not be assessed because of the lack of an adaptive inflammatory response since the experiments were carried out *in vitro.* Other studies, however, report that in addition to the changes of the gut microbiota caused by the host inflammatory reaction, *S.* Typhimurium exerts changes in the biota composition via pathogen-commensal interactions [324]. DGGE results obtained here however did not show any change in the faecal bacterial profile whilst in mixed culture with *S.* Typhimurium.

The work presented here demonstrates the quantitative and functional impact that faecal bacteria exerts over *S.* Typhimurium; however, further research is necessary to ascertain what are the exact mechanisms behind the inactivation of *S.* Typhimurium by contact with faecal bacteria.

6 Chapter 6 – Concluding remarks

The study of bacterial interactions is essential to advance our understanding of the complex interplay between different bacterial species and provide insights into the mechanisms underpinning bacterial relationships within microbial communities. The gut of a healthy human harbours a diverse microbiota that is an essential barrier against exogenous bacteria [107] such as *S.* Typhimurium, which is responsible for causing localised infection of the intestinal mucosa of healthy individuals and invasive NTS in immunocompromised patients [325, 326]. However, *S.* Typhimurium has to overcome the host's defenses, including the intestinal microbiota, before it is able to colonise and cause disease. This study set out to explore interactions between *S.* Typhimurium and GI bacteria, obtained from culture collections and from fresh faecal samples, and to measure the impact of GI bacteria on the growth and survival of this pathogen. The study also sought to investigate the molecular activity of *S.* Typhimurium in the gut environment and also molecular profiling of faecal bacteria with and without the presence of *S.* Typhimurium.

S. Typhimurium is an intracellular pathogen that affects mainly the distal parts of the ileum, colon and caecum [327]; however, it can result in a carrier state with 'healthy' patients excreting the bacteria in their faeces for lengthy periods of time, following asymptomatic encounter or acute disease [202]. There is comprehensive understanding, for example, of the mechanisms used by *Salmonella* to penetrate into host cells and how *Salmonella* interacts with immune defense cells during infectious processes; however, not much has been published about the population dynamics of *Salmonella* in the distal colon environment during persistent infections. The overriding purpose of this work was to establish what kind of interactions between *S.* Typhimurium and the GIT bacteria affect the concentration of this pathogen in the gut environment. This chapter reports on the main findings and conclusions resulted from this study as well as the study limitations and areas for future research.

In vitro models, particularly the multi-compartment models, are important tools to simulate the microbial environment of the human intestine not only with respect to composition but also with regards to bacterial activity. The results obtained using these models are comparable to those obtained in *in vivo* studies and are used to test the influence of foods and/or drugs on the bacterial composition and activity as well as pathogens such as *Salmonella* [210, 328]. These *in vitro* models have been validated in numerous studies [329-331] using modern molecular techniques that have demonstrated that not only the gut microbiota can be maintained *in vitro* but also

the whole distinctive range of microbes that are specific to individuals. However, this study was restricted to *in vitro* models, which were not equipped with epithelial and immune cells and therefore the interactions with the host were missing. Given the importance of these interactions and the feedback mechanisms by the host, using *in vitro* models on enterocytes or including the mucosa in them, combining both cell cultures and the mucus fraction, may broaden the scope of applications of these models and achieve a more accurate mimicking of the GIT.

Here, the interactions between *S.* Typhimurium and GI bacteria described in chapter 3 showed that the net specific growth rate and survival of *S.* Typhimurium was not affected by any of the selected GI bacteria or by a simplified model microbiota including all the selected GI bacteria in batch cultures mimicking the colon conditions. However, the concentration of *S.* Typhimurium decreased sharply when inoculated with fresh human faecal samples in batch cultures under the same conditions. In addition, the fate of total facultative aerobes and *Enterobacteriaceae* from the faecal samples was parallel to that of *S.* Typhimurium both in separated and mixed cultures of faecal bacteria with the pathogen. It has been reported that competition between bacteria can be regulated by the composition of the background community; changes in, for instance, the concentration of a limiting substrate, could alter the interaction between different strains. In other words, the microbial species that define the environment can modify the interaction between competing strains [230], which implies that the dominance of a pathogen such as *S.* Typhimurium is dependent on the composition of the gut microbiota. The fact that the survival of *S.* Typhimurium in mixed cultures with fresh human faecal samples differed from its survival in a defined mixed culture of selected GI bacteria, obtained from culture collections, is attributed then to the different bacterial environment. On the other hand, it has been reported that many genes may be expressed only in the presence of other organisms [332]; moreover, genes of some commensals such as *B. fragilis* are thought to be expressed preferably in the presence of mucosal tissue [105] and are not expressed if originated from a laboratory culture - colonisation only being possible when *B. fragilis* were recovered directly from animals [105]. Therefore, these contrasting effects of GI bacteria, obtained from culture collections or fresh faecal samples, on *S.* Typhimurium might well be due to the lack of expression of genes of GI bacteria that can alter *S.* Typhimurium; the reason being that the microorganisms or mucosal compounds required to facilitate that gene expression were not present in the defined mixed microbiota that was obtained from culture collections. A limitation of this study might be that the defined mixed culture of selected GI bacteria included only seven strains

as opposed to the diverse microecosystem and numerous metabolites, proteins and surfactants that can be found in the gut and might have an influence in the bacterial response; other 'defined microbiotas', such as the Altered Schaedler Flora (ASF) [333], used to inoculate germfree rodents with a known microbiota, also include a similar number of strains.

When *S.* Typhimurium was inoculated with fresh human faecal samples in a continuous model system mimicking the colon conditions, the result was different from batch cultures as the gut bacteria had an effect on the pathogen's growth rather than on its survival. In batch cultures with fresh faecal samples, *S.* Typhimurium concentration had a sharp reduction of several logarithmic units, followed by a pronounced tail in the survival curve. This reduction in the concentration of *S.* Typhimurium was not detected in the chemostat. However, in the chemostat system, the growth of *S.* Typhimurium was affected and, as opposed to the faecal bacteria present in the continuous culture, could not grow at the rate required to maintain a constant concentration under the continuous flow of the medium and it was finally excluded from the system. Unlike in batch cultures where the levels of nutrients and metabolites fluctuate [230], the chemostat system maintained a constant intake of fresh media and continuous disposal of waste providing optimum conditions for the growth of *S.* Typhimurium. Thus, there was an inhibitory effect on *S.* Typhimurium due to the presence of faecal bacteria both in batch and chemostat cultures, but in batch culture, *S.* Typhimurium was inactivated whereas in chemostat culture its growth was inhibited. This difference could be attributable to difficulties in establishing the conditions required for inactivation under a continuous flow and renewal of the medium. These results are in line with reports that maintain that 'pathogen clearance' is mediated by the gut microbiota rather than by the host's immune mechanisms [201]. The mentioned study demonstrated that a disturbed bacterial community failed to eliminate *S.* Typhimurium from the gut in experiments with mice; however, the restoration of a normal rodent microbiota managed to clear the pathogen from the intestine, even when it was in high concentrations.

Although the difficulty to recover colonic microbiota is commonly acknowledged [233, 235], future work should endeavour to identify the bacterial strains or microbial groups of the gut microbiota that are involved in the inhibition of *S.* Typhimurium. Further research should also be carried out to elucidate whether the prolonged tail of survival of *S.* Typhimurium in batch cultures is due to an adaptation process of the pathogen or because of changes in the bacterial environment. Future work should also find out

what would the result be if *Salmonella* from those survival colonies were isolated and inoculated in batch cultures of fresh faecal bacteria. The answer to these questions might help understand why *S.* Typhimurium is not always eliminated from the GIT in long-term asymptomatic 'carriers' following an infection.

The main objective of chapter 4 was to study the nature of the interactions between *S.* Typhimurium and the faecal bacteria. No inhibition of *S.* Typhimurium was observed when using the supernatants of fresh faecal sample cultures, inactivated faecal bacterial cells or when *S.* Typhimurium was incubated in a culture of faecal bacteria, but within a membrane that prevented the direct contact with GI bacterial cells. It was only when *S.* Typhimurium was in direct contact or close proximity with the GI bacteria that its culturability was effectively inhibited. Based on this work, the production of bacteriocins, antibiotics or metabolites that antagonise with *S.* Typhimurium cannot be dismissed, but if such substances were generated, they were not effective or they were too dilute in the medium to provoke the observed inhibition. Furthermore, metabolomic analysis was carried out on the media outside and inside the membrane, containing faecal bacteria and *S.* Typhimurium and *S.* Typhimurium only respectively, and no significant differences in metabolite profiles were found. In addition, a mathematical model was developed to describe the rate of loss of culturability of *S.* Typhimurium as a function of the frequency of encounters between GI bacteria and *S.* Typhimurium and the probability of inactivation after encounter. The frequency of encounters was determined by the concentration of each bacterial population; when the product of the concentrations of these two populations was below a certain value, mainly as a consequence of the inhibition of *S.* Typhimurium, the pathogen was no longer affected, explaining the tailing off pattern of the *S.* Typhimurium inhibition. The model was validated using several bacterial concentrations and inoculation protocols and the hypothesis, that the probability of inactivation after encounter should be constant under identical experimental conditions, was proved. In the different inoculation protocols, it was observed that when the faecal samples were incubated for 24 h prior to the inoculation of *S.* Typhimurium, the decrease of the concentration of the latter was detected immediately after its inoculation. In contrast, when *S.* Typhimurium was inoculated simultaneously or 24 h prior to the faecal bacteria, there was a delay before the inhibition of the pathogen was observed. Exploring the underlying reasons for this delay of *Salmonella* inactivation, depending on the inoculation protocol, is an intriguing subject for further research as this would contribute to the understanding of the defence mechanism used by faecal bacteria.

The introductory chapter described multiple mechanisms of cell-cell interaction that require contact or close proximity between bacterial cells such as membrane vesicles, the development of cytoplasmic connections, conjugation, the use of T5SS and T6SS, nanotubular channels etc. Here, the inhibition of *S.* Typhimurium might be dependent on the mechanism of bacterial interplay; however this mechanism was not elucidated in this work. It is unknown if *S.* Typhimurium was inhibited by a single or several factors or there was a synergic effect between them.

Microarrays and DGGE techniques were used as described in chapter 5. The purpose was to study the (i) gene expression of *S.* Typhimurium immediately prior to its growth arrest in mixed cultures with faecal bacteria and (ii) possible changes on the composition of faecal bacteria in these mixed cultures, respectively.

DNA genomic hybridisation revealed that up to 60% of the array features cross-hybridised with genomic samples of faecal cultures free of *S.* Typhimurium; despite this, the results obtained demonstrated that gene expression microarray experiments in complex mixed cultures can be performed. Two groups of microarray signal intensities were found upon competitive hybridisation between cultures of faecal bacteria and *S.* Typhimurium and cultures of faecal bacteria only. One of the groups corresponded to genes that had been expressed by *S.* Typhimurium whereas the second group related to transcripts expressed by faecal bacteria. However, it is unknown whether the group thought to correspond to *Salmonella* transcripts includes transcripts generated from genes of faecal bacteria that might have been expressed in response to the presence of *S.* Typhimurium in the mixed culture. Another limitation of this study was that the group of intensities corresponding to *Salmonella* transcripts was only observed for samples obtained from cultures that had been incubated for less than 9 h. When the mixed cultures of faecal bacteria and *S.* Typhimurium had been incubated longer than 9 h, the transcripts expressed by *S.* Typhimurium were not distinguishable from those expressed by the faecal bacteria - the amount of *Salmonella* RNA extracted from cultures after 9 h of incubation was not enough as the concentration of the pathogen started to decrease.

The analysis of the functions expressed by *S.* Typhimurium in mixed cultures with faecal bacteria showed that many of these functions were related to a stress response- the pathogen was being inhibited at the time the samples were collected. Two-component systems and heat shock proteins were two of the functions expressed by the pathogen.

No evidence of changes in the faecal bacteria profile when in mixed culture with S. Typhimurium was shown by DGGE analysis.

In this thesis some consistent and robust findings on the interactions between *S.* Typhimurium and faecal bacteria are shown. The work demonstrates the existence of a novel way of interaction between the gut microbiota an *S.* Typhimurium that requires cell contact and leads to loss of culturability of *S.* Typhimurium. Additionally, this was the first study pointing out that the effect of GI bacteria on *S.* Typhimurium depends on the origin of the former, i.e. culture collections or faecal samples. This suggests that details of the bacterial environment of faecal samples, not reproducible by a simplified microbiota model, are needed for full characterisation. This lends itself to the conclusion that describing the full dynamics would require system thinking, with many agents of disparate kind and complex links between them. This is how relatively minor and, at the moment unknown changes, may have profound effects on the outcome, and ultimately on our understanding of the remarkable ability of our GIT to cope with pathogenesis.

7 References

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8 Appendices

8.1 Appendix 1: Growth parameters of *S.* Typhimurium and GI bacteria in mixed and separated cultures

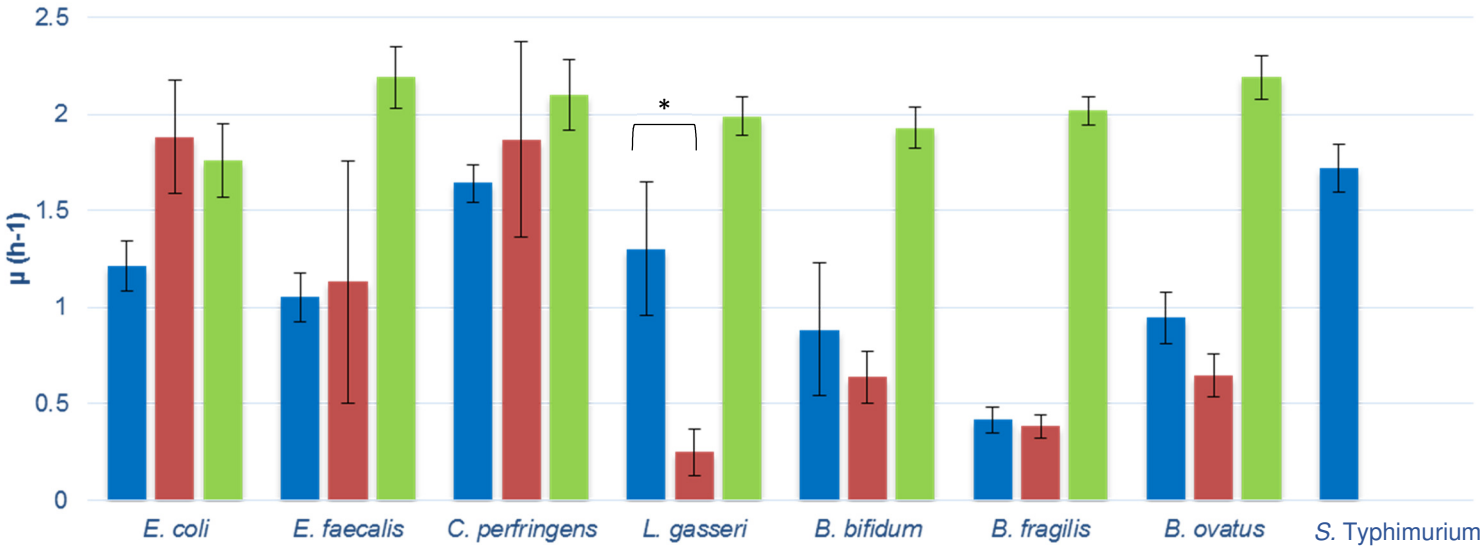

Figure A. 1. 1. Net specific growth rate, μ, (h^{-1}) of *S.* Typhimurium and culture collection bacterial strains of GI origin in exponential growth in a two-compartment system separated by a 0.45 µm pore membrane and in pure cultures. Columns represent the net growth rate of all strains in pure cultures (blue) and the rates of each GI bacteria (red) and of *S.* Typhimurium (green) in co-culture. The results for *S.* Typhimurium in pure cultures were obtained from four independent curves. Vertical bars indicate the standard error of the fitted parameter. Parameters estimated in pure culture were compared to those estimated in co-cultures. Horizontal bars and stars denote significant differences ($p < 0.05$).

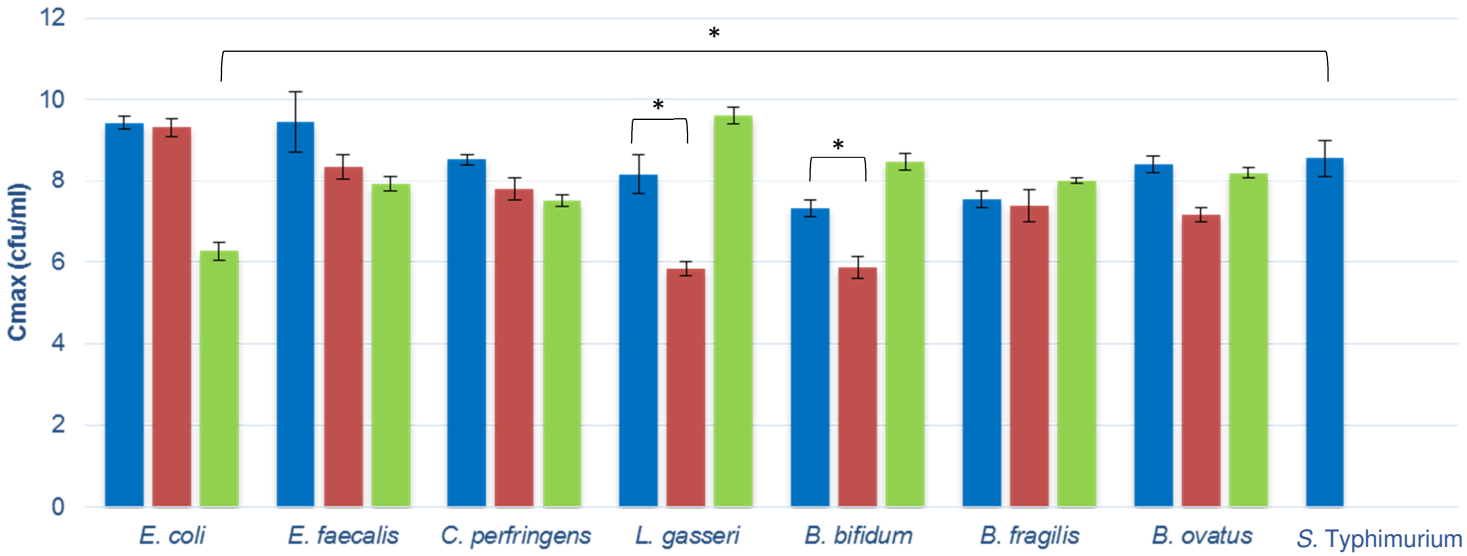

Figure A. 1. 2. Maximum concentration, Cmax, (cfu/ml) of *S.* Typhimurium and culture collection bacterial strains of GI origin in exponential growth in a two-compartment system separated by a 0.45 µm pore membrane and in pure cultures. Columns represent the $\log_{10}$ Cmax of all strains in pure cultures (blue) and of each GI bacteria (red) and *S.* Typhimurium (green) in co-culture. Vertical bars indicate- the standard error of the fitted parameter. Parameters estimated in pure culture were compared to those estimated in co-cultures. Horizontal bars and stars denote significant differences ($p < 0.05$).

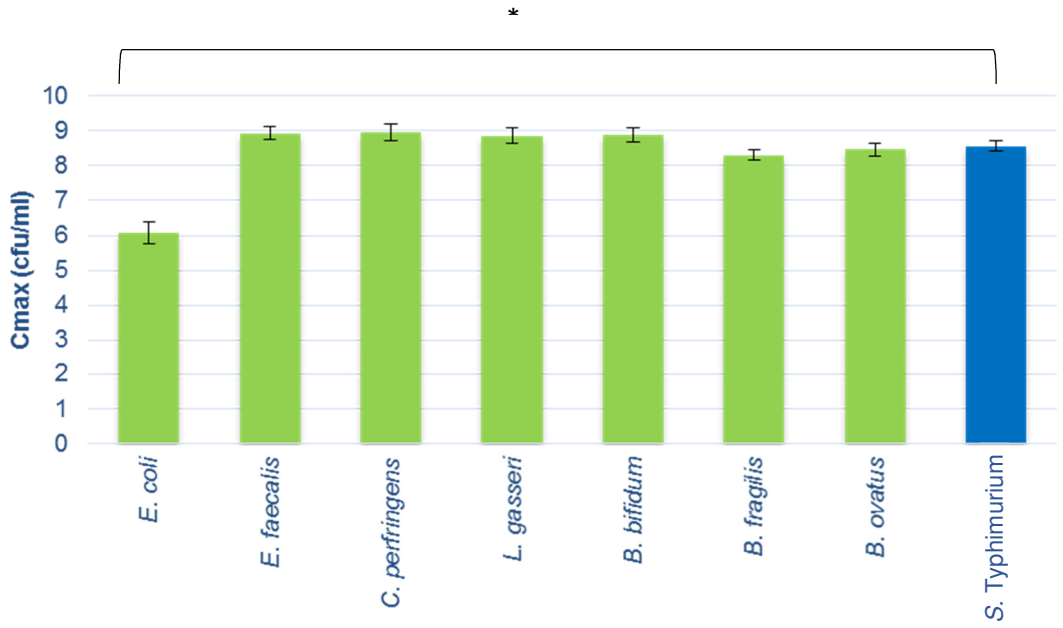

Figure A. 1. 3. Maximum concentration, Cmax, (cfu/ml) reached by *S.* Typhimurium when inoculated with bacterial strains of GI origin in stationary phase in a two-compartment system separated by a 0.45 µm pore membrane and in pure culture. Columns represent the $\log_{10}$ Cmax of *S.* Typhimurium in co-culture with each of the GI bacteria (green) and in pure culture (blue). Vertical bars indicate the standard error of the fitted parameter. Parameters estimated in pure culture were compared to those estimated in co-cultures. Horizontal bars and stars denote significant differences ($p < 0.05$).

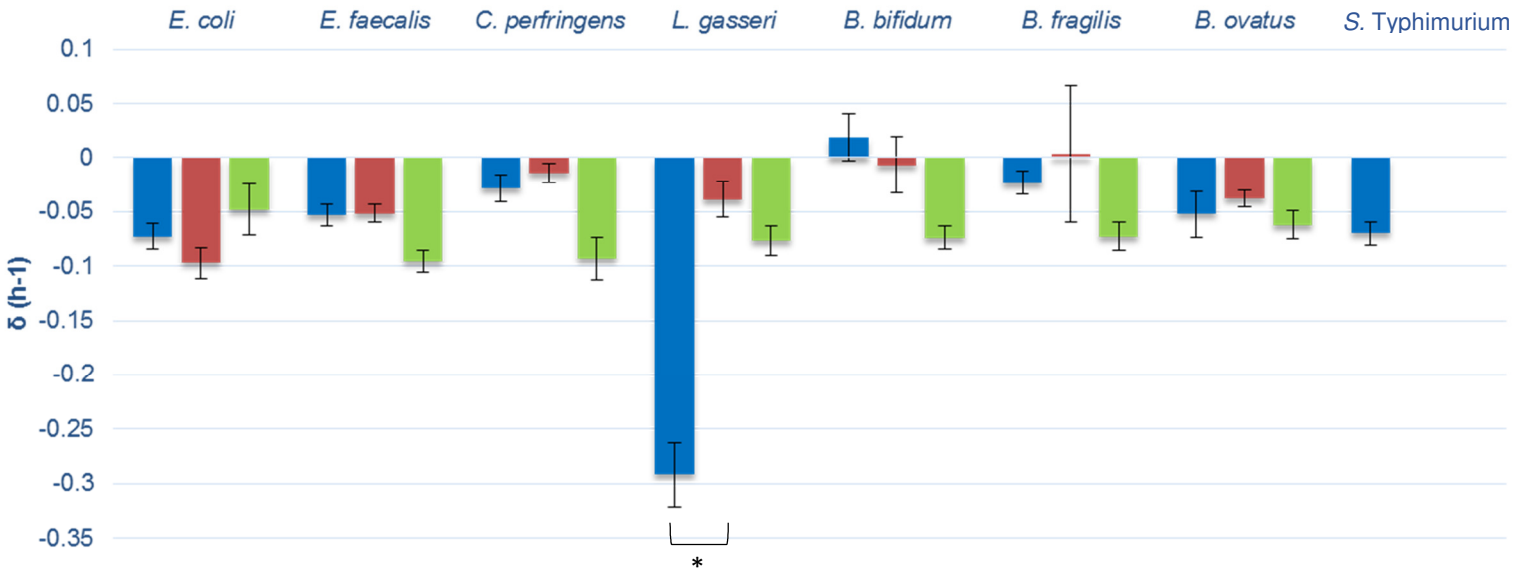

Figure A. 1. 4. Net specific decay rate, δ, (h^{-1}) of *S.* Typhimurium and culture collection bacterial strains of GI origin in stationary phase in a two-compartment system separated by a 0.45 µm pore membrane and in pure cultures. Columns represent the net decay rate of all strains in pure cultures (blue) and the rates of each GI bacteria (red) and of *S.* Typhimurium (green) in co-culture. Vertical bars indicate the standard error of the fitted parameter. Parameters estimated in pure culture were compared to those estimated in co-cultures. Horizontal bars and stars denote significant differences ($p < 0.05$).

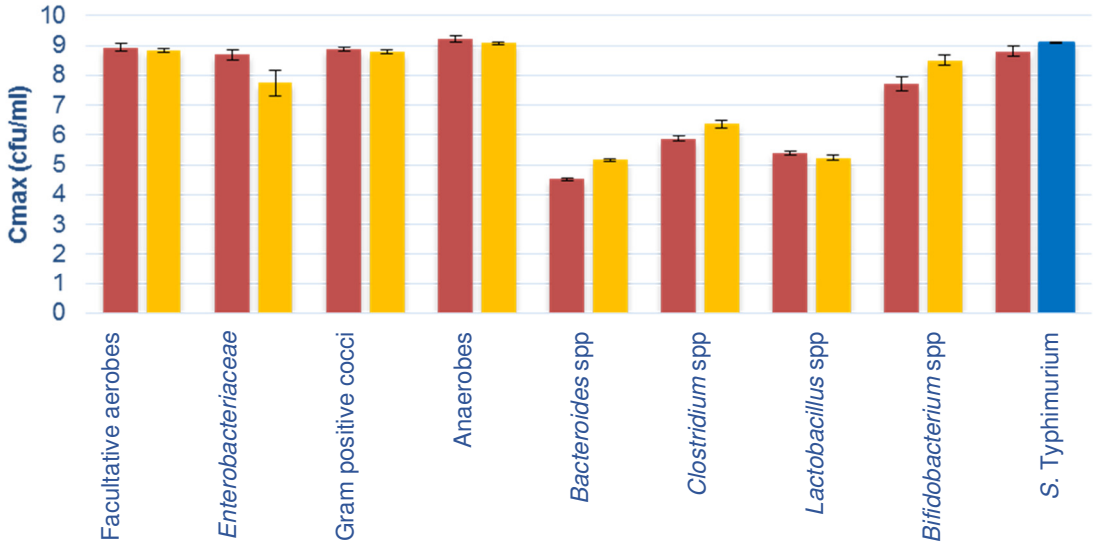

Figure A. 1. 5. Maximum concentration, Cmax, (cfu/ml) of *S.* Typhimurium and GI bacterial groups of a simplified gut microbiota model, from bacterial collections, in mixed and separated one-compartment batch cultures. Columns represent the $\log_{10}$ Cmax of all GI bacterial groups and *S.* Typhimurium in mixed culture (red), the $\log_{10}$ Cmax of all GI bacterial groups in mixed culture but with no *S.* Typhimurium (orange) and $\log_{10}$ Cmax of *S.* Typhimurium in pure culture (blue). Vertical bars indicate the standard error of the fitted parameter.

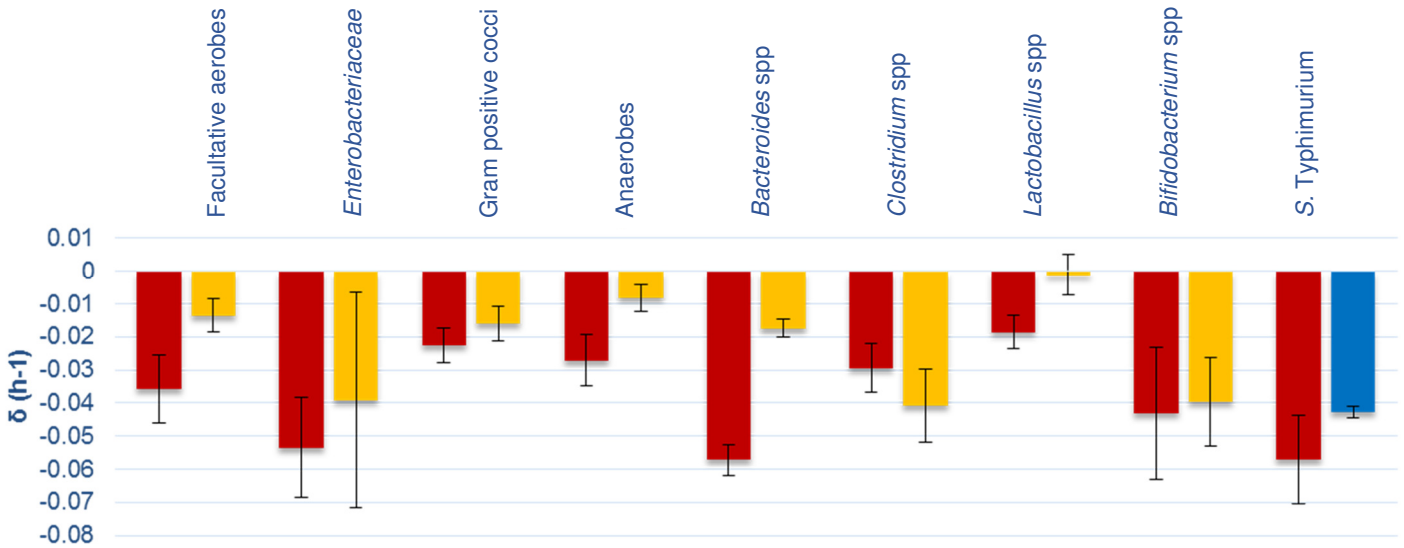

Figure A. 1. 6. Net specific decay rate, δ, (h^{-1}) of *S.* Typhimurium and GI bacterial groups of a simplified gut microbiota model, from bacterial collections, in mixed and separated one-compartment batch cultures. Columns represent the net decay rate of all GI bacterial groups and *S.* Typhimurium in mixed cultures (red), the net decay rate of all GI bacterial groups in mixed culture but with no *S.* Typhimurium (orange) and net decay rate of *S.* Typhimurium in pure culture (blue). Vertical bars indicate the standard error of the fitted parameter.

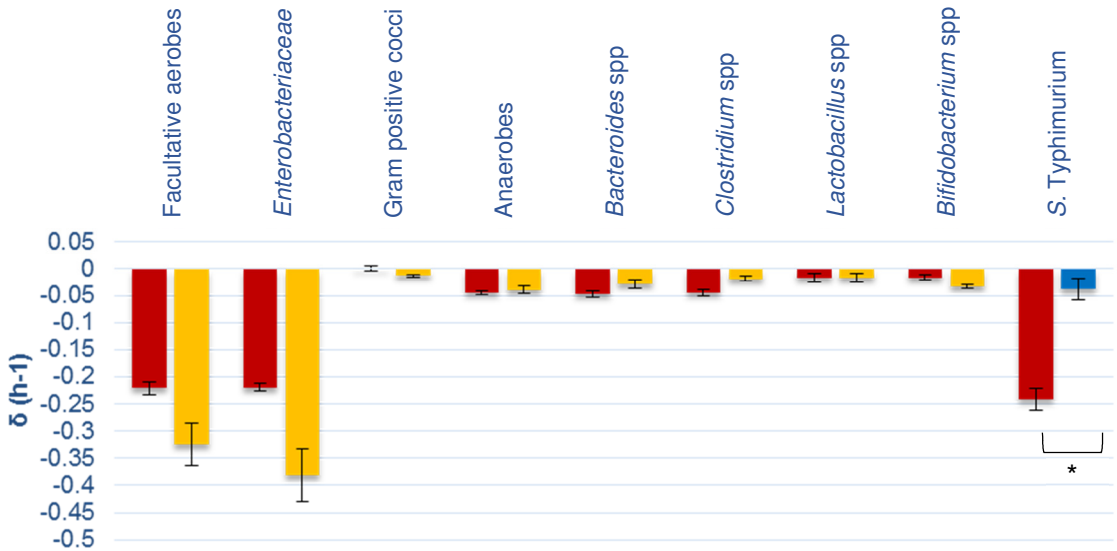

Figure A. 1. 7. Net specific decay rate, δ, (h^{-1}) of *S.* Typhimurium and GI bacterial groups, from faecal samples, in mixed and separated one-compartment batch cultures. Columns represent the net decay rate of all faecal bacteria groups and *S.* Typhimurium in mixed cultures (red), the net decay rate of all faecal bacteria groups in mixed culture but with no *S.* Typhimurium (orange) and net decay rate of *S.* Typhimurium in pure culture (blue). Vertical bars indicate the standard error of the fitted parameter. Parameters estimated in mixed cultures of faecal bacteria and *S.* Typhimurium were compared to those estimated in mixed culture of faecal bacteria without *S.* Typhimurium and to those estimated in pure culture of *S.* Typhimurium. Horizontal bars and stars denote significant differences ($p < 0.05$).

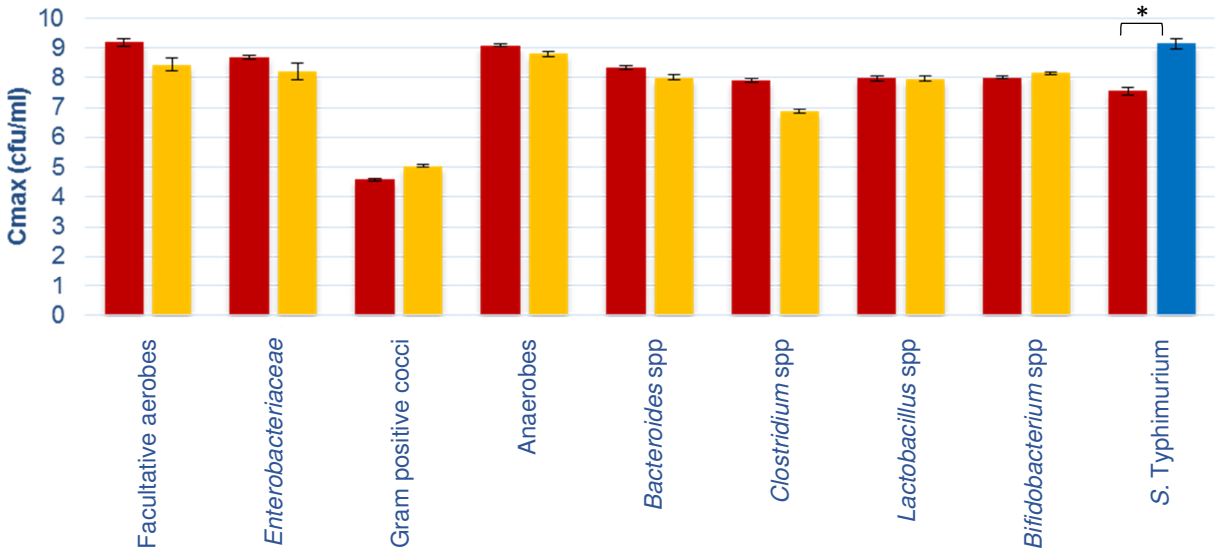

Figure A. 1. 8. Maximum concentration, Cmax, (cfu/ml) of *S.* Typhimurium and GI bacterial groups, from faecal samples, in mixed and separated one-compartment batch cultures. Columns represent the $\log_{10}$ Cmax of all faecal bacteria groups and *S.* Typhimurium in mixed cultures (red), the $\log_{10}$ Cmax of all faecal bacteria groups in mixed culture but with no *S.* Typhimurium (orange) and $\log_{10}$ Cmax of *S.* Typhimurium in pure culture (blue). Vertical bars indicate the standard error of the fitted parameter. Parameters estimated in mixed cultures of faecal bacteria and *S.* Typhimurium were compared to those estimated in mixed culture of faecal bacteria without *S.* Typhimurium and to those estimated in pure culture of *S.* Typhimurium. Horizontal bars and stars denote significant differences ($p < 0.05$).

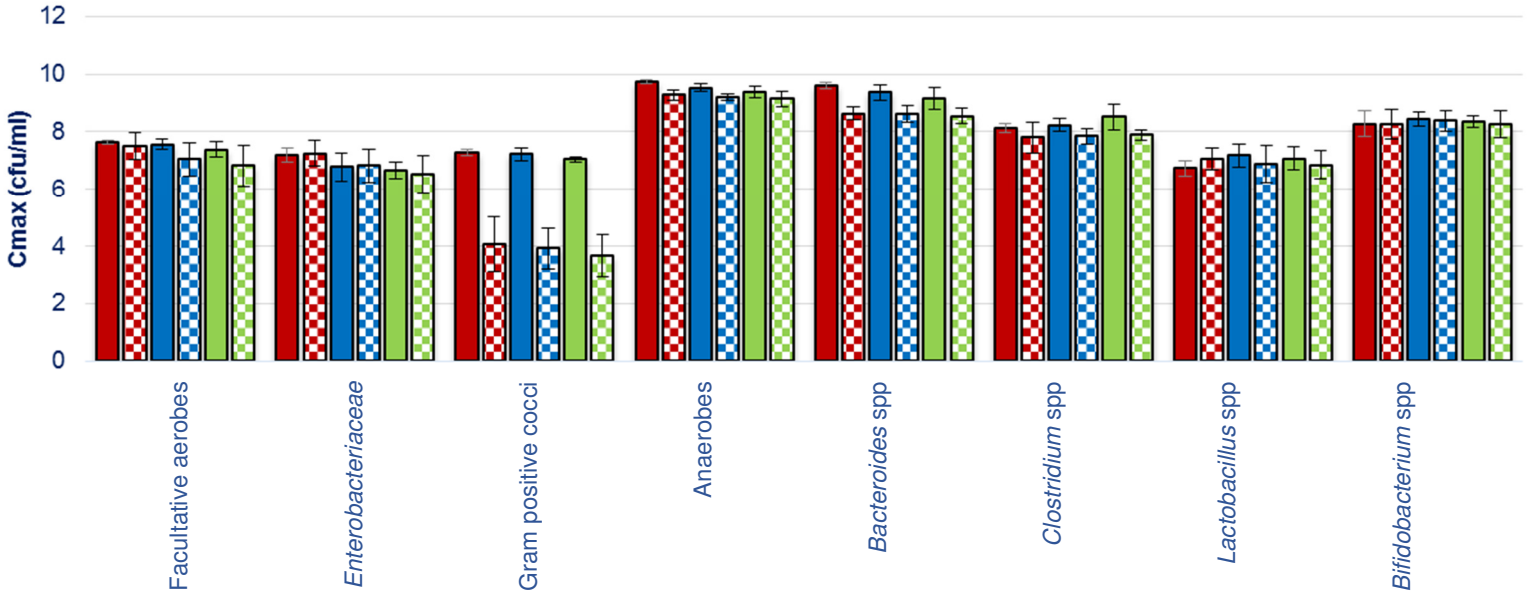

Figure A. 1. 9. Average values and standard deviation of the concentration of GI bacterial groups, from faecal samples, in a continuous system with and without *S.* Typhimurium. The continuous system consisted of three vessels representing the ascending (red columns), transverse (blue columns) and descending (green columns) sections of the colon. Smooth pattern columns denote the mixed cultures of faecal bacteria and *S.* Typhimurium and square pattern columns denote the control culture with no *S.* Typhimurium.

8.2 Appendix 2: Profile of gene expression in each culture condition and sampling time

ID_SL	S+C 3.5h	S+C 6h	S+C 9h	S 1h	S 8h	F 3.5h	F 6h	F 9h	F 24h
SL0306			1			1		1	
SL0870		1	1			1		1	
SL1004		1	1			1		1	
SL1059	1	1	1			1		1	
SL1367	1	1	1			1		1	
SL1464		1	1					1	
SL1613	1	1	1			1		1	
SL1648	1	1	1					1	
SL1721			1					1	
SL1773			1					1	
SL2042		1	1			1		1	
SL2046		1	1					1	
SL2082		1	1			1		1	
SL2098	1	1	1					1	
SL2290			1			1		1	
SL2370		1	1			1		1	
SL2485		1	1			1		1	
SL2494			1					1	
SL2517		1	1			1		1	
SL2542			1			1		1	
SL2543	1	1	1					1	
SL2654		1	1			1		1	
SL2668			1			1		1	
SL2828			1			1		1	
SL2910			1			1		1	
SL3109		1	1					1	
SL3120			1			1		1	
SL3139			1					1	
SL3255			1			1		1	
SL3343			1			1		1	

ID_SL	S+C 3.5h	S+C 6h	S+C 9h	S 1h	S 8h	F 3.5h	F 6h	F 9h	F 24h
SL2177			1			1	1	1	1
SL2181			1			1	1	1	1
SL2183				1		1		1	
SL2195									1
SL2196		1	1			1	1	1	1
SL2197	1	1	1						
SL2198						1		1	1
SL2200	1	1							
SL2201						1		1	
SL2206	1					1			
SL2219								1	
SL2222	1	1							
SL2223	1	1	1						
SL2224	1	1	1	1					
SL2225				1					
SL2226		1	1	1				1	
SL2228		1	1						
SL2229				1					
SL2230							1		
SL2232	1	1	1						
SL2233	1								
SL2234			1			1		1	1
SL2236	1	1	1			1	1	1	1
SL2237						1		1	
SL2239								1	1
SL2248								1	
SL2251	1								
SL2252						1		1	1
SL2253	1			1	1				
SL2254	1	1							

Table A. 1

SL3518	1	1	1					1	
SL3562	1	1	1					1	
SL3870		1	1			1		1	
SL3876			1			1		1	
SL3898		1	1			1		1	
SL3954			1			1		1	
SL4010		1	1			1		1	
SLP2_0014	1	1	1					1	
SLP2_0029		1	1			1		1	
SLP2_0036		1	1					1	
SL0001						1	1	1	1
SL0007					1	1		1	
SL0009						1			
SL0012				1	1	1		1	
SL0013			1						
SL0022		1							
SL0024		1							
SL0026		1							
SL0028						1		1	1
SL0032						1	1		1
SL0035	1	1							
SL0044				1					
SL0045	1	1							
SL0046	1								
SL0053									1
SL0055	1	1							
SL0056	1		1			1	1	1	1
SL0059						1			
SL0062						1			
SL0065		1	1			1			
SL0070	1								
SL0074	1	1							
SL0077						1		1	1
SL0078		1							

SL2257	1	1							
SL2266					1				
SL2267	1	1							
SL2275	1	1							
SL2276								1	
SL2278		1	1			1		1	1
SL2281			1			1		1	1
SL2283	1								
SL2289		1	1			1		1	1
SL2291			1						
SL2292		1	1			1		1	1
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SL2295			1			1		1	1
SL2296		1	1						
SL2299					1				
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SL2310								1	
SL2314		1							
SL2315						1			1
SL2321		1	1			1		1	1
SL2323			1			1			1
SL2333		1							
SL2334	1	1							
SL2341						1		1	1
SL2342	1	1							
SL2344									1
SL2347		1	1						
SL2348								1	1
SL2350			1			1		1	1
SL2351			1			1	1	1	1

Table A. 2

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SL0090		1	1						
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SL0093		1							
SL0095	1	1							
SL0101	1	1							
SL0105		1	1						
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SL0109	1	1							
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SL0118		1	1			1			
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SL0121	1	1	1						
SL0122						1		1	1
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SL0126		1	1			1	1	1	1
SL0129		1	1						
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SL0133						1		1	
SL0134	1				1	1		1	1
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SL0137	1	1	1			1		1	1
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SL0147						1			
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SL0151								1	
SL0153						1		1	
SL0155						1			1
SL0156	1	1							
SL0161			1						
SL0165	1	1							

SL2353	1								
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SL2436								1	
SL2437						1		1	
SL2438			1			1		1	1
SL2439		1	1			1		1	1
SL2441	1								

Table A. 2

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SL0230	1	1	1			1		1	1
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SL2479	1	1	1						
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Table A. 2

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SL0379	1	1							
SL0380		1							
SL0382								1	
SL0383		1							
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SL0403			1						

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SL2583	1								
SL2585	1								
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SL2627	1					1			1
SL2632		1							
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SL2648				1	1	1		1	

Table A. 2

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SL0423									1
SL0424						1			
SL0427		1	1			1		1	1
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SL0437	1								
SL0440	1							1	
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SL0442			1	1	1				
SL0444						1		1	
SL0445			1			1		1	1
SL0449			1			1	1	1	1
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SL0454		1							
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SL0501		1	1			1		1	1

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SL2678						1			
SL2680	1	1							
SL2682	1								
SL2683	1	1							
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SL2701		1						1	1
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SL2742						1		1	
SL2750			1						1
SL2753								1	1
SL2768	1	1							
SL2778									1
SL2779	1	1							
SL2783				1		1		1	
SL2785		1							
SL2786								1	
SL2790		1	1			1		1	1
SL2797								1	
SL2800	1	1							
SL2801	1								

Table A. 2

SL0503		1	1			1		1	1
SL0504		1	1			1	1	1	1
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SL0518		1	1			1		1	1
SL0520						1			
SL0525		1	1			1	1	1	1
SL0526						1	1		
SL0528		1	1			1	1	1	1
SL0529				1		1		1	
SL0536	1			1					
SL0539						1			
SL0544		1							
SL0548						1			
SL0551		1	1			1	1	1	1
SL0556		1	1						
SL0563								1	1
SL0567	1								
SL0574									1
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SL0578						1		1	1
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SL0583	1	1							
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SL0592	1		1			1		1	1
SL0596						1		1	
SL0597								1	
SL0600						1			
SL0602			1		1				
SL0603		1	1			1			1
SL0610		1	1			1	1	1	1

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SL2824	1	1							
SL2826						1		1	
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SL2883								1	1
SL2895		1	1			1			
SL2897			1			1		1	1
SL2900			1			1		1	1
SL2903					1			1	
SL2905	1		1						
SL2908	1	1	1			1		1	1
SL2926	1	1	1			1		1	1
SL2929	1	1							
SL2931				1	1	1	1	1	
SL2932						1		1	
SL2933		1	1			1	1	1	1
SL2937	1		1						

Table A. 2

SL0614						1			
SL0617				1	1	1		1	1
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SL0621		1	1						
SL0624					1				
SL0629						1		1	1
SL0630						1			
SL0636		1	1	1	1	1		1	
SL0641								1	
SL0647	1	1							
SL0649									1
SL0650	1								
SL0655						1			
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SL0698		1							
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SL2960	1	1							
SL2964		1	1			1	1	1	1
SL2968		1							
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SL2971									1
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SL2974		1	1			1		1	1
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SL2986	1	1							
SL2987	1	1						1	1
SL2989						1	1		
SL2991		1	1			1	1	1	1
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SL2997		1	1			1		1	1
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SL3006									1
SL3007	1	1							
SL3016			1	1	1	1			
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SL3033			1			1	1	1	1
SL3034						1			
SL3035						1	1	1	1

Table A. 2

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SL0744	1	1	1			1	1	1	1
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SL0750		1	1						
SL0751								1	
SL0752	1		1						
SL0753								1	
SL0758					1				
SL0765								1	1
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SL0767								1	
SL0769						1	1		
SL0771	1								
SL0775	1	1	1			1		1	1
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SL0783	1								
SL0790						1	1	1	1
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SL0797						1			
SL0805						1		1	
SL0806								1	1
SL0807	1								
SL0808						1		1	
SL0823			1			1		1	1
SL0827						1			1
SL0829	1	1	1		1	1	1	1	1
SL0830	1								
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SL3049		1							
SL3050	1	1							
SL3052	1								
SL3053						1			1
SL3055		1	1			1			
SL3058	1								
SL3065			1			1		1	1
SL3067		1				1			
SL3072									1
SL3079	1	1				1		1	
SL3081						1		1	
SL3083						1		1	
SL3088	1		1			1		1	1
SL3096						1			1
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SL3110									1
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SL3119	1		1						
SL3124	1								
SL3127		1	1						
SL3130	1	1							
SL3134			1					1	1
SL3141						1		1	
SL3143	1	1				1			1
SL3144								1	1
SL3148			1			1			1
SL3164						1		1	
SL3170	1	1							
SL3175								1	

Table A. 2

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SL0853						1			
SL0858						1		1	1
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SL0868		1	1						
SL0872	1		1			1		1	1
SL0877			1			1		1	1
SL0880							1		
SL0882	1				1			1	1
SL0883	1	1							
SL0884					1	1		1	
SL0885	1	1							
SL0894		1				1			
SL0896		1	1						
SL0898			1			1			1
SL0901						1		1	
SL0903	1	1	1			1		1	1
SL0907			1						
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SL0911			1	1	1	1	1	1	1
SL0913			1						
SL0915			1						
SL0918						1		1	
SL0920	1	1	1			1		1	1
SL0930		1	1						
SL0931			1			1		1	1
SL0941								1	1
SL0945	1	1							
SL0948	1								
SL0950	1	1							
SL0954	1	1							

SL3176		1	1			1	1	1	1
SL3178			1			1			
SL3179			1			1	1	1	1
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SL3183				1	1	1		1	
SL3184	1								
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SL3186			1			1		1	1
SL3188	1	1							
SL3193						1			
SL3202	1	1	1			1		1	1
SL3209								1	1
SL3218	1	1							
SL3219	1	1							
SL3221			1			1			
SL3229	1								
SL3232							1		1
SL3241	1	1	1						
SL3246			1						
SL3253				1	1	1			
SL3254				1	1	1		1	1
SL3256				1	1	1		1	
SL3261	1			1					
SL3264			1	1		1		1	
SL3267	1	1							
SL3271		1							
SL3273	1	1	1			1		1	1
SL3275				1	1	1		1	
SL3276				1	1	1		1	
SL3291		1	1						
SL3293				1	1				
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SL3316						1		1	
SL3317				1	1	1		1	

Table A. 2

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SL0970		1							
SL0971		1							
SL0976			1			1	1	1	1
SL0980	1	1							
SL0981		1	1			1		1	1
SL0992		1	1			1	1	1	1
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SL1002	1								
SL1006								1	
SL1010	1			1	1	1	1	1	
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SL1015		1							
SL1018			1						
SL1019	1	1							
SL1023	1	1							
SL1026	1								
SL1032	1								
SL1040	1	1							
SL1042		1							
SL1047	1								
SL1050	1	1							
SL1051						1		1	
SL1057	1	1			1				
SL1058	1	1	1					1	1
SL1061	1	1							
SL1064	1		1						
SL1070		1	1			1	1	1	1

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SL3356	1	1						1	1
SL3362	1								
SL3373	1								
SL3374		1							
SL3377								1	
SL3378	1			1					
SL3381	1			1	1	1	1	1	
SL3382				1	1	1		1	
SL3383				1	1	1	1	1	
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SL3385				1	1	1	1	1	
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SL3387				1	1	1		1	
SL3388				1	1	1		1	1
SL3389				1	1	1		1	
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SL3391				1	1	1		1	
SL3392				1	1	1		1	
SL3393			1	1	1	1		1	
SL3394				1	1	1		1	
SL3395				1	1	1		1	
SL3396				1	1	1	1	1	
SL3397	1			1	1	1	1	1	
SL3398				1	1	1		1	
SL3399		1	1	1	1	1		1	
SL3400			1	1	1	1		1	
SL3401			1	1	1	1		1	
SL3402				1	1	1		1	
SL3403			1	1	1	1		1	
SL3404				1	1				

Table A. 2

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SL1092		1							
SL1093		1							
SL1097	1	1							
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SL1123		1	1			1	1	1	1
SL1127				1	1	1		1	
SL1128				1	1	1		1	
SL1129	1	1							
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SL1132						1		1	
SL1133				1	1	1	1	1	
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SL1135	1	1	1			1	1	1	1
SL1138		1	1			1	1		
SL1139		1	1			1		1	1
SL1142		1			1				
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SL1149	1								
SL1150								1	1
SL1151					1	1		1	1
SL1157			1			1	1	1	1
SL1163	1								
SL1166	1	1							

SL3405				1	1	1	1	1	
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SL3407				1	1	1		1	
SL3408				1	1	1	1	1	
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SL3415				1	1	1		1	
SL3419	1	1	1						
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SL3441			1	1					
SL3446	1	1							
SL3449	1	1	1			1		1	1
SL3452		1		1					
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SL3455		1	1			1		1	1
SL3457	1	1							
SL3471								1	
SL3478						1		1	1
SL3480		1	1						
SL3486			1						
SL3493	1				1				
SL3501						1		1	
SL3506			1		1			1	
SL3508			1			1		1	1
SL3509	1								
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SL3521						1			
SL3525	1	1							
SL3533	1	1	1		1				
SL3534						1			
SL3537	1								

Table A. 2

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SL1175	1								
SL1180		1							
SL1181			1			1		1	1
SL1183						1		1	1
SL1187	1	1			1				
SL1197						1		1	
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SL1215	1	1	1						
SL1217	1	1							
SL1220	1	1							
SL1221		1	1			1	1	1	1
SL1224	1	1							
SL1225				1	1	1	1	1	1
SL1226								1	
SL1229									1
SL1230		1	1					1	1
SL1232	1	1							
SL1240	1		1			1		1	1
SL1244		1							1
SL1245	1								
SL1249	1	1							
SL1251	1								
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SL1267						1		1	
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SL1269				1	1	1		1	1
SL1270						1		1	1
SL1273				1	1	1		1	1

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SL3549		1							
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SL3551		1	1			1		1	1
SL3555		1	1			1	1	1	1
SL3556	1	1			1			1	
SL3558		1							
SL3574						1		1	1
SL3577		1							
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SL3586		1	1			1	1	1	1
SL3594			1			1		1	1
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SL3660						1			
SL3664	1								
SL3667					1				
SL3670			1	1		1		1	
SL3676						1		1	
SL3693	1					1		1	
SL3694				1	1	1		1	
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SL3737		1							
SL3741			1			1	1	1	1
SL3749	1								
SL3751	1	1							
SL3761		1				1			
SL3769		1							

Table A. 2

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SL1290	1	1							
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SL1307	1								
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SL1312				1		1		1	1
SL1313						1		1	
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SL1317		1	1			1		1	1
SL1320		1	1			1	1	1	1
SL1321		1							
SL1323	1	1							
SL1326	1								
SL1334		1							
SL1357		1	1			1	1	1	1
SL1359								1	
SL1363					1			1	
SL1366						1			
SL1372		1							
SL1377						1		1	
SL1384			1						
SL1385		1	1			1		1	1
SL1389						1	1		
SL1394						1		1	
SL1396	1								
SL1397	1	1				1		1	1
SL1398		1							
SL1400	1								
SL1402		1	1				1	1	1

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SL3777	1	1							
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SL3782	1	1							
SL3783				1					
SL3785	1	1		1					
SL3795						1			
SL3798		1	1						
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SL3832						1		1	
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SL3834						1		1	
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SL3837						1		1	
SL3838						1		1	
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SL3846						1			
SL3849		1	1						
SL3855						1		1	1

Table A. 2

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SL1410		1	1						
SL1415						1	1	1	1
SL1417						1	1		
SL1418			1					1	1
SL1419						1			
SL1423	1	1				1		1	
SL1426								1	1
SL1427	1	1	1			1		1	1
SL1433		1		1	1				
SL1434								1	1
SL1435	1								
SL1436	1								
SL1438		1							
SL1439		1							
SL1443			1					1	1
SL1447			1			1		1	1
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SL1455						1			
SL1468						1		1	
SL1472						1		1	
SL1475	1	1	1			1		1	1
SL1479	1								
SL1480	1								
SL1488	1								
SL1493	1	1							
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SL1498						1			
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SL1503	1	1						1	

SL3859				1					
SL3874						1		1	
SL3875				1	1	1		1	
SL3880		1	1			1	1	1	1
SL3882						1			
SL3888		1							
SL3895	1								
SL3900		1	1	1	1	1	1	1	1
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SL3917						1		1	1
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SL3922		1	1			1		1	1
SL3927				1					
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SL3937	1	1							
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SL3943	1								
SL3949	1	1	1	1	1			1	1
SL3951		1	1			1			
SL3955				1					
SL3965	1								
SL3974	1		1						
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SL3991	1								
SL4006						1		1	1
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SL4013	1	1							
SL4024								1	1
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SL4032	1	1							
SL4033		1							
SL4034								1	1

Table A. 2

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SL1534						1			
SL1538	1								
SL1540	1	1	1			1		1	1
SL1541		1							
SL1542						1		1	
SL1544			1						
SL1549	1	1							
SL1553						1		1	1
SL1554	1	1							
SL1557		1							
SL1558						1		1	
SL1560	1	1				1			
SL1562						1			
SL1575						1		1	1
SL1576						1			
SL1577					1				
SL1578	1		1			1		1	1
SL1589	1	1							
SL1591	1			1	1				
SL1592								1	1
SL1596			1			1		1	1
SL1603	1	1							
SL1604	1	1	1						
SL1605	1	1							
SL1611	1	1							
SL1612			1						
SL1616	1	1							

SL4037				1	1	1		1	
SL4038								1	
SL4040		1	1						
SL4041				1					
SL4056		1	1					1	1
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SL4062		1	1						
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SL4065	1	1							
SL4067	1	1							
SL4072		1	1			1			
SL4075			1					1	1
SL4078						1			
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SL4090				1	1	1	1	1	
SL4091				1	1	1	1	1	
SL4092			1						
SL4093			1	1		1		1	
SL4100						1			
SL4102			1			1	1	1	1
SL4106		1							
SL4109				1	1	1	1	1	
SL4125			1			1		1	1
SL4133	1								
SL4137	1								
SL4145	1								
SL4146		1	1			1	1	1	1
SL4158		1	1	1		1	1	1	1
SL4173								1	

Table A. 2

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SL1645						1		1	1
SL1649						1			
SL1651			1			1		1	1
SL1653						1		1	1
SL1655								1	1
SL1671			1			1		1	1
SL1680				1	1	1	1	1	
SL1682				1	1	1	1	1	
SL1683			1						
SL1690				1					
SL1691	1		1						
SL1693	1	1							
SL1696	1	1							
SL1698								1	1
SL1702	1								
SL1703	1	1							
SL1709		1	1			1		1	1
SL1710	1	1	1			1	1	1	1
SL1712		1							
SL1719						1			
SL1720								1	
SL1724						1		1	1
SL1725		1	1						
SL1730	1	1							

SL4185	1								
SL4188		1	1			1		1	1
SL4191	1								
SL4205							1		1
SL4207								1	
SL4212		1							
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SL4263						1		1	
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SL4267				1	1	1		1	
SL4271						1		1	
SL4279						1		1	
SL4280						1		1	
SL4285						1			
SL4287	1	1				1			
SL4289	1	1							
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SL4292		1	1						1
SL4295					1	1		1	
SL4299						1		1	
SL4301		1	1						
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SL4324			1	1	1	1		1	
SL4325				1	1	1		1	

Table A. 2

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SL1767					1	1		1	
SL1768	1	1							
SL1780			1						
SL1796	1								
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SL1805	1	1	1						1
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SL1816		1	1					1	1
SL1817						1	1	1	1
SL1820								1	1
SL1828		1	1			1	1	1	1
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SL1836			1			1	1	1	1
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SL1849						1			
SL1856						1		1	1
SL1857		1	1			1	1	1	1
SL1858		1							
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SL1869						1		1	
SL1871						1		1	

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SL4373	1	1							
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SL4452	1	1							
SL4459	1	1							
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SL4463								1	
SL4475	1								
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SL4496				1					
SL4497			1	1					
SL4508			1			1	1	1	1
SL4511	1								

Table A. 2

SL1873	1	1							
SL1875		1	1						
SL1877	1	1							
SL1878						1			
SL1879	1	1				1			
SL1884	1					1			
SL1886	1	1							
SL1888	1								
SL1889	1	1				1			
SL1892			1			1		1	1
SL1899		1	1			1	1	1	1
SL1903		1	1			1		1	1
SL1906						1			1
SL1911	1								
SL1913	1	1	1			1		1	1
SL1916	1	1							
SL1917	1	1	1						
SL1922						1	1	1	1
SL1929		1							
SL1931		1							
SL1936	1	1							
SL1940	1	1							
SL1941								1	1
SL1942	1								
SL1943						1		1	1
SL1953	1	1				1		1	1
SL1957		1	1			1		1	1
SL1959								1	1
SL1962	1	1							
SL1964	1	1							
SL1972	1	1							
SL1976	1	1							
SL1977			1				1	1	1
SL1979		1	1				1	1	1

SL4516	1					1			
SL4525				1		1	1	1	1
SL4526				1					
SLP1_0001	1	1							
SLP1_0002	1	1							
SLP1_0005	1	1							
SLP1_0006	1	1							
SLP1_0008		1	1		1				
SLP1_0009	1	1		1	1				
SLP1_0010	1								
SLP1_0011	1	1							
SLP1_0014	1	1				1			
SLP1_0020		1							
SLP1_0021		1							
SLP1_0022	1	1							
SLP1_0024	1	1							
SLP1_0025	1								
SLP1_0040	1	1							
SLP1_0041		1	1						
SLP1_0043	1	1							
SLP1_0045	1	1	1						
SLP1_0049	1	1							
SLP1_0051	1	1							
SLP1_0052	1	1							
SLP1_0057		1				1		1	
SLP1_0058	1	1							
SLP1_0059	1	1							
SLP1_0060		1							
SLP1_0061	1	1			1				
SLP1_0064	1	1							
SLP1_0069		1	1			1		1	1
SLP1_0072	1	1		1	1				
SLP1_0073	1	1							
SLP1_0076	1	1							

Table A. 2

SL1994	1	1	1			1	1	1	1
SL1998		1				1			
SL2000									1
SL2003		1							
SL2004						1			
SL2015			1			1	1	1	1
SL2016	1	1							
SL2017	1								
SL2018								1	1
SL2023						1		1	
SL2026			1			1		1	1
SL2027		1							
SL2030		1	1			1	1	1	1
SL2035					1	1		1	
SL2041	1	1							
SL2045						1			
SL2047	1	1	1						
SL2048			1		1			1	
SL2049					1				
SL2050		1			1				
SL2051					1				
SL2052			1		1	1		1	1
SL2053					1				
SL2054		1	1		1	1			
SL2055					1				
SL2058						1	1	1	
SL2064						1			
SL2068		1	1			1		1	1
SL2076								1	
SL2081	1	1	1			1		1	1
SL2083						1			
SL2086						1	1	1	1
SL2087						1			
SL2090	1								

SLP1_0077									1
SLP1_0078	1	1	1					1	1
SLP1_0079	1	1							
SLP1_0082				1					
SLP1_0089	1	1							
SLP1_0090		1	1					1	1
SLP1_0096		1							
SLP1_0098	1	1							
SLP1_0099	1	1	1						
SLP1_0101	1	1							
SLP1_0102				1	1				
SLP2_0002	1	1	1						
SLP2_0003	1	1							
SLP2_0006	1	1							
SLP2_0008		1	1						
SLP2_0015		1	1					1	1
SLP2_0016	1	1							
SLP2_0020	1	1							
SLP2_0023	1	1	1						
SLP2_0027		1	1						
SLP2_0031		1	1						
SLP2_0033	1		1						
SLP2_0035	1	1	1			1		1	1
SLP2_0042	1	1	1						
SLP2_0043	1	1	1						
SLP2_0044	1	1		1	1	1		1	1
SLP2_0045	1	1	1						
SLP2_0049	1	1							
SLP2_0050	1	1							
SLP2_0053	1	1	1	1		1		1	1
SLP2_0054	1	1	1	1		1		1	1
SLP2_0055	1	1							
SLP2_0058	1	1							
SLP2_0064		1							

Table A. 2

SL2092	1		1						
SL2099	1								
SL2101			1			1		1	1
SL2102		1	1			1		1	1
SL2104		1	1			1		1	1
SL2117		1	1			1	1	1	1
SL2119								1	
SL2120	1	1							
SL2123		1	1			1		1	1
SL2124			1			1		1	1
SL2125									1
SL2131			1						
SL2135		1							
SL2139		1	1	1	1	1	1	1	1
SL2140						1			1
SL2143			1			1		1	1
SL2144	1								
SL2145									1
SL2146		1							
SL2147						1			
SL2153	1								
SL2154	1	1							
SL2171	1					1			1
SL2175		1	1			1	1	1	1

SLP2_0065	1								
SLP2_0066	1	1							
SLP2_0069	1	1							
SLP2_0075			1			1	1	1	1
SLP2_0076	1	1							
SLP2_0088		1							
SLP2_0093	1	1							
SLP2_0094	1	1							
SLP2_0095		1	1						
SLP2_0098		1							
SLP2_0101		1							
SLP3_0001		1	1	1	1	1		1	
SLP3_0002	1	1							
SLP3_0003	1	1							
SLP3_0004		1	1			1	1	1	1
SLP3_0005	1		1			1		1	1
SLP3_0006	1	1							
SLP3_0007	1	1							
SLP3_0008	1	1							
SLP3_0009	1	1							
SLP3_0010	1	1							
SLP3_0011	1	1							
SLP3_0012		1	1					1	1
SLP3_0013			1	1		1	1	1	1
SLP3_0014	1	1	1		1				

Table A. 2. Profile of gene expression in each culture condition and sampling time. The first column includes the genes of *S.* Typhimurium SL 1344 (ID SL). *S.* Typhimurium in co-culture with faecal bacteria is denoted by 'S+C'. *S.* Typhimurium in pure culture is denoted by 'S'. Cultures of faecal bacteria free from *S.* Typhimurium are denoted by 'F'.

8.3 Appendix 3: Cellular functions and metabolic pathways expressed by *S.* Typhimurium in mixed culture with faecal bacteria

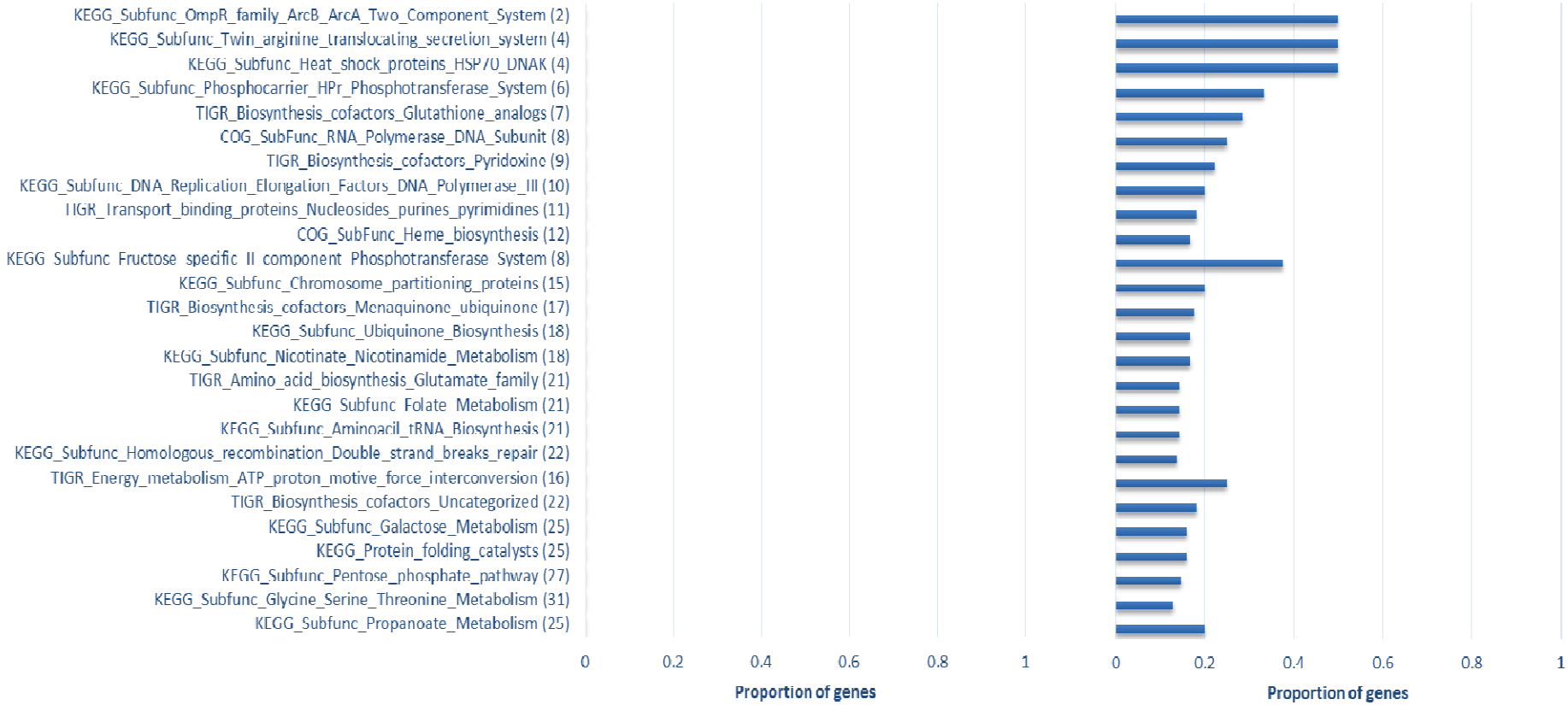


Figure A. 3. 1


KEGG_Subfunc_Glyoxylate_Dicarboxylate_Metabolism (30)
KEGG_Other_Amino_acids_Metabolism (52)
TIGR_Cellular_processes_Cell_division (36)
TIGR_Energy_metabolism_Fermentation (46)
COG_Defense_mechanisms (48)
TIGR_Cell_envelope_Biosynthesis_degradation_murein_sacculus_peptidoglycan (51)
TIGR_Energy_metabolism_Amino_acids_amines (52)
TIGR_Energy_metabolism_Sugars (55)
TIGR_Cell_envelope_Surface_structures (60)
KEGG_Phosphotransferase_System (63)
COG_Secondary_metabolites_biosynthesis_transport_catabolism (68)
KEGG_ABC_transporters_Phosphate_amino_acid (42)
TIGR_Fatty_acid_phospholipid_metabolism_Biosynthesis (46)
KEGG_Subfunc_Fructose_Mannose_Metabolism (58)
KEGG_Subfunc_Amino_sugar_Nucleotide_sugar_Metabolism (64)
KEGG_Subfunc_Arginine_Proline_Metabolism (49)
KEGG_Lipid_Metabolism (59)
TIGR_Transport_binding_proteins_Cations_iron_carrying_compounds (95)
TIGR_Cell_envelope_Biosynthesis_degradation_surface_polysaccharides_lipopolysaccharides (85)
TIGR_Energy_metabolism_Glycolysis (41)
TIGR_Protein_fate_Degradation_proteins_peptides_glycopeptides (76)
KEGG_Subfunc_Purine_Metabolism (77)
KEGG_Transcription (107)
COG_Cell_motility (125)
KEGG_Nucleotide_Metabolism (101)
TIGR_Energy_metabolism_Uncategorized (88)
TIGR_Energy_metabolism_Electron_transport (110)
TIGR_Transport_binding_proteins_Amino_acids_peptides_amines (178)
COG_Signal_transduction_mechanisms (179)
KEGG_Cofactors_Vitamins_Metabolism (121)
TIGR_Mobile_extrachromosomal_element_functions_Prophage_functions (160)
TIGR_Transport_binding_proteins_Uncategorized (165)
COG_Coenzyme_transport_metabolism (177)
COG_Inorganic_ion_transport_metabolism (198)
TIGR_DNA_metabolism_DNA_replication_recombination_repair (126)
0
0.2
0.4
0.6
0.8
1
Proportion of genes
0
0.2
0.4
0.6
0.8
1
Proportion of genes

Figure A. 3. 1


COG_Intracellular_trafficking_secretion (140)
TIGR_Protein_synthesis_tRNA_aminoacylation (238)
COG_Replication_recombination_repair (161)
TIGR_Cell_envelope_Uncategorized (271)
COG_Transcription (329)
COG_Cell_wall_membrane_biogenesis (253)
COG_Amino_acid_transport_metabolism (356)
COG_Carbohydrate_transport_metabolism (388)
KEGG_Carbohydrate_Metabolism (299)
KEGG_Subfunc_Chromosome_Nucleoid_associated_proteins_Histone_like (2)
KEGG_Subfunc_Heat_shock_proteins_HSP60_Chaperonin (1)
KEGG_Subfunc_Heat_shock_proteins_HSP10 (1)
TIGR_DNA_metabolism_Chromosome_associated_proteins (2)
KEGG_Subfunc_Ribosomal_Proteins_Small_Subunit (22)
COG_SubFunc_Ribosomal_large_subunit (33)
KEGG_Subfunc_Ribosomal_Proteins_Large_Subunit (35)
TIGR_Protein_synthesis_Ribosomal_proteins_synthesis_modification (63)
COG_SubFunc_Glycolysis (12)
KEGG_Subfunc_Chromosome_Nucleoid_associated_proteins_Integration_Host_Factor (2)
COG_SubFunc_Gluconeogenesis (11)
KEGG_Translation (113)
KEGG_Subfunc_Non_homologous_endjoying_Double_strand_breaks_repair (17)
COG_Translation (186)
KEGG_Subfunc_DNA_Replication_Initiation_Factors (10)
TIGR_Protein_synthesis_Translation_factors (33)
KEGG_Subfunc_Glycolysis (37)
KEGG_Subfunc_Transcription_factors (25)
KEGG_Subfunc_Phenylalanine_Tyrosine_Tryptophan_Biosynthesis (23)
KEGG_Subfunc_Heat_shock_proteins_HSP27 (3)
KEGG_Subfunc_Enzyme_I_Phosphotransferase_System (3)
KEGG_Subfunc_Transcription_factors_Helix_turn_helix_Fur_Family (2)
TIGR_Amino_acid_biosynthesis_Histidine_family (9)
TIGR_Cellular_processes_Adaptations_to_atypical_conditions (32)
KEGG_Subfunc_OmpR_family_RstB_RstA_Two_Component_System (2)
KEGG_Subfunc_Multidrug_ABC2_transporter (2)
0
0.2
0.4
0.6
0.8
1
Proportion of genes
0
0.2
0.4
0.6
0.8
1
Proportion of genes

Figure A. 3. 1

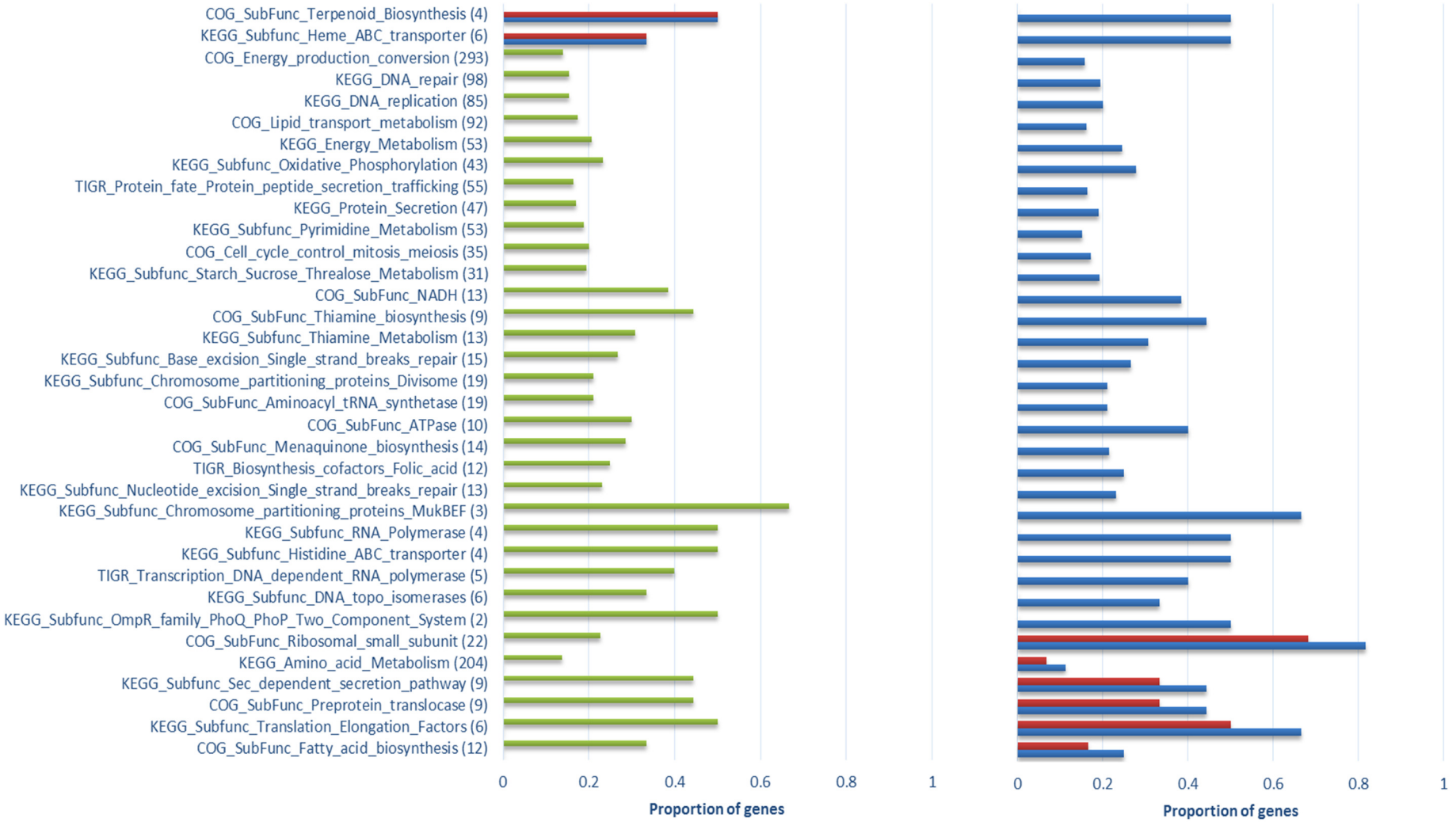
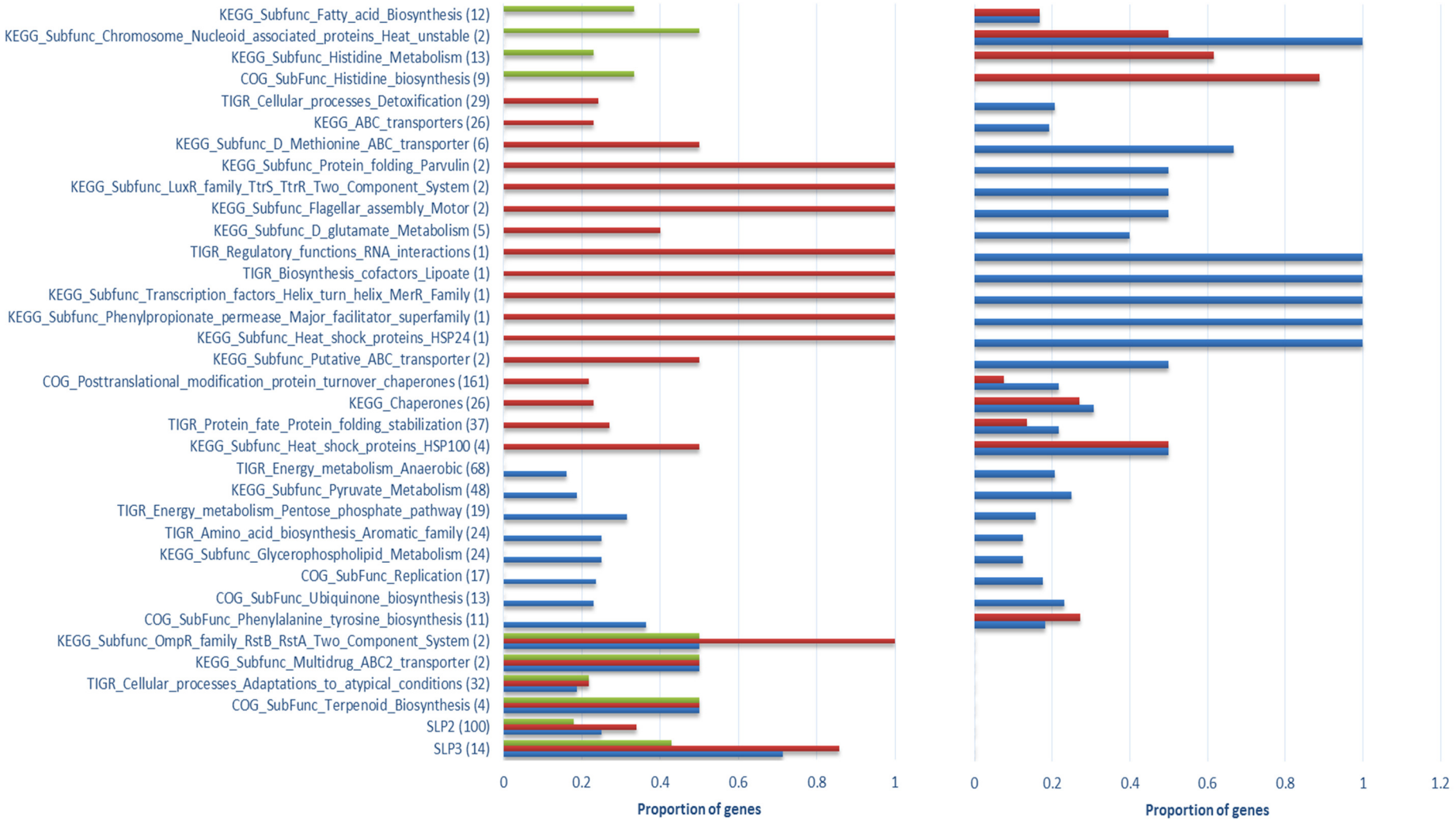

COG_SubFunc_Terpenoid_Biosynthesis (4)
KEGG_Subfunc_Heme_ABC_transporter (6)
COG_Energy_production_conversion (293)
KEGG_DNA_repair (98)
KEGG_DNA_replication (85)
COG_Lipid_transport_metabolism (92)
KEGG_Energy_Metabolism (53)
KEGG_Subfunc_Oxidative_Phosphorylation (43)
TIGR_Protein_fate_Protein_peptide_secretion_trafficking (55)
KEGG_Protein_Secretion (47)
KEGG_Subfunc_Pyrimidine_Metabolism (53)
COG_Cell_cycle_control_mitosis_meiosis (35)
KEGG_Subfunc_Starch_Sucrose_Threalose_Metabolism (31)
COG_SubFunc_NADH (13)
COG_SubFunc_Thiamine_biosynthesis (9)
KEGG_Subfunc_Thiamine_Metabolism (13)
KEGG_Subfunc_Base_excision_Single_strand_breaks_repair (15)
KEGG_Subfunc_Chromosome_partitioning_proteins_Divisome (19)
COG_SubFunc_Aminoacyl_tRNA_synthetase (19)
COG_SubFunc_ATPase (10)
COG_SubFunc_Menaquinone_biosynthesis (14)
TIGR_Biosynthesis_cofactors_Folic_acid (12)
KEGG_Subfunc_Nucleotide_excision_Single_strand_breaks_repair (13)
KEGG_Subfunc_Chromosome_partitioning_proteins_MukBEF (3)
KEGG_Subfunc_RNA_Polymerase (4)
KEGG_Subfunc_Histidine_ABC_transporter (4)
TIGR_Transcription_DNA_dependent_RNA_polymerase (5)
KEGG_Subfunc_DNA_topo_isomerases (6)
KEGG_Subfunc_OmpR_family_PhoQ_PhoP_Two_Component_System (2)
COG_SubFunc_Ribosomal_small_subunit (22)
KEGG_Amino_acid_Metabolism (204)
KEGG_Subfunc_Sec_dependent_secretion_pathway (9)
COG_SubFunc_Preprotein_translocase (9)
KEGG_Subfunc_Translation_Elongation_Factors (6)
COG_SubFunc_Fatty_acid_biosynthesis (12)
0
0.2
0.4
0.6
0.8
1
Proportion of genes
0
0.2
0.4
0.6
0.8
1
Proportion of genes

Figure A. 3. 1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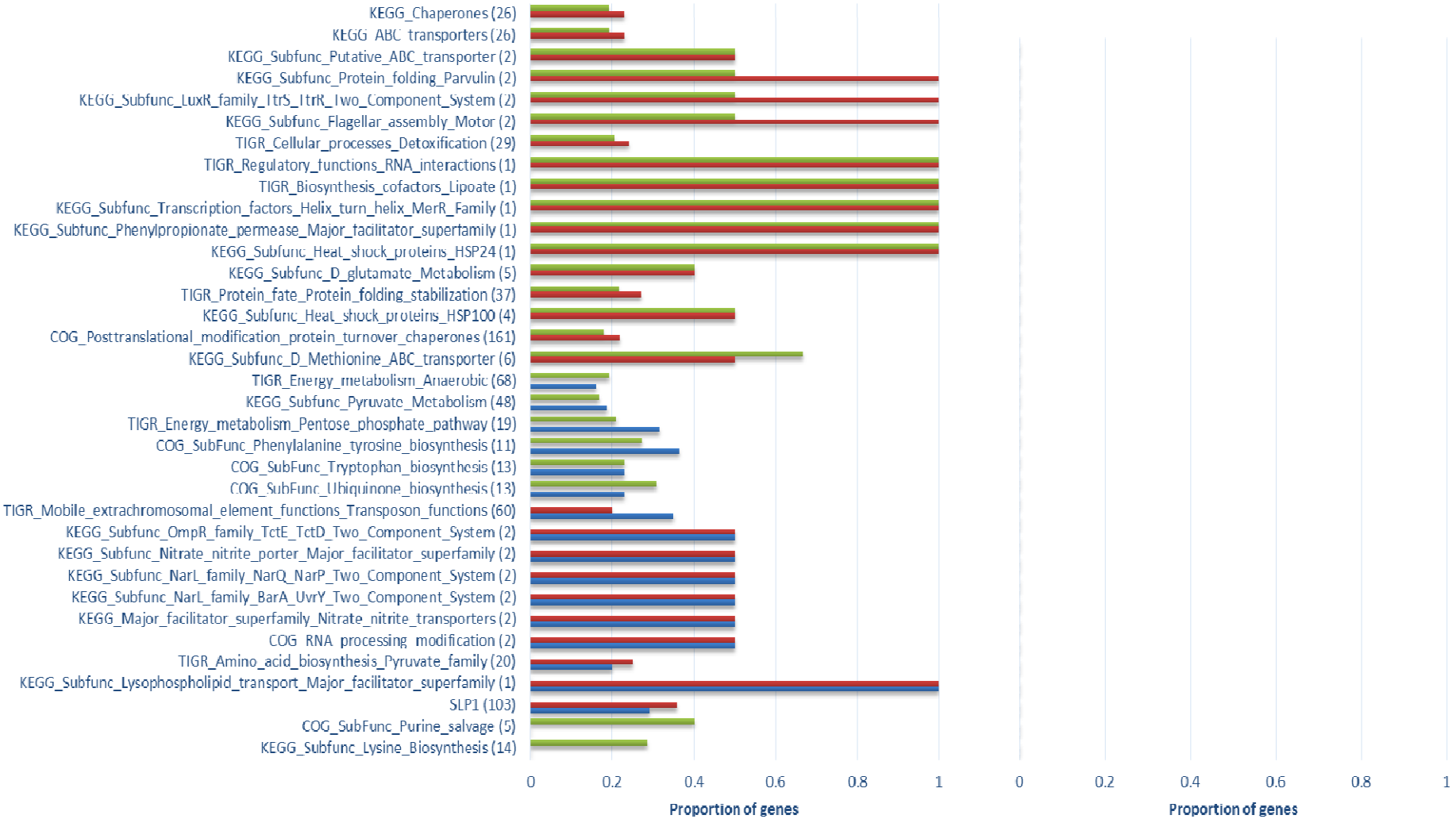
KEGG_Subfunc_Chromosome_Nucleoid_associated_proteins_Heat_unstable (2)
KEGG_Subfunc_Histidine_Metabolism (13)
COG_SubFunc_Histidine_biosynthesis (9)
TIGR_Cellular_processes_Detoxification (29)
KEGG_ABC_transporters (26)
KEGG_Subfunc_D_Methionine_ABC_transporter (6)
KEGG_Subfunc_Protein_folding_Parvulin (2)
KEGG_Subfunc_LuxR_family_TtrS_TtrR_Two_Component_System (2)
KEGG_Subfunc_Flagellar_assembly_Motor (2)
KEGG_Subfunc_D_glutamate_Metabolism (5)
TIGR_Regulatory_functions_RNA_interactions (1)
TIGR_Biosynthesis_cofactors_Lipoate (1)
KEGG_Subfunc_Transcription_factors_Helix_turn_helix_MerR_Family (1)
KEGG_Subfunc_Phenylpropionate_permease_Major_facilitator_superfamily (1)
KEGG_Subfunc_Heat_shock_proteins_HSP24 (1)
KEGG_Subfunc_Putative_ABC_transporter (2)
COG_Posttranslational_modification_protein_turnover_chaperones (161)
KEGG_Chaperones (26)
TIGR_Protein_fate_Protein_folding_stabilization (37)
KEGG_Subfunc_Heat_shock_proteins_HSP100 (4)
TIGR_Energy_metabolism_Anaerobic (68)
KEGG_Subfunc_Pyruvate_Metabolism (48)
TIGR_Energy_metabolism_Pentose_phosphate_pathway (19)
TIGR_Amino_acid_biosynthesis_Aromatic_family (24)
KEGG_Subfunc_Glycerophospholipid_Metabolism (24)
COG_SubFunc_Replication (17)
COG_SubFunc_Ubiquinone_biosynthesis (13)
COG_SubFunc_Phenylalanine_tyrosine_biosynthesis (11)
KEGG_Subfunc_OmpR_family_RstB_RstA_Two_Component_System (2)
KEGG_Subfunc_Multidrug_ABC2_transporter (2)
TIGR_Cellular_processes_Adaptations_to_atypical_conditions (32)
COG_SubFunc_Terpenoid_Biosynthesis (4)
SLP2 (100)
SLP3 (14)
0
0.2
0.4
0.6
0.8
1
Proportion of genes
0
0.2
0.4
0.6
0.8
1
1.2
Proportion of genes

Figure A. 3. 1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KEGG_ABC_transporters (26)
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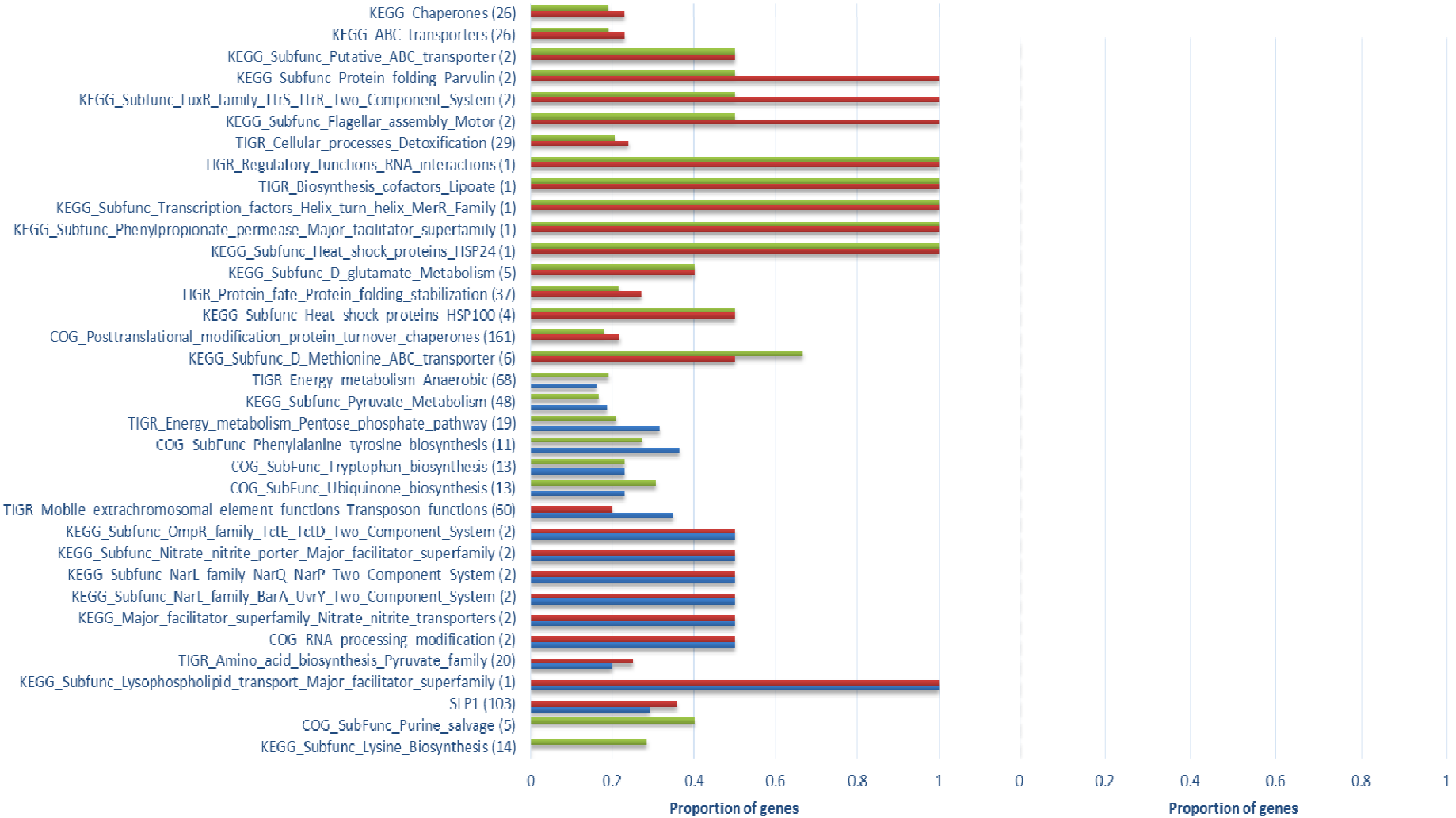
KEGG_Subfunc_Protein_folding_Parvulin (2)
KEGG_Subfunc_LuxR_family_ItrS_ItrR_Two_Component_System (2)
KEGG_Subfunc_Flagellar_assembly_Motor (2)
TIGR_Cellular_processes_Detoxification (29)
TIGR_Regulatory_functions_RNA_interactions (1)
TIGR_Biosynthesis_cofactors_Lipoate (1)
KEGG_Subfunc_Transcription_factors_Helix_turn_helix_MerR_Family (1)
KEGG_Subfunc_Phenylpropionate_permease_Major_facilitator_supertamily (1)
KEGG_Subfunc_Heat_shock_proteins_HSP24 (1)
KEGG_Subfunc_D_glutamate_Metabolism (5)
TIGR_Protein_fate_Protein_folding_stabilization (37)
KEGG_Subfunc_Heat_shock_proteins_HSP100 (4)
COG_Posttranslational_modification_protein_turnover_chaperones (161)
KEGG_Subfunc_D_Methionine_ABC_transporter (6)
TIGR_Energy_metabolism_Anaerobic (68)
KEGG_Subfunc_Pyruvate_Metabolism (48)
TIGR_Energy_metabolism_Pentose_phosphate_pathway (19)
COG_SubFunc_Phenylalanine_tyrosine_biosynthesis (11)
COG_SubFunc_Tryptophan_biosynthesis (13)
COG_SubFunc_Ubiquinone_biosynthesis (13)
TIGR_Mobile_extrachromosomal_element_functions_Transposon_functions (60)
KEGG_Subfunc_OmpR_family_TctE_TctD_Two_Component_System (2)
KEGG_Subfunc_Nitrate_nitrite_porter_Major_facilitator_superfamily (2)
KEGG_Subfunc_NarL_family_NarQ_NarP_Two_Component_System (2)
KEGG_Subfunc_NarL_family_BarA_UvrY_Two_Component_System (2)
KEGG_Major_facilitator_superfamily_Nitrate_nitrite_transporters (2)
COG_RNA_processing_modification (2)
TIGR_Amino_acid_biosynthesis_Pyruvate_family (20)
KEGG_Subfunc_Lysophospholipid_transport_Major_facilitator_superfamily (1)
SLP1 (103)
COG_SubFunc_Purine_salvage (5)
KEGG_Subfunc_Lysine_Biosynthesis (14)
0
0.2
0.4
0.6
0.8
1
Proportion of genes
0
0.2
0.4
0.6
0.8
1
Proportion of genes

Picture A. 3. 1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KEGG_ABC_transporters (26)
KEGG_Subfunc_Putative_ABC_transporter (2)
KEGG_Subfunc_Protein_folding_Parvulin (2)
KEGG_Subfunc_LuxR_family_ItrS_ItrR_Two_Component_System (2)
KEGG_Subfunc_Flagellar_assembly_Motor (2)
TIGR_Cellular_processes_Detoxification (29)
TIGR_Regulatory_functions_RNA_interactions (1)
TIGR_Biosynthesis_cofactors_Lipoate (1)
KEGG_Subfunc_Transcription_factors_Helix_turn_helix_MerR_Family (1)
KEGG_Subfunc_Phenylpropionate_permease_Major_facilitator_superfamily (1)
KEGG_Subfunc_Heat_shock_proteins_HSP24 (1)
KEGG_Subfunc_D_glutamate_Metabolism (5)
TIGR_Protein_fate_Protein_folding_stabilization (37)
KEGG_Subfunc_Heat_shock_proteins_HSP100 (4)
COG_Posttranslational_modification_protein_turnover_chaperones (161)
KEGG_Subfunc_D_Methionine_ABC_transporter (6)
TIGR_Energy_metabolism_Anaerobic (68)
KEGG_Subfunc_Pyruvate_Metabolism (48)
TIGR_Energy_metabolism_Pentose_phosphate_pathway (19)
COG_SubFunc_Phenylalanine_tyrosine_biosynthesis (11)
COG_SubFunc_Tryptophan_biosynthesis (13)
COG_SubFunc_Ubiquinone_biosynthesis (13)
TIGR_Mobile_extrachromosomal_element_functions_Transposon_functions (60)
KEGG_Subfunc_OmpR_family_TctE_TctD_Two_Component_System (2)
KEGG_Subfunc_Nitrate_nitrite_porter_Major_facilitator_superfamily (2)
KEGG_Subfunc_NarL_family_NarQ_NarP_Two_Component_System (2)
KEGG_Subfunc_NarL_family_BarA_UvrY_Two_Component_System (2)
KEGG_Major_facilitator_superfamily_Nitrate_nitrite_transporters (2)
COG_RNA_processing_modification (2)
TIGR_Amino_acid_biosynthesis_Pyruvate_family (20)
KEGG_Subfunc_Lysophospholipid_transport_Major_facilitator_superfamily (1)
SLP1 (103)
COG_SubFunc_Purine_salvage (5)
KEGG_Subfunc_Lysine_Biosynthesis (14)
0
0.2
0.4
0.6
0.8
1
Proportion of genes
0
0.2
0.4
0.6
0.8
1
Proportion of genes

Figure A. 3. 1

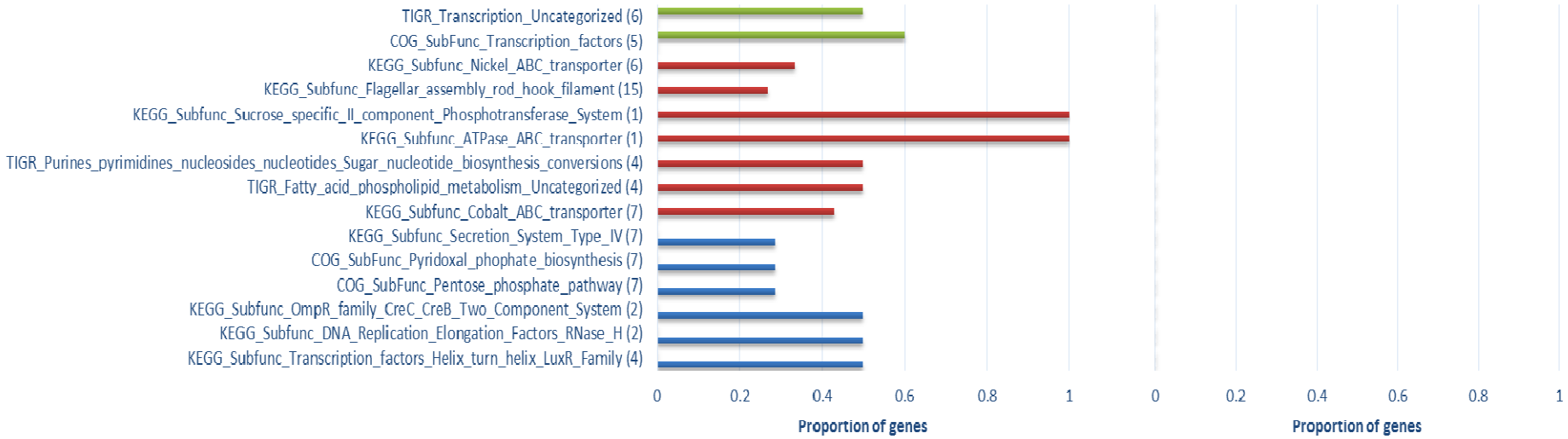


Figure. A. 3. 1. Functions expressed by faecal bacteria and *S.* Typhimurium in mixed cultures (left) and by *S.* Typhimurium in pure cultures (right). The numbers in brackets denote the number of genes regulating each function. The bars represent the proportion of genes that expressed the function at 3.5 h , 6 h and 9 h in mixed cultures and 1 h and 8 h in pure *S.* Typhimurium cultures.

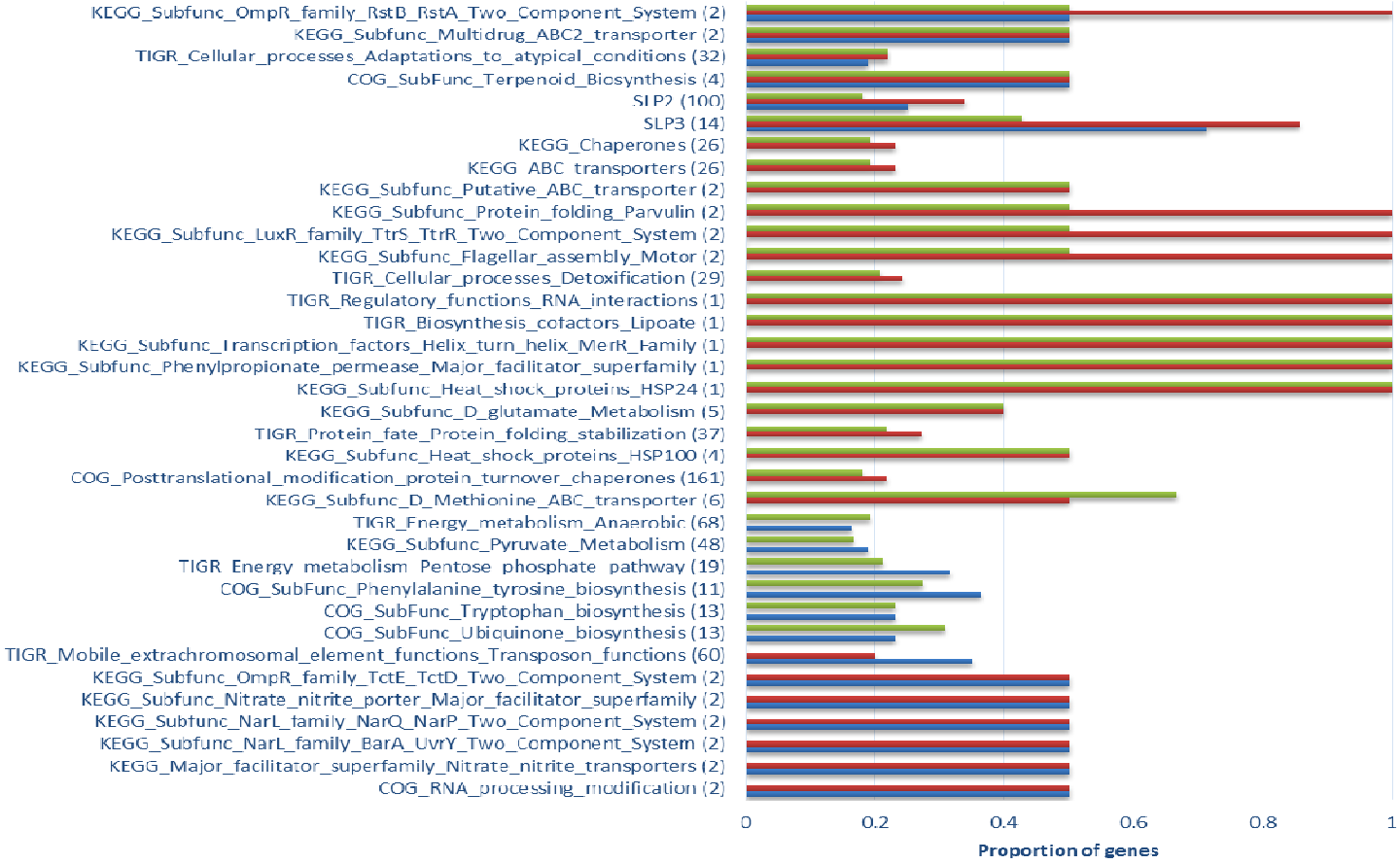

KEGG_Subfunc_OmpR_family_RstB_RstA_Two_Component_System (2)
KEGG_Subfunc_Multidrug_ABC2_transporter (2)
TIGR_Cellular_processes_Adaptations_to_atypical_conditions (32)
COG_SubFunc_Terpenoid_Biosynthesis (4)
SLP2 (100)
SLP3 (14)
KEGG_Chaperones (26)
KEGG_ABC_transporters (26)
KEGG_Subfunc_Putative_ABC_transporter (2)
KEGG_Subfunc_Protein_folding_Parvulin (2)
KEGG_Subfunc_LuxR_family_TtrS_TtrR_Two_Component_System (2)
KEGG_Subfunc_Flagellar_assembly_Motor (2)
TIGR_Cellular_processes_Detoxification (29)
TIGR_Regulatory_functions_RNA_interactions (1)
TIGR_Biosynthesis_cofactors_Lipoate (1)
KEGG_Subfunc_Transcription_factors_Helix_turn_helix_MerR_family (1)
KEGG_Subfunc_Phenylpropionate_permease_Major_facilitator_superfamily (1)
KEGG_Subfunc_Heat_shock_proteins_HSP24 (1)
KEGG_Subfunc_D_glutamate_Metabolism (5)
TIGR_Protein_fate_Protein_folding_stabilization (37)
KEGG_Subfunc_Heat_shock_proteins_HSP100 (4)
COG_Posttranslational_modification_protein_turnover_chaperones (161)
KEGG_Subfunc_D_Methionine_ABC_transporter (6)
TIGR_Energy_metabolism_Anaerobic (68)
KEGG_Subfunc_Pyruvate_Metabolism (48)
TIGR_Energy_metabolism_Pentose_phosphate_pathway (19)
COG_SubFunc_Phenylalanine_tyrosine_biosynthesis (11)
COG_SubFunc_Tryptophan_biosynthesis (13)
COG_SubFunc_Ubiquinone_biosynthesis (13)
TIGR_Mobile_extrachromosomal_element_functions_Transposon_functions (60)
KEGG_Subfunc_OmpR_family_TctE_TctD_Two_Component_System (2)
KEGG_Subfunc_Nitrate_nitrite_porter_Major_facilitator_superfamily (2)
KEGG_Subfunc_NarL_family_NarQ_NarP_Two_Component_System (2)
KEGG_Subfunc_NarL_family_BarA_UvrY_Two_Component_System (2)
KEGG_Major_facilitator_superfamily_Nitrate_nitrite_transporters (2)
COG_RNA_processing_modification (2)
0
0.2
0.4
0.6
0.8
1
Proportion of genes

Figure A. 3. 2

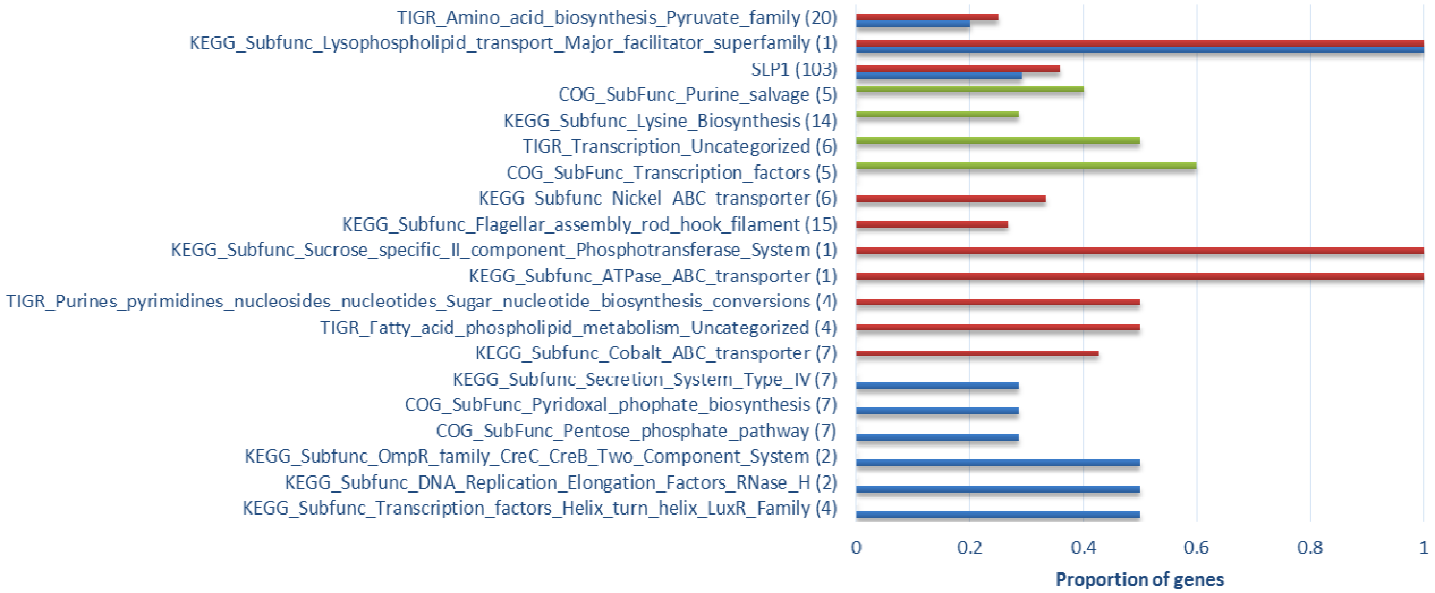


Figure A. 3. 2. Functions expressed by *S.* Typhimurium in mixed cultures with faecal bacteria having removed the functions that are also expressed in pure cultures. Numbers in brackets after each function name denote the total number of genes found to be associated to that function. The bars represent the proportion of genes belonging to that function expressed at 3.5 h , 6 h and 9 h .

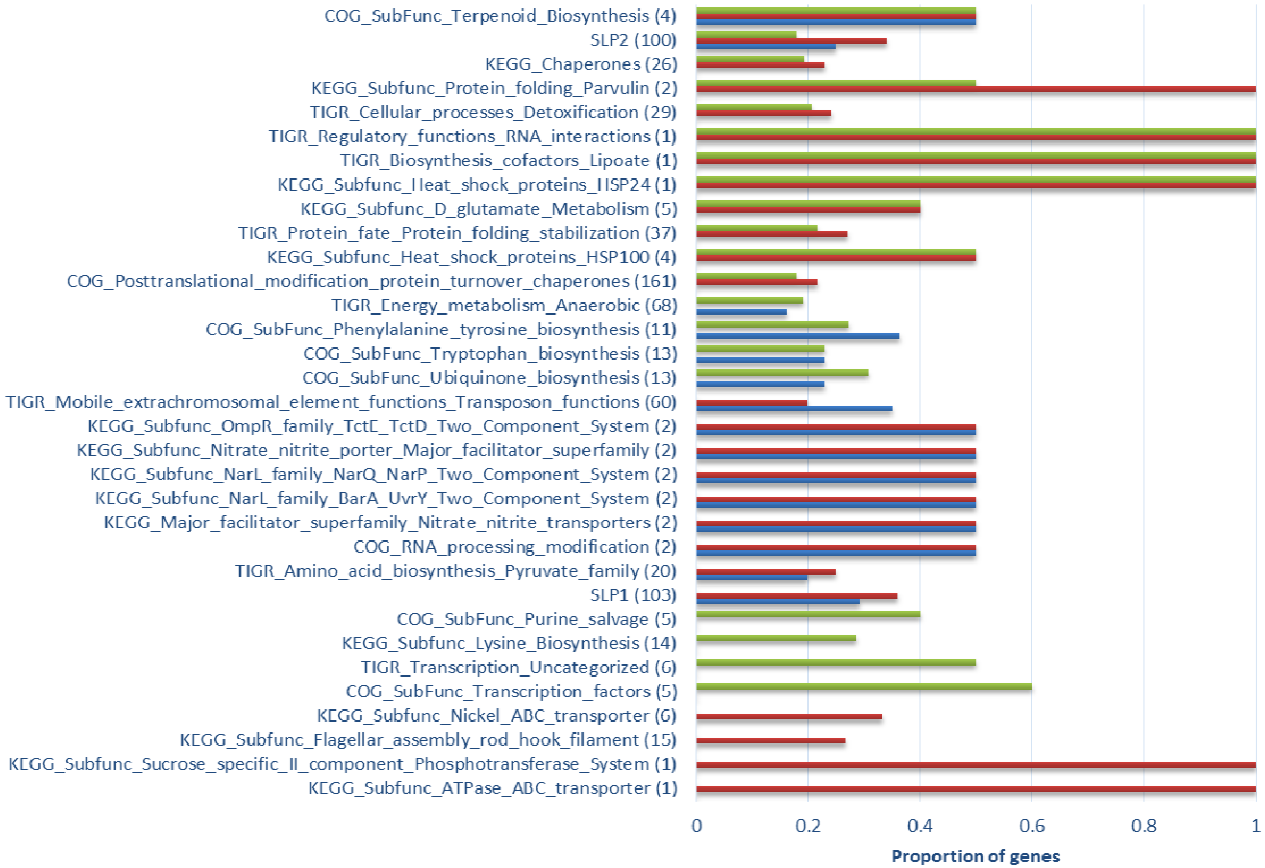

COG_SubFunc_Terpenoid_Biosynthesis (4)
SLP2 (100)
KEGG_Chaperones (26)
KEGG_Subfunc_Protein_folding_Parvulin (2)
TIGR_Cellular_processes_Detoxification (29)
TIGR_Regulatory_functions_RNA_interactions (1)
TIGR_Biosynthesis_cofactors_Lipoate (1)
KEGG_Subfunc_Heat_shock_proteins_HSP24 (1)
KEGG_Subfunc_D_glutamate_Metabolism (5)
TIGR_Protein_fate_Protein_folding_stabilization (37)
KEGG_Subfunc_Heat_shock_proteins_HSP100 (4)
COG_Posttranslational_modification_protein_turnover_chaperones (161)
TIGR_Energy_metabolism_Anaerobic (68)
COG_SubFunc_Phenylalanine_tyrosine_biosynthesis (11)
COG_SubFunc_Tryptophan_biosynthesis (13)
COG_SubFunc_Ubiquinone_biosynthesis (13)
TIGR_Mobile_extrachromosomal_element_functions_Transposon_functions (60)
KEGG_Subfunc_OmpR_family_TctE_TctD_Two_Component_System (2)
KEGG_Subfunc_Nitrate_nitrite_porter_Major_facilitator_superfamily (2)
KEGG_Subfunc_NarL_family_NarQ_NarP_Two_Component_System (2)
KEGG_Subfunc_NarL_family_BarA_UvrY_Two_Component_System (2)
KEGG_Major_facilitator_superfamily_Nitrate_nitrite_transporters (2)
COG_RNA_processing_modification (2)
TIGR_Amino_acid_biosynthesis_Pyruvate_family (20)
SLP1 (103)
COG_SubFunc_Purine_salvage (5)
KEGG_Subfunc_Lysine_Biosynthesis (14)
TIGR_Transcription_Uncategorized (6)
COG_SubFunc_Transcription_factors (5)
KEGG_Subfunc_Nickel_ABC_transporter (6)
KEGG_Subfunc_Flagellar_assembly_rod_hook_filament (15)
KEGG_Subfunc_Sucrose_specific_II_component_Phosphotransferase_System (1)
KEGG_Subfunc_ATPase_ABC_transporter (1)
0
0.2
0.4
0.6
0.8
1
Proportion of genes

Figure A. 3. 3

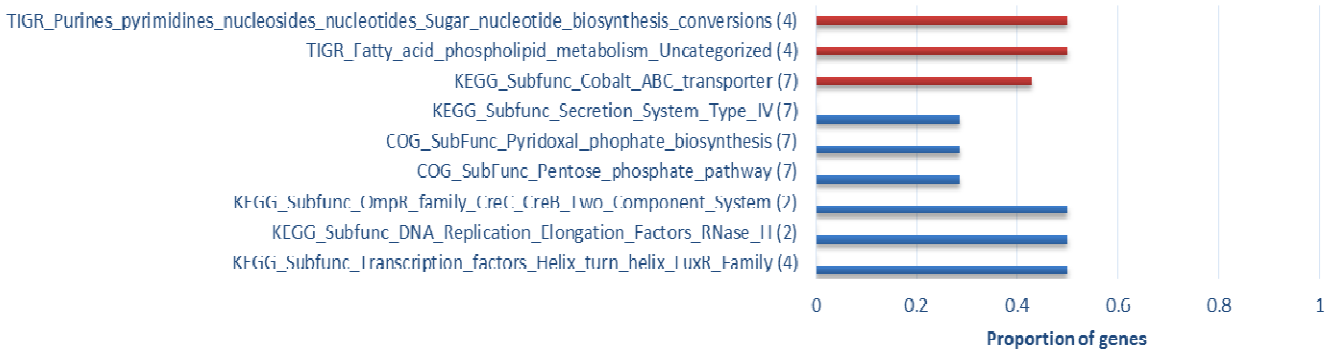


Figure A. 3. 3. Functions expressed by *S.* Typhimurium in mixed cultures with faecal bacteria having removed the functions that were also expressed in pure cultures of *S.* Typhimurium and the functions expressed in faecal cultures after 24 h of incubation. Numbers in brackets after each function name denote the total number of genes found to be associated to that function. The bars represent the proportion of genes belonging to that function expressed at 3.5 h , 6 h and 9 h .

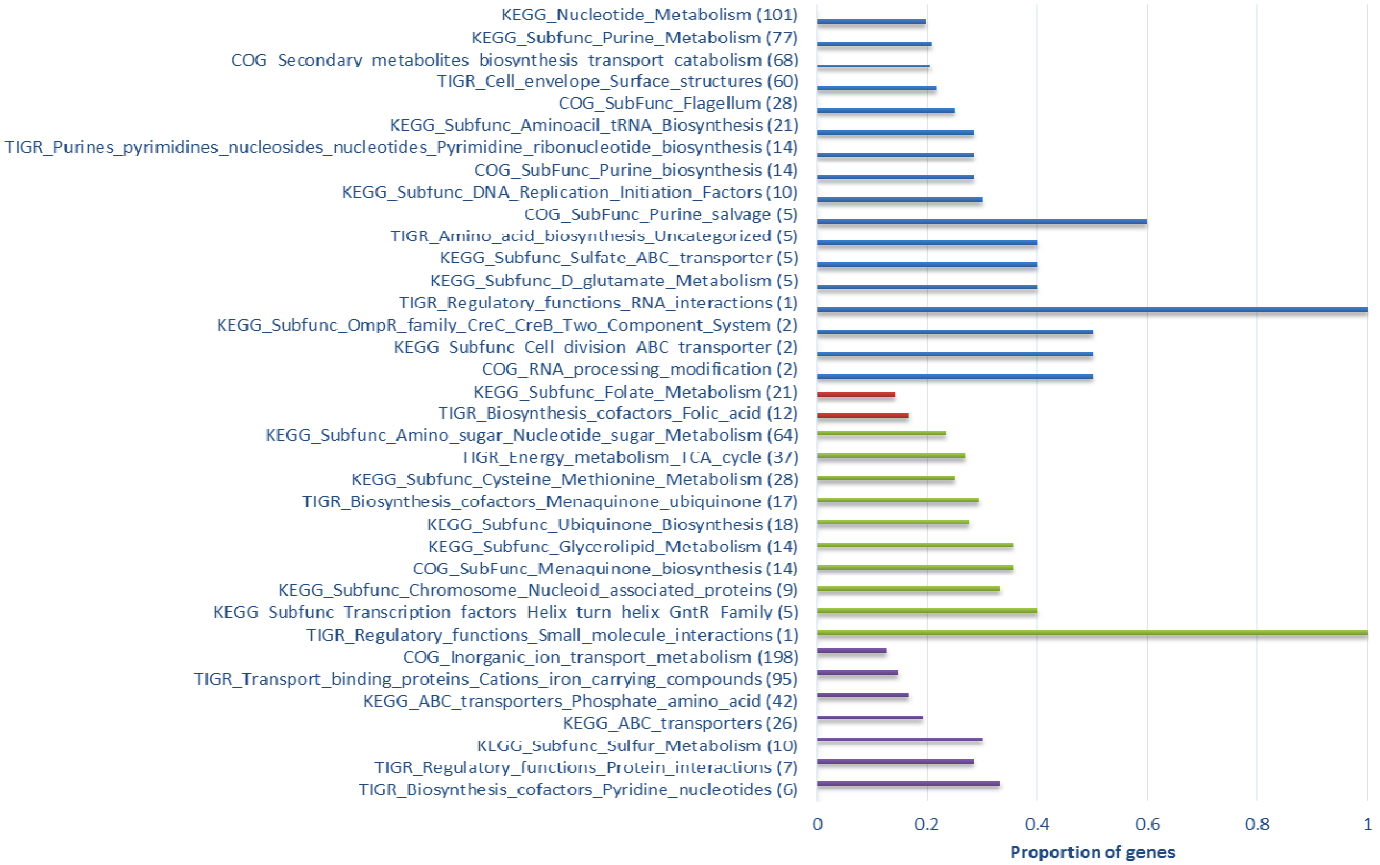
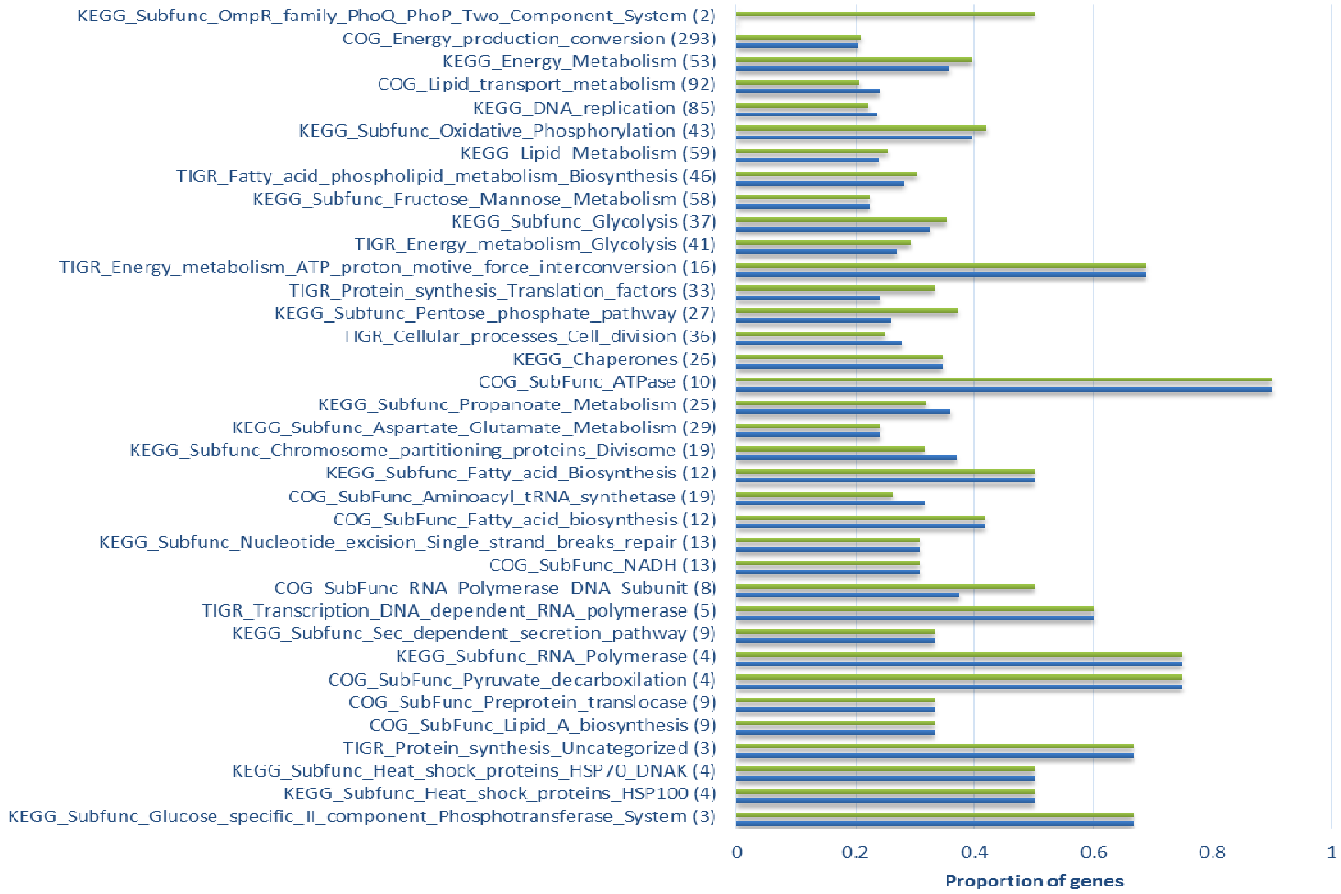

KEGG_Nucleotide_Metabolism (101)
KEGG_Subfunc_Purine_Metabolism (77)
COG Secondary metabolites biosynthesis transport catabolism (68)
TIGR_Cell_envelope_Surface_structures (60)
COG_SubFunc_Flagellum (28)
KEGG_Subfunc_Aminoacil_tRNA_Biosynthesis (21)
TIGR_Purines_pyrimidines_nucleosides_nucleotides_Pyrimidine_ribonucleotide_biosynthesis (14)
COG_SubFunc_Purine_biosynthesis (14)
KEGG_Subfunc_DNA_Replication_Initiation_Factors (10)
COG_SubFunc_Purine_salvage (5)
TIGR_Amino_acid_biosynthesis_Uncategorized (5)
KEGG_Subfunc_Sulfate_ABC_transporter (5)
KEGG_Subfunc_D_glutamate_Metabolism (5)
TIGR_Regulatory_functions_RNA_interactions (1)
KEGG_Subfunc_OmpR_family_CreC_CreB_Two_Component_System (2)
KEGG Subfunc Cell division ABC transporter (2)
COG_RNA_processing_modification (2)
KEGG_Subfunc_Folate_Metabolism (21)
TIGR_Biosynthesis_cofactors_Folic_acid (12)
KEGG_Subfunc_Amino_sugar_Nucleotide_sugar_Metabolism (64)
TIGR_Energy_metabolism_TCA_cycle (37)
KEGG_Subfunc_Cysteine_Methionine_Metabolism (28)
TIGR_Biosynthesis_cofactors_Menaquinone_ubiquinone (17)
KEGG_Subfunc_Ubiquinone_Biosynthesis (18)
KEGG_Subfunc_Glycerolipid_Metabolism (14)
COG_SubFunc_Menaquinone_biosynthesis (14)
KEGG_Subfunc_Chromosome_Nucleoid_associated_proteins (9)
KEGG Subfunc Transcription factors Helix turn helix GntR Family (5)
TIGR_Regulatory_functions_Small_molecule_interactions (1)
COG_Inorganic_ion_transport_metabolism (198)
TIGR_Transport_binding_proteins_Cations_iron_carrying_compounds (95)
KEGG_ABC_transporters_Phosphate_amino_acid (42)
KEGG_ABC_transporters (26)
KEGG_Subfunc_Sulfur_Metabolism (10)
TIGR_Regulatory_functions_Protein_interactions (7)
TIGR_Biosynthesis_cofactors_Pyridine_nucleotides (6)
0
0.2
0.4
0.6
0.8
1
Proportion of genes

Figure A. 3. 4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COG_Energy_production_conversion (293)
KEGG_Energy_Metabolism (53)
COG_Lipid_transport_metabolism (92)
KEGG_DNA_replication (85)
KEGG_Subfunc_Oxidative_Phosphorylation (43)
KEGG_Lipid_Metabolism (59)
TIGR_Fatty_acid_phospholipid_metabolism_Biosynthesis (46)
KEGG_Subfunc_Fructose_Mannose_Metabolism (58)
KEGG_Subfunc_Glycolysis (37)
TIGR_Energy_metabolism_Glycolysis (41)
TIGR_Energy_metabolism_ATP_proton_motive_force_interconversion (16)
TIGR_Protein_synthesis_Translation_factors (33)
KEGG_Subfunc_Pentose_phosphate_pathway (27)
TIGR_Cellular_processes_Cell_division (36)
KEGG_Chaperones (26)
COG_SubFunc_ATPase (10)
KEGG_Subfunc_Propanoate_Metabolism (25)
KEGG_Subfunc_Aspartate_Glutamate_Metabolism (29)
KEGG_Subfunc_Chromosome_partitioning_proteins_Divisome (19)
KEGG_Subfunc_Fatty_acid_Biosynthesis (12)
COG_SubFunc_Aminoacyl_tRNA_synthetase (19)
COG_SubFunc_Fatty_acid_biosynthesis (12)
KEGG_Subfunc_Nucleotide_excision_Single_strand_breaks_repair (13)
COG_SubFunc_NADH (13)
COG_SubFunc_RNA_Polymerase_DNA_Subunit (8)
TIGR_Transcription_DNA_dependent_RNA_polymerase (5)
KEGG_Subfunc_Sec_dependent_secretion_pathway (9)
KEGG_Subfunc_RNA_Polymerase (4)
COG_SubFunc_Pyruvate_decarboxilation (4)
COG_SubFunc_Preprotein_translocase (9)
COG_SubFunc_Lipid_A_biosynthesis (9)
TIGR_Protein_synthesis_Uncategorized (3)
KEGG_Subfunc_Heat_shock_proteins_HSP70_DNAK (4)
KEGG_Subfunc_Heat_shock_proteins_HSP100 (4)
KEGG_Subfunc_Glucose_specific_II_component_Phosphotransferase_System (3)
0
0.2
0.4
0.6
0.8
1
Proportion of genes

Figure A. 3. 4

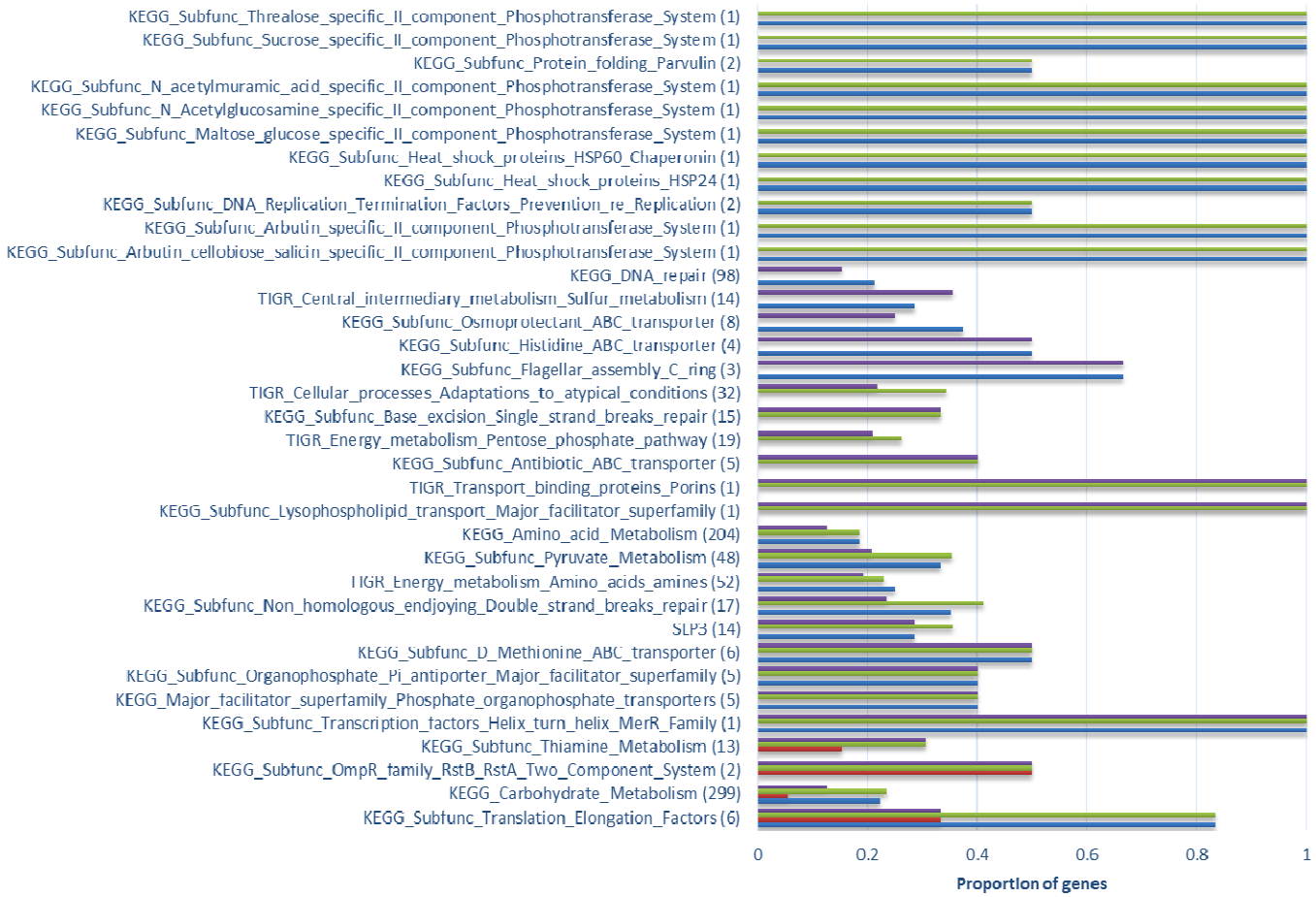
KEGG_Subfunc_Threalose_specific_II_component_Phosphotransferase_System (1)
KEGG_Subfunc_Sucrose_specific_II_component_Phosphotransferase_System (1)
KEGG_Subfunc_Protein_folding_Parvulin (2)
KEGG_Subfunc_N_acetylmuramic_acid_specific_II_component_Phosphotransferase_System (1)
KEGG_Subfunc_N_Acetylglucosamine_specific_II_component_Phosphotransferase_System (1)
KEGG_Subfunc_Maltose_glucose_specific_II_component_Phosphotransferase_System (1)
KEGG_Subfunc_Heat_shock_proteins_HSP60_Chaperonin (1)
KEGG_Subfunc_Heat_shock_proteins_HSP24 (1)
KEGG_Subfunc_DNA_Replication_Termination_Factors_Prevention_re_Replication (2)
KEGG_Subfunc_Arbutin_specific_II_component_Phosphotransferase_System (1)
KEGG_Subfunc_Arbutin_cellobiose_salicin_specific_II_component_Phosphotransferase_System (1)
KEGG_DNA_repair (98)
TIGR_Central_intermediary_metabolism_Sulfur_metabolism (14)
KEGG_Subfunc_Osmoprotectant_ABC_transporter (8)
KEGG_Subfunc_Histidine_ABC_transporter (4)
KEGG_Subfunc_Flagellar_assembly_C_ring (3)
TIGR_Cellular_processes_Adaptations_to_atypical_conditions (32)
KEGG_Subfunc_Base_excision_Single_strand_breaks_repair (15)
TIGR_Energy_metabolism_Pentose_phosphate_pathway (19)
KEGG_Subfunc_Antibiotic_ABC_transporter (5)
TIGR_Transport_binding_proteins_Porins (1)
KEGG_Subfunc_Lysophospholipid_transport_Major_facilitator_superfamily (1)
KEGG_Amino_acid_Metabolism (204)
KEGG_Subfunc_Pyruvate_Metabolism (48)
TIGR_Energy_metabolism_Amino_acids_amines (52)
KEGG_Subfunc_Non_homologous_endjoying_Double_strand_breaks_repair (17)
SLP3 (14)
KEGG_Subfunc_D_Methionine_ABC_transporter (6)
KEGG_Subfunc_Organophosphate_Pi_antiporter_Major_facilitator_superfamily (5)
KEGG_Major_facilitator_superfamily_Phosphate_organophosphate_transporters (5)
KEGG_Subfunc_Transcription_factors_Helix_turn_helix_MerR_Family (1)
KEGG_Subfunc_Thiamine_Metabolism (13)
KEGG_Subfunc_OmpR_family_RstB_RstA_Two_Component_System (2)
KEGG_Carbohydrate_Metabolism (299)
KEGG_Subfunc_Translation_Elongation_Factors (6)
0
0.2
0.4
0.6
0.8
1
Proportion of genes

Figure A. 3. 4

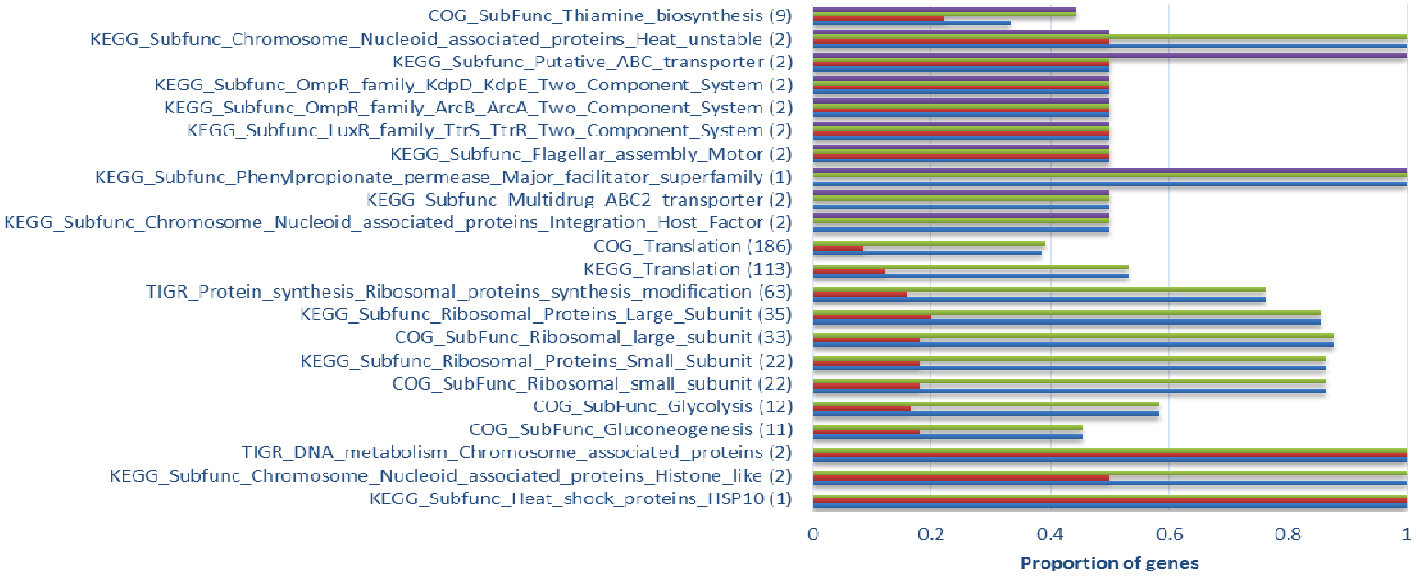

Figure A. 3. 4. Cellular functions and metabolic pathways expressed by faecal bacteria in cultures free from *S.* Typhimurium. Numbers in brackets after each function name denote the total number of genes found to be associated to that function. The bars represent the proportion of genes belonging to that function expressed at 3.5 h, 6 h, 9 h and 24 h.

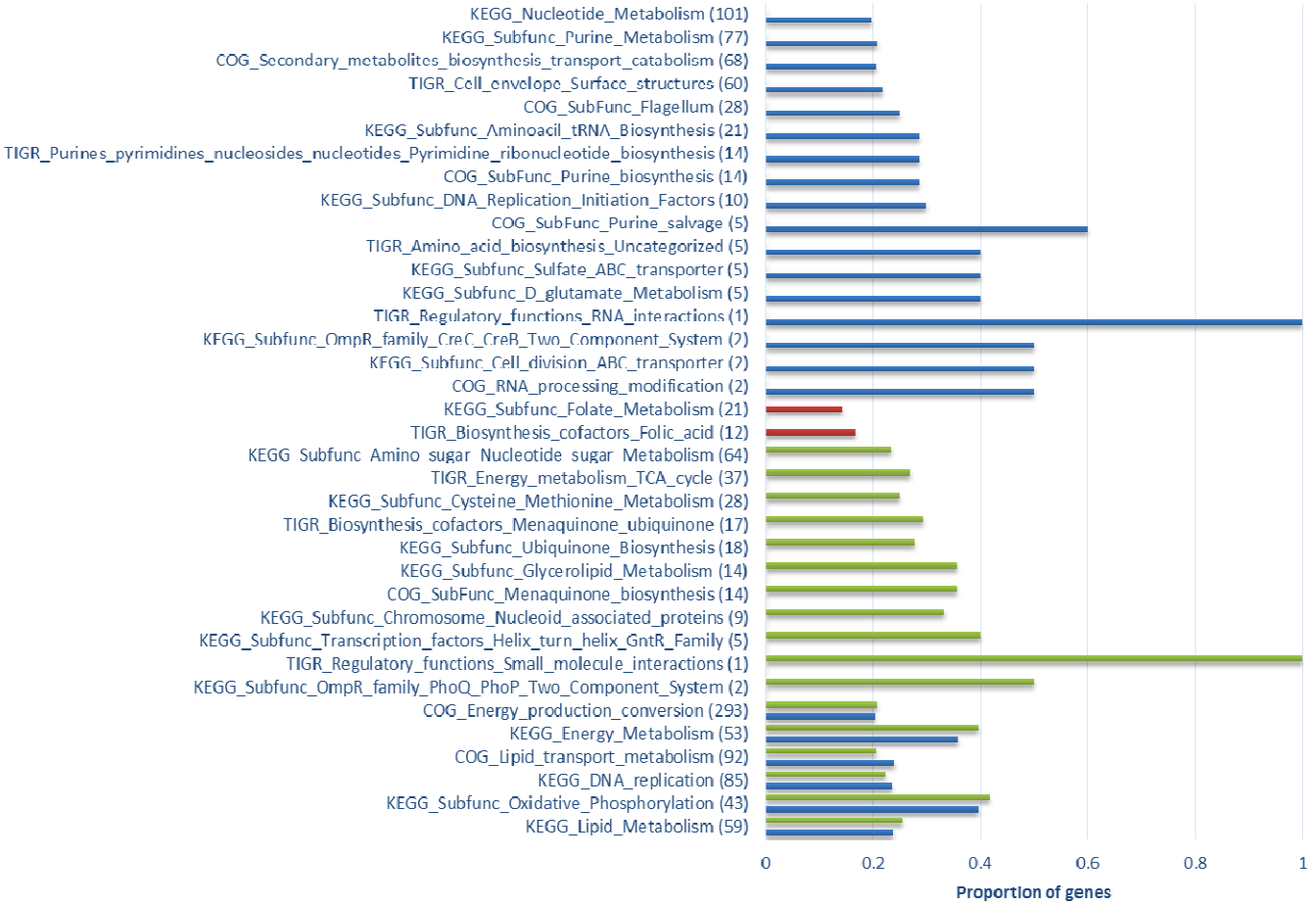
KEGG_Nucleotide_Metabolism (101)
KEGG_Subfunc_Purine_Metabolism (77)
COG_Secondary_metabolites_biosynthesis_transport_catabolism (68)
TIGR_Cell_envelope_Surface_structures (60)
COG_SubFunc_Flagellum (28)
KEGG_Subfunc_Aminoacil_tRNA_Biosynthesis (21)
TIGR_Purines_pyrimidines_nucleosides_nucleotides_Pyrimidine_ribonucleotide_biosynthesis (14)
COG_SubFunc_Purine_biosynthesis (14)
KEGG_Subfunc_DNA_Replication_Initiation_Factors (10)
COG_SubFunc_Purine_salvage (5)
TIGR_Amino_acid_biosynthesis_Uncategorized (5)
KEGG_Subfunc_Sulfate_ABC_transporter (5)
KEGG_Subfunc_D_glutamate_Metabolism (5)
TIGR_Regulatory_functions_RNA_interactions (1)
KEGG_Subfunc_OmpR_family_CreC_CreB_Two_Component_System (2)
KEGG_Subfunc_Cell_division_ABC_transporter (2)
COG_RNA_processing_modification (2)
KEGG_Subfunc_Folate_Metabolism (21)
TIGR_Biosynthesis_cofactors_Folic_acid (12)
KEGG Subfunc Amino sugar Nucleotide sugar Metabolism (64)
TIGR_Energy_metabolism_TCA_cycle (37)
KEGG_Subfunc_Cysteine_Methionine_Metabolism (28)
TIGR_Biosynthesis_cofactors_Menaquinone_ubiquinone (17)
KEGG_Subfunc_Ubiquinone_Biosynthesis (18)
KEGG_Subfunc_Glycerolipid_Metabolism (14)
COG_SubFunc_Menaquinone_biosynthesis (14)
KEGG_Subfunc_Chromosome_Nucleoid_associated_proteins (9)
KEGG_Subfunc_Transcription_factors_Helix_turn_helix_GntR_Family (5)
TIGR_Regulatory_functions_Small_molecule_interactions (1)
KEGG_Subfunc_OmpR_family_PhoQ_PhoP_Two_Component_System (2)
COG_Energy_production_conversion (293)
KEGG_Energy_Metabolism (53)
COG_Lipid_transport_metabolism (92)
KEGG_DNA_replication (85)
KEGG_Subfunc_Oxidative_Phosphorylation (43)
KEGG_Lipid_Metabolism (59)
0
0.2
0.4
0.6
0.8
1
Proportion of genes

Figure A. 3. 5

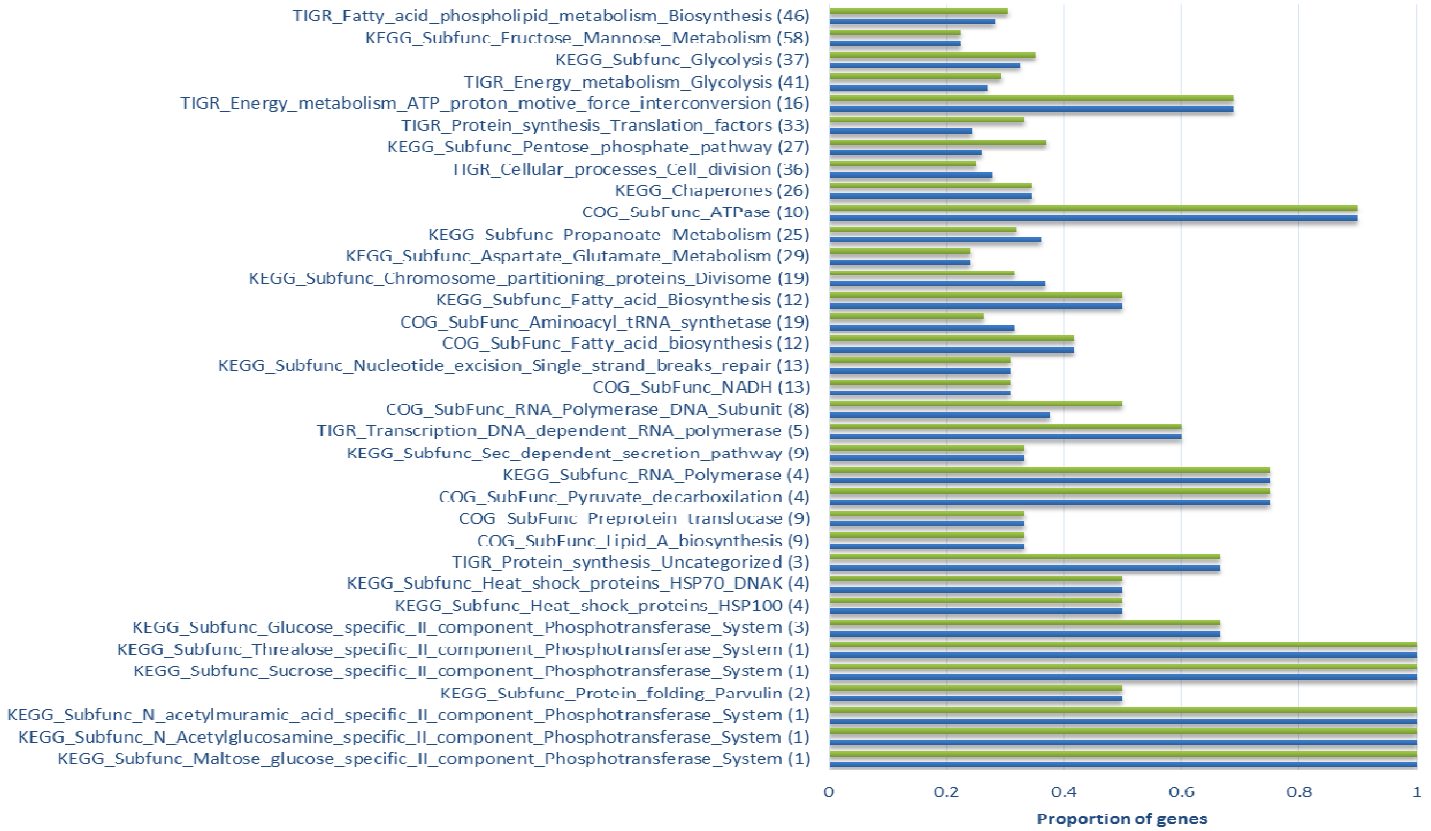
TIGR_Fatty_acid_phospholipid_metabolism_Biosynthesis (46)
KEGG_Subfunc_Fructose_Mannose_Metabolism (58)
KEGG_Subfunc_Glycolysis (37)
TIGR_Energy_metabolism_Glycolysis (41)
TIGR_Energy_metabolism_ATP_proton_motive_force_interconversion (16)
TIGR_Protein_synthesis_Translation_factors (33)
KEGG_Subfunc_Pentose_phosphate_pathway (27)
TIGR_Cellular_processes_Cell_division (36)
KEGG_Chaperones (26)
COG_SubFunc_ATPase (10)
KEGG_Subfunc_Propanoate_Metabolism (25)
KEGG_Subfunc_Aspartate_Glutamate_Metabolism (29)
KEGG_Subfunc_Chromosome_partitioning_proteins_Divisome (19)
KEGG_Subfunc_Fatty_acid_Biosynthesis (12)
COG_SubFunc_Aminoacyl_tRNA_synthetase (19)
COG_SubFunc_Fatty_acid_biosynthesis (12)
KEGG_Subfunc_Nucleotide_excision_Single_strand_breaks_repair (13)
COG_SubFunc_NADH (13)
COG_SubFunc_RNA_Polymerase_DNA_Subunit (8)
TIGR_Transcription_DNA_dependent_RNA_polymerase (5)
KEGG_Subfunc_Sec_dependent_secretion_pathway (9)
KEGG_Subfunc_RNA_Polymerase (4)
COG_SubFunc_Pyruvate_decarboxilation (4)
COG_SubFunc_Preprotein_translocase (9)
COG_SubFunc_Lipid_A_biosynthesis (9)
TIGR_Protein_synthesis_Uncategorized (3)
KEGG_Subfunc_Heat_shock_proteins_HSP70_DNAK (4)
KEGG_Subfunc_Heat_shock_proteins_HSP100 (4)
KEGG_Subfunc_Glucose_specific_II_component_Phosphotransferase_System (3)
KEGG_Subfunc_Threalose_specific_II_component_Phosphotransferase_System (1)
KEGG_Subfunc_Sucrose_specific_II_component_Phosphotransferase_System (1)
KEGG_Subfunc_Protein_folding_Parvulin (2)
KEGG_Subfunc_N_acetylmuramic_acid_specific_II_component_Phosphotransferase_System (1)
KEGG_Subfunc_N_Acetylglucosamine_specific_II_component_Phosphotransferase_System (1)
KEGG_Subfunc_Maltose_glucose_specific_II_component_Phosphotransferase_System (1)
0
0.2
0.4
0.6
0.8
1
Proportion of genes

Figure A. 3. 5

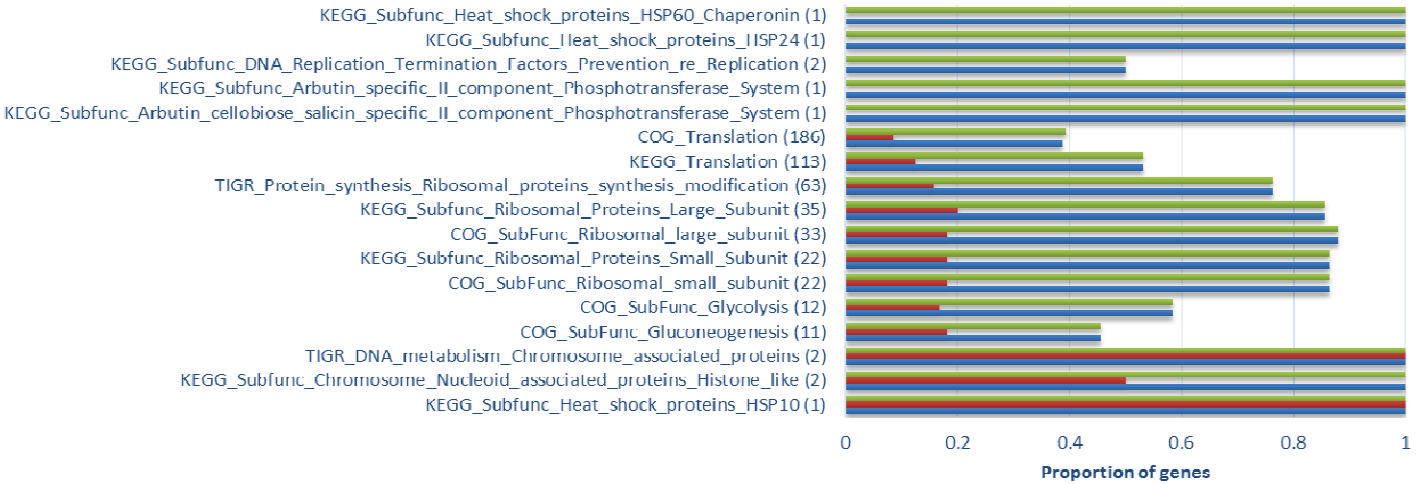

Figure A. 3. 5. Functions expressed by faecal cultures free from *S.* Typhimurium after removing the functions expressed exclusively after 24 h of incubation. Numbers in brackets after each function name denote the total number of genes found to be associated to that function. The bars represent the proportion of genes belonging to that function expressed at 3.5 h , 6 h and 9 h .

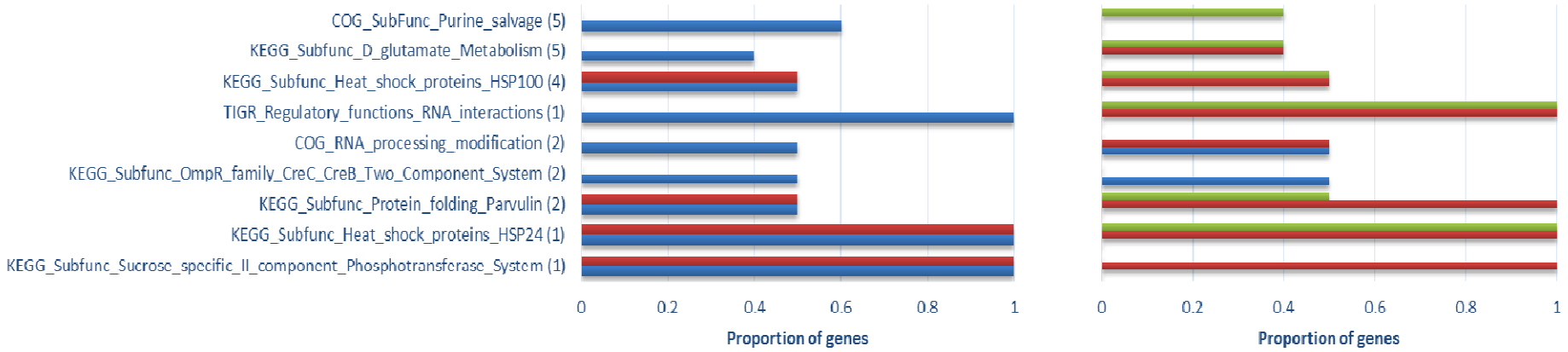

Figure A. 3. 6. Comparison of coincidental functions expressed by *S.* Typhimurium in mixed cultures with faecal bacteria but not in pure cultures (left) with the functions expressed in faecal cultures (right) during the first 9 h of incubation, i.e. immediately prior to contact killing. Numbers in brackets after each function name denote the total number of genes found to be associated to that function. The bars represent the proportion of genes belonging to that function expressed at 3.5 h, 6 h and 9 h.

8.4 Appendix 4: Publications