INVESTIGATION OF EFFECTS OF TURKISH BLACK TEA POLYPHENOLS ON HUMAN GUT MICROBIOTA IN *IN-VITRO* FERMENTER CULTURES

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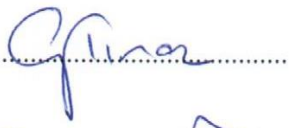
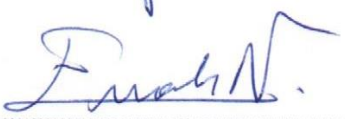
Prof. Dr. Gülgün Tınaz ..... 

Assist. Prof. Dr. İlkem Emrah Nikerel ..... 

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To all tea lovers…

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ABSTRACT

INVESTIGATION OF EFFECTS OF TURKISH BLACK TEA POLYPHENOLS ON HUMAN GUT MICROBIOTA IN *IN-VITRO* FERMENTER CULTURES

Human gut microbiota has a highly complex microbial composition and it functions in many processes related to human well-being. Of many endogeneous and exogeneous conditions affecting gut microbial population, diet is the one, which has the most impact. The aim of the study was to evaluate the influence of Turkish black tea polyphenols (BTP) on gut microbiota of a Turkish individual. The effects of Turkish BTPs on gut microbiota of a Turkish individual was determined in samples collected from pH controlled fecal batch fermenters at 7 time points (0, 4, 8, 10, 24, 30, 48 h) in the presence or absence of water extracted BTP (1000 mg/L) by using real-time PCR (Q-PCR) and fluorescent *in-situ* hybridization (FISH) analysis. While the mean values of three fermenter samples analyzed by Q-PCR analysis did not show any significant differences between the bacterial populations of *Atopobium, Bacteroides-Prevotella-Porphyromonas, C. coccoides, Bifidobacterium, Enterobacteriaceae, Enterococcus* and *Lactobacillus* groups and total bacterial count in the presence of BTP compared to control samples, the growth of *C. coccoides-E. rectale* and *Lactobacillus-Enterococcus* groups increased significantly ($P<0.01$) in black tea added cultures relative to control cultures in FISH analysis. Our data indicated that BTP might have an ability to modulate the composition of specific gut bacteria. This ability might be due to specific metabolic transformations of BTP by gut microbiota and/or antimicrobial properties of BTP on specific gut bacterial groups. However, further *in-vivo* animal and human intervention studies followed by metagenomic and metabolomic analyses are needed.

ÖZET

TÜRK SİYAH ÇAYI POLİFENOLLERİNİN İNSAN KALIN BAĞIRSAK MİKROBİYOTASINA ETKİSİNİN *IN-VITRO* FERMENTÖR KÜLTÜRLERİNDE İNCELENMESİ

İnsan bağırsak mikrobiyotası oldukça karmaşık bir içeriğe sahip olup insan yaşamında olumlu birçok işlemde görev almaktadır. Beslenme, bağırsakta bulunan mikrobiyal popülasyonu etkileyen birçok iç ve dış etmenlerin en başında gelmektedir. Bu çalışmada, Türkiye'de en çok tüketilen ürünlerden biri olan Türk siyah çayı polifenollerinin Türk bireyin bağırsağındaki mikrobiyal popülasyonuna etkisi; Türk siyah çayı su ekstraktının (1000 mg/L) varlığında veya yokluğunda, pH kontrollü fekal kesikli fermentörlerden 7 farklı saatte (0, 4, 8, 10, 24, 30, 48) toplanan örnekler ile; kantitatif polimeraz zincir reaksiyonu (Q-PCR) ve floresan *in-situ* hibridizasyon (FISH) yöntemleri kullanılarak incelenmiştir. Üç fermentör örneğinin Q-PCR analizlerinin ortalama değerlerinde; *Atopobium, Bacteroides-Prevotella-Porphyromonas, C. coccoides, Bifidobacterium, Enterobacteriaceae, Enterococcus, Lactobacillus* grupları ve toplam bakteri sayımında kontrole göre kayda değer bir değişim gözlenmezken, FISH analizinde *C. coccoides-E. rectale* ve *Lactobacillus-Enterococcus* gruplarının üremelerinin siyah çay varlığında kayda değer bir şekilde arttığı ($P<0.01$) gözlenmiştir. Elde edilen verilere göre, Türk siyah çayı polifenollerinin bağırsakta bulunan spesifik bakteri gruplarının kompozisyonlarını değiştirebilme yetisine sahip olabileceği tespit edilmiştir. Bu yetinin, Türk siyah çayı polifenollerinin bağırsak bakterileri tarafından metabolik dönüşümü ve/veya siyah çayı polifenollerinin bazı bağırsak bakterileri üzerindeki antimiktobiyal etkilerinden dolayı olabileceği düşünülmektedir. Ancak, konuyla ilgili destekleyici *in-vivo* hayvan ve insan çalışmalarına ek olarak ileri metagenomik ve metabolobik analizler gerekmektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

ANOVA	Analysis of variance
Bac 303	*Bacteroides-Prevotella* group
BHI	Brain Heart Infusion
Bif 164	*Bifidobacterium* group
BSA	Bovine serum albumin
BTP	Black tea polyphenols
CFU	Colony forming unit
Ct	Cycle threshold
DI-MS	Direct infusion-mass spectrometry
DNA	Deoxyribonucleic acid
DGGE	Denaturing gradient gel electrophoresis
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
Erec 482	*Clostridium coccoides-Eubacterium rectale* group
EUB 338	Total bacteria
EUB338II	Total bacteria
EUB338III	Total bacteria
F	Forward primer
FISH	Fluorescence *in-situ* hybridization
GAM	Gifu anaerobic medium
GABA	Gamma-aminobutyric acid
GC	Gallocatechin
GC-MS	Gas chromatography-mass spectrometry
GC-TOFMS	Gas chromatography-time of flight mass spectrometry
HITchip	Human Intestinal Tract Chip
HuGChip	The Human Gut Chip
HPLC	High performance liquid chromatography
ISB	Index of specific bacteria

LAB	Lactic acid bacteria
Lab 158	*Lactobacillus−Enterococcus* group
LC-MS	Liquid chromatography-mass spectrometry
MRS	de Man, Rogosa and Sharpe
MS	Mass spectrophotometry
NGS	Next generation sequencing
NMR	Nuclear magnetic resonance
NTC	No template control
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDA	Photodiode array
Q-PCR	Real time polymerase chain reaction
R	Reverse primer
RNA	Ribonucleic acid
SHIME	Simulator of Human Intestinal Microbial Ecosystem
SIMGI	SIMulator Gastro-Intestinal
TGGE	Temperature gradient gel electrophoresis
TIM	TNO Intestinal Model
UPLC-ESI-TQ-MS	Ultra-performance liquid chromatography-electrospray ionization-tandem quadrupole mass spectrometry
VBNC	Viable but not culturable

1. INTRODUCTION

Human digestive tract houses an extremely complex bacterial community which includes more than 1000 species making up to 10^{12} CFU/mL feces in number and has many impacts on human health. Human gut microbial composition is altered by many conditions of which diet is one of the most effective exogenous one. Therefore, many *in-vivo* and *in-vitro* studies have been conducted to investigate the impacts of different metabolites on gut microbiota. *In-vitro* studies are based either batch fermenter models or continuous culture systems which both mimic physiological conditions of gut. Alternatively, *in-vivo* studies focus on human intervention trials or animal models having specific diets for certain time periods.

With the collected samples from either *in-vivo* or *in-vitro* studies, gut microbiota analysis is performed by culture-dependent and culture-independent methods. Culture-dependent methods are based on isolation of microorganisms from samples and then further identification of microorganisms by morphological and biochemical analyses. Culture-independent methods target to nucleic acids of microorganisms. Dot blot hybridization, microarray analysis, fluorescent *in-situ* hybridization (FISH), PCR-denaturing/temperature gradient gel electrophoresis (PCR-DGGE/TGGE), quantitative real-time polymerase chain reaction (Q–PCR) and next generation sequencing (NGS) can be listed as culture-independent methods for gut microbiota analysis.

Among dietary substances, polyphenols are one of the most studied metabolites that affect gut microbial composition. Turkish black tea is one of the most consumed beverages in Turkey and a rich source of polyphenols. Despite studies regarding the effects of different teas on gut microbiota, studies regarding the effects of Turkish black tea on gut microbiota of a Turkish individual is poorly studied. Therefore, this study focused on investigating the impacts on Turkish black tea polyphenols on human gut microbiota of a Turkish individual in 48 h *in-vitro* fecal batch fermenter cultures in the presence and absence of black tea extract by Q-PCR and FISH methods with samples provided from 7 time points of fermentation (0, 4, 8, 10, 24, 30 & 48 h).

2. LITERATURE REVIEW

2.1. HUMAN DIGESTIVE TRACT

The human digestive tract harbors a complex and diverse microbial population. It houses more than 1000 bacterial species, which are mostly obligate anaerobes [1, 2]. Due to the low movement rate, most of the bacterial communities locate in large intestine, containing up to 10^{11}- 10^{12} CFU/mL feces making 40- 45% of all fecal material by weight [3, 4]. Human gut microbiota is dominated by four main phyla which are *Firmicutes* (including *Clostridium*, *Lactobacillus* and *Enterococcus* genera), *Bacteroidetes* (including *Bacteroides*, *Prevotella* and *Porphyromonas* genera), *Actinobacteria* (including *Atopobium* and *Bifidobacterium* genera) and *Proteobacteria* (including *Enterobacteriaceae* family) [1, 5]. The majority of bacteria belong either to the phylum *Firmicutes* or to the phylum *Bacteroidetes* which constitute over 90% of the bacterial population found in the human intestine [3].

Bacteroidetes are numerically one of the most important phyla. They are gram negative, obligate anaerobe, non-sporulating rods. This phyla is divided to 3 groups including *Bacteroides* (saccharolytic, non-pigmenting bacteria), *Prevotella* (moderately saccharolytic, bile-sensitive) which are dominantly found in oral microflora, and *Porphyromonas* (non-saccharolytic, black pigmenting). Bacteria of *Clostridium* group are gram-positive, anaerobic, spore-forming rods. Both symbionts and pathogens are present in this group. Metabolically, they cannot reduce sulphate. The numbers of *Clostridium* in gut can be as high as 10^{10} CFU/g feces [2]. Lactic acid bacteria (LAB) are one of the beneficial bacterial groups in gut microbiota including *Lactobacillus, Lactococcus, Streptococcus* and *Enterococcus* species. They are gram positive, non spore-forming, carbohydrate-fermenting, lactic acid-producing, acid-tolerant and catalase-negative. Intestinal *Lactobacillus* species generally found at 10^{9} CFU/g feces in gut [2]. *Bifidobacterium* group is another beneficial group in gut microbiota. They are gram positive, anaerobic and saccharolytic rods. *Enterobacteriaceae* group consists of both symbionts and pathogens. They are gram negative, aerobic, facultative anaerobic, sugar fermenting rods. The most important member of *Enterobacteriaceae* is *Escherichia coli*. [2].

Human gut microbiota which functions in many metabolic, nutritional, immunological, and physiological processes contributing to well-being of the host can get affected dramatically by endogenous (enzymes, hormones, disease, age, parts of gut, colonic transit time etc.) and exogenous conditions (diet, medication, geographical regions etc.) [3, 5-7]. According to a study, 1000 fold change in gut microbial count was obtained in 12-month period. The same study also showed that British and American population had higher *Bifidobacterium* and *Bacteroides* number; whereas Ugandans, Japanese and Indians had higher *Enterococcus* and *Enterobacteria* number compared to each other [2]. Moreover, in a study comparing intestinal microbiota of children from Burkina Faso and Europe, it was stated that the amounts of *Firmicutes* and *Enterobacteriaceae* were significantly lower and amount of *Bacteroidetes* was much higher in gut microflora of children from Burkina Faso than Europe. It was also stated that unique bacterial species were detected in children from Burkina Faso related to their diets [8].

2.2. INTERACTIONS BETWEEN HUMAN GUT MICROBIOTA AND DIETARY SUBSTANCES

Human gut microbiota can get affected by many endogenous and exogenous conditions as stated above. Diet, one of the exogenous conditions, is the one which affects gut microbial composition the most [9].

According to reasons stated above, studies regarding human intestinal microflora analysis focused on the investigation of these conditions on composition of human gut microbiota. *In-vitro* & *in-vivo* studies have been performed for investigation of the effects of different variables on human gut microbiota. *In-vitro* studies mostly focus on fermenter models aiming to mimic the intestinal physiological conditions by using fecal samples as inocula [10-14]. Batch cultures are one of the simple fermenter models to study human gut microflora. However, only short-term colonization can be performed in batch cultures [15]. As an alternative, continuous culture systems such as Simulator of Human Intestinal Microbial Ecosystem (SHIME) [11], TNO Intestinal Model (TIM) [16] and SIMulator Gastro-Intestinal (SIMGI) [13] are used. Those systems create a complete gastrointestinal tract model enabling to study long term colonization of bacteria in a dynamic model [15,

17]. However, *in-vitro* models are limited since they do not represent *in-vivo* conditions of gut.

In-vivo studies are based on human intervention trials in which fecal samples of healthy volunteers are collected to be analyzed directly. Human intervention studies are performed by controlling volunteers' diet for a long period of time followed by collecting samples from blood, urine, stool and tissues [18]. *In vivo* studies are also being performed with human flora-associated animals such as rats and mice [19-21]; and rarely other animals such as birds and pigs [22, 23]. *In vivo* studies are good alternatives to *in-vitro* studies and so far the best ways to investigate the parameters that may affect gut microbial composition despite the limitations like high-cost, time consumption and ethical concerns [4, 18, 24, 25].

With either collected fecal samples from *in-vitro* fermenter cultures or fecal, urine and/or blood samples from *in-vivo* studies, the effects of gut microbiota on dietary substances can be analyzed by analytical methods [26, 27]. Methods include high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), gas chromatography (GC) and mass spectroscopy (MS) [14, 28, 29]. Combinations and variations of those methods such as GS-MS, HPLC-MS, UPLC-ESI-TQ-MS which is an UPLC system coupled to an electrospray interface photodiode array detector and an tandem quadruple mass spectrometer, DI-MS, GC-TOFMS and LC-MS have been used as well according to the target of metabolites [13, 30-32]. While the effects of human gut microbiota on dietary substances are analyzed by analytical methods, the effects of dietary substances and their metabolites on gut microbial profile can be analyzed by culture-dependent and -independent methods.

2.3. ANALYSIS OF HUMAN GUT MICROBIOTA

To investigate the impact of metabolites on gut microbiota, culture- dependent and culture-independent methods have been used. While culture-dependent methods are based on the cultivation, isolation and quantification of microorganisms, culture-independent methods basically target the analysis of nucleic acids such as dot blot hybridization, microarray analysis, fluorescent *in-situ* hybridization (FISH), PCR-denaturing/temperature gradient

gel electrophoresis (PCR-DGGE/TGGE), quantitative real-time polymerase chain reaction (Q–PCR) and next generation sequencing (NGS) [4, 33, 34].

2.3.1. Culture-dependent Methods

Culture-dependent methods, firstly used for intestinal microflora analysis, have been the standard methods based on isolation of microorganisms from fecal samples using selective media and then further identification of colonies by several morphological and biochemical analysis [4, 25, 35, 36]. However, culture-dependent methods are tedious, labor intensive and time consuming as well as they require large amount of materials [4, 37]. Also, not all bacteria in gut can be cultivated (viable but not culturable, VBNC) due to unknown culturing conditions [36]. Identification tests are not enough for certain classification of bacterial species or not effective enough to observe the relatedness between isolated species [37]. Therefore, quantification and diversity of gut microbiota is underestimated by culture methods. Although all the disadvantages stated above, culture-dependent methods are still useful due to studies requiring pure cultures and their physiologies.

2.3.2. Culture-independent Methods

2.3.2.1. Dot Blot Hybridization

Dot blot analysis is one of the methods used for detection of intestinal microflora based on hybridization technique [38]. In dot blot analysis method, extracted DNA or RNA is generally directly fixed on membrane without amplification and then, hybridization is performed with radioactively or non-radioactively labeled probes [4]. The relative abundance of each bacterial group can be calculated by quantifying the signal intensity of probes, making dot blot a semi-quantitative analysis method [34]. A limitation of dot blot method on other quantification methods is that since amplification is not generally processed, the sensitivity of the system decreases in non-amplified samples [38].

Gut microbial studies using dot blot analysis are rare. Studies of Sghir et al. [39] and Smith and Mackie [21] determined gut microbiota of individuals having western-type diet and

tannin-rich diet, respectively concluding that dot blot was a useful method for microbial detection although the exact numbers of bacterial groups could not be investigated.

2.3.2.2. Microarray Analysis

DNA microarray analysis is a powerful semi quantitative microflora investigation method based on hybridization as well. Microarray enables simultaneous analysis of thousands of target DNA in a single experiment [40]. Although microarray technology is originally used for gene expression studies, they can also be used for phylogenetic identification and quantification [33, 41]. In microarray analysis, isolated and amplified DNA from samples are hybridized with fluorescent labeled probes fixed on membrane and bacterial quantification analysis is performed by obtained fluorescent signal intensity [36]. Microarray is a high-throughput, rapid and user-friendly technique for microbial analysis, while hybridization biases and detection of low level bacteria are the limitations of this method [33, 37, 40]. Moreover, the sequences of the target organism must be known in order to design probes for analysis [36].

Various microarrays such as “HuGChip” [40], “Microbiota Array” [42] and “HITCip” [43] have been designed for gut microbiota analysis. Results indicated that all the arrays are able to rapidly detect intestinal bacteria with high specificity. With the confirmation of suitability, gut microbiota studies regarding the effects of dietary contents on gut microbiota are further being expected.

2.3.2.3. Fluorescent In-situ Hybridization (FISH)

Fluorescent *in-situ* hybridization (FISH) is another hybridization method which enables detection of organisms *in-situ*. The procedure involves fixation of samples followed by permeabilization of cells, enabling designed probes to enter the cell. Then, probes are allowed to hybridize to target nucleic acid (DNA or RNA) and excess unbound probes are washed after hybridization. Hybridized cells are observed under confocal microscopy or via flow cytometry. FISH is a semi-quantitative method that enables distribution analysis of the microbiota with no PCR bias [37]. Specificity is another advantageous point of

FISH. However, some microorganisms cannot be detected due to their impermeability or lack of probes complementary to their nucleic acid sequences [36].

Several gut microbiota analysis studies were performed by FISH including the effects of polyphenols [13, 14, 30, 31, 44-47] as well as the effects of prebiotics [12, 48] and other dietary substrates [19, 20, 49] as indicated in Table 2.1. *In-vitro* and *in-vivo* studies analyzing the effects of food products such as pomegranate by-product, black tea, grape seed extract and cocoa, and their bioactive food compounds such as dahlia inulin, anthocyanins, flavanols and many more on gut bacterial groups concluded that FISH was a useful method for microbial detection and quantification, with the advantage of being *in-situ*.

For example, *in-vitro* fecal fermentation of grape seed flavan-3-ol monomer (GSE-M) and oligomer (GSE-O) fractions [13], EGCG, GCG, EGCG3”Me isolated from oolong tea [14], anthocyanins [30] and pomegranate by-product [46] increased the growth of *Lactobacillus* and *Enterococcus* groups compared to control fermenters cultures. *Bifidobacterium* showed higher numbers in fermenters supplemented with isomalto-oligosaccharides and fructo-oligosaccharides [12], EGCG, GCG, EGCG3”Me isolated from oolong tea [14], anthocyanins [30], (-)-epicatechin [45] and pomegranate by-product [46]. Moreover, (-)-epicatechin and (+)-catechin [45] stimulated the growth of *C. coccoides–E. rectale* group in fecal fermenter cultures compared to control fermenter cultures. Fermenter cultures supplemented with dahlia inulins [49] stimulated the growth of *R. flavefaciens, R.bromii* and *E. cylindroides* groups, while (+)-catechin [45] and pomegranate by-product [46] increased the growth of *E. coli* and total bacterial count, respectively compared to control vessels. On the other hand, grape seed flavan-3-ol monomer (GSE-M) and oligomer (GSE-O) fractions [13], polyphenols of red wine extract containing catechin, epicatechin, gallic acid and epicatechin-3-O-gallate) [31] and (+)-catechin [45] inhibited the growth of *C. histolyticum* group in fecal fermentor cultures compared to control. The numbers of *C. histolyticum* as well as *Bacteroides–Prevotella* and *Clostridium-Eubacterium* groups decreased in the presence of oolong tea polyphenols (EGCG, GCG, EGCG3”Me) compared to control in fecal fermentation [14].

In human intervention studies, stimulation in the microbial population including *Bifidobacterium* and *Lactobacillus* groups were observed after ingestion of high-cocoa flavanol compared to placebo treatment [44]. Intervention study on intake of black tea [47] extract and high cocoa flavanol [44] decreased the growth of *Clostridium* group and total bacterial count compared to placebo, respectively.

Animal studies which were performed with rats revealed that consumption of blackcurrant extract stimulated the growth of *Bifidobacterium* and *Lactobacillus* groups and inhibited the growth of *Bacteroides* and *Clostridium* groups compared to untreated rats [20]. Moreover, cocoa rich diet inhibited the growth of *Bacteroides, Clostridium* and *Staphylococcus* groups in rat gut compared to normal diet consumption [19].

Those studies showed that different food sources had different effects on gut microbiota suggesting that bioactive compounds might have possible stimulative or inhibitive effects on gut microbial groups.

Table 2.1. Studies concluding the effects of different dietary components on different intestinal bacterial groups by FISH.

Dietary component	**Positively affected bacterial groups**	**Negatively affected bacterial groups**	**Reference**
Grape seed extract	*Lactobacillus, Enterococcus*	*C. histolyticum*	[13]
Dahlia inulin	*R. flavefaciens, R. bromii, E. cylindroides*	-	[49]
Oolong polyphenols	*Bifidobacterium, Lactobacillus-Enterococcus*	*Bacteroides–Prevotella, C. histolyticum, Clostridium-Eubacterium*	[14]
Anthocyanins	*Bifidobacterium, Lactobacillus-Enterococcus*	-	[30]

Red wine extract		*C. histolyticum*	[31]
(+)-catechin	*C. coccoides–E. rectale*, *Bifidobacterium*, *E. coli*	*C. histolyticum*	[45]
(-)-epicatechin	*C. coccoides–E. rectale*	-	[45]
High-cocoa flavanol	*Bifidobacterium*, *Lactobacillus*	*Clostridium*	[44]
Pomegranate by-product	Total bacteria, *Bifidobacterium*, *Lactobacillus*	-	[46]
Black tea	-	Total bacteria	[47]
Isomaltooligosaccharides	*Bifidobacterium*	-	[12]
Fructo-oligosaccharides	*Bifidobacterium*	-	[12]
Cocoa	-	*Bacteroides*, *Clostridium*, *Staphylococcus*	[19]
Blackcurrant extract	*Bifidobacterium*, *Lactobacillus*	*Bacteroides*, *Clostridium*	[20]

2.3.2.4. PCR-Denaturing/Temperature Gradient Gel Electrophoresis (PCR-DGGE/TGGE)

PCR-DGGE/TGGE are semi quantitative molecular fingerprinting methods that allow detection of microbial diversity. In DGGE, amplified 16S rDNA gene samples by PCR are separated in gels including gradient of chemical denaturing agents. During PCR, a GC-clamp is added to amplicons in order to prevent complete denaturation while DGGE process. After gel separation, bands in different positions appear according to their different denaturation rates, indicating different bacterial species [50]. Similarly, the principle of TGGE is based on temperature gradient instead of chemical gradient [36]. DGGE/TGGE are useful and fast methods for microbial community profiling. Likewise, separated bands can be used for further studies [33]. However, these methods are not sensitive enough for sub-dominant species detection and similar melting points of different

DNA fragments may cause false outcomes [36, 50]. Furthermore, these techniques lack quantification and phylogenetic identification properties [4, 36]. That's why, DGGE and TGGE methods are generally performed in combination of other culture-independent methods such as NGS and Q-PCR.

Several studies are present regarding the effects of different dietary components such as polyphenols [11, 24, 47, 51] and oligosaccharides [35, 52-54] on gut microbial community profile by PCR-DGGE/TGGE. Recently, Nakatsu et al. [51] determined the effects of soy isoflavones on gut microflora of postmenopausal women in an *in-vivo* study. For microbial community profiling, PCR-DGGE and pyro-sequencing was performed. DGGE results show that although there was a high inter-individual variation in intestinal microbial communities, significant differences were obtained when samples before and after diet were compared. However, differences of bacterial numbers between treatments could be observed by additional methods such as pyro-sequencing, in this case. In the study of Queipo-Ortuńo et al. [24], DGGE as well as Q-PCR were performed to analyze the effects of red wine polyphenols on human gut microbiota in an *in-vivo* study suggesting that although DGGE could detect the variations of bacterial composition between samples, additional quantitative analysis of gut microbiota are needed.

2.3.2.5. Quantitative Real-time PCR (Q-PCR)

Q-PCR is a quantitative method in which fluorescence labeled probes such as Taqman probes or nonsequence-specific DNA-binding dye like SYBR green are used during amplification process. These fluorophores are captured by the thermal cycler device and converted to data by the software. This system allows continuous measurement and quantification of target DNA [36]. Q-PCR is the most powerful method for bacterial quantification and it has been widely used due to its rapidness, high accuracy, high sensitivity, relatively low cost, and high specificity [4, 37]. However, there are some limitations of Q-PCR analysis. Nucleic acid extraction efficiency of Q-PCR template is an important parameter since low DNA efficiency may lead to biased Q-PCR results. The presence of PCR inhibitors is another parameter leading underestimated results. Moreover, primer design is a parameter that has to be taken into account as primer design might affect primer specificity and hybridization efficiency, affecting quantitative assessments. Also,

Q-PCR is inapplicable for unknown species of microbial population due to its dependence on the primer design [33, 36].

Several Q-PCR studies were conducted for quantification of gut microbial communities and determination of the effects of dietary components on gut microbiota in *in-vitro* [11, 17, 52, 54, 55] and *in-vivo* human intervention [18, 24, 48, 56] as well as animal studies [53, 57-61] as presented in Table 2.2. Studies analyzing the effects of different dietary compounds such as arabinogalactan, fructo-oligosaccharides and inulin, as well as plant sources of dietary compounds such as gold & green kiwifruit, red wine and green tea on gut microbial groups suggested that Q-PCR is a powerful method for quantification of gut bacterial groups.

Representatively, *in-vitro* batch fecal fermentation of gold and green kiwifruit lead to higher numbers of *Bifidobacterium* and *Bacteroides-Prevotella-Porphyromonas* groups compared to control fermenter cultures [55] Continuous fermentation models also studied the effects of different food compounds on gut microbial groups [11, 17, 52, 54]. While black tea and red wine grape extract supplementation stimulated the growth of *Klebsiella, Enterococcus, Alistipes, Cloacibacillus, Victivallis* and *Akkermansia* groups [11], arabinogalactan supplementation increased the numbers of *Bacteroidetes, Faecalibacterium prausnitzii* and *Bifidobacterium* groups and total bacterial count [52] compared to non-treated time interval in continuous fermentation systems. Moreover, fructo-oligosaccharide [52] and inulin supplementation [54] in continuous fermentation systems lead to higher numbers of *Lactobacillus* and *Bifidobacterium* groups, respectively compared to non-treated time interval. On the other hand, black tea and red wine grape extract supplementation inhibited the growth of *Bifidobacterium, C. coccoides, Anaeroglobus, Victivallis, Subdoligranulum,* and *Bacteroides* groups in SHIME fermentation model [11]. While red wine inhibited the growth of total bacterial count [17], the presence of arabinogalactan lead to lower numbers of group *C. perfringens* [52] in continuous fermentation systems, compared to washout period.

In an *in-vivo* human intervention study, increase in the growth of *Enterococcus, Prevotella, Bacteroides, Bifidobacterium, Bacteroides uniformis, Eggerthella lenta* and *C.coccoides*–

E. Rectale groups was observed after the ingestion of red wine polyphenols containing catechin, epicatechin and gallic acid compared to placebo treatment [24].

Moreover, animal studies showed that diet rich in pectic oligosaccharides [53], conjugated linoleic acid and saponins from ginseng [58] stimulated the growth of *Bacteroides* and *Prevotella* groups in mice. While intervention of pectic oligosaccharides [53] and saponins from ginseng [58] stimulated the growth of *Bifidobacterium* group, pectic oligosaccharides [53], conjugated linoleic acid [57] and saponins from ginseng and *Gynostemma pentaphyllum* [58] stimulated the growth of *Roseburia, Akkermansia muciniphila, Lactobacillus* and *F. prausnitzii* respectively in mice. On the other hand, diet rich in conjugated linoleic acid [56] and fermented green tea extract [61] inhibited the growth of *Bifidobacterium* group and *Firmicutes/Bacteroidetes* ratio respectively in mice, compared to non-ingested mice.

Those studies revealed that different food sources lead to different impacts on gut microbiota suggesting that bioactive compounds might have possible stimulative or inhibitive effects on gut microbial groups.

Table 2.2. Studies concluding the effects of different dietary components on different intestinal bacterial groups by Q-PCR.

Dietary component	**Positively affected bacterial groups**	**Negatively affected bacterial groups**	**Reference**
Red wine	-	Total bacteria	[17]
Black tea	*Klebsiella, Enterococcus, Akkermansia*	*Bifidobacterium, C. coccoides, Anaeroglobus, Victivallis*	[11]
Red wine grape extract	*Klebsiella, Alistipes, Cloacibacillus, Victivallis, Akkermansia*	*Bifidobacterium, C. coccoides, Anaeroglobus, Subdoligranulum, Bacteroides*	[11]
Arabinogalactan	Total bacteria,	*C. perfringens*	[52]

	Bacteroidetes, Faecalibacterium prausnitzii, Bifidobacterium		
Fructo-oligosaccharides	*Lactobacillus*	-	[52]
Gold & green kiwifruit	*Bifidobacterium, Bacteroides-Prevotella-Porphyromonas*	-	[55]
Inulin	*Bifidobacterium*	-	[55]
Inulin	*Bifidobacterium*	-	[54]
Red wine	*Enterococcus, Prevotella, Bacteroides, Bifidobacterium, Bacteroides uniformis, Eggerthella lenta, C. coccoides–E. rectale*	-	[24]
Pectic oligosaccharides	*Bifidobacterium, Roseburia, Bacteroides*	-	[53]
Conjugated linoleic acid	*Bacteroidetes-Prevotella, Akkermansia muciniphila*	*Bifidobacterium*	[57]
Saponins of ginseng	*Bacteroides, Lactobacillus, Bifidobacterium*	-	[58]
Saponins of *Gynostemma pentaphyllum*	*F. prausnitzii*	-	[58]
Fermented green tea extract	-	*Firmicutes/Bacteroidetes* ratio	[61]

2.3.2.6. Next Generation Sequencing (NGS)

Next generation sequencing based on determining the nucleotide order in DNA of whole genome, is a powerful method to analyze microbial composition and diversity with high-throughput efficiency, sensitivity and high speed [36, 37]. However, computational downstream analyses of data are needed [37]. Despite the growth in output-per-cost ratio, high cost of genome sequencing is still a critical drawback for NGS [62].

There are several studies in which NGS technology is used for phylogenetic identification of gut microbiota [8, 11, 51, 53, 54, 62, 63]. Nam et al. [63] aimed to investigate the intestinal microbiota composition of Korean people and to compare with Japanese, Chinese and American individuals by 454-pyrosequensing. Results show that gut microbiota of Korean people were dominated by *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria* and *Proteobacteria* and overall microbial composition of Korean people was stable despite individual variations. When gut microbial composition of Korean people was compared with individuals from China, Japan and USA, intestinal microflora of each country included *Firmicutes, Bacteroidetes* and *Actinobacteria.* However the proportion of those phyla differed between countries. As a result, overall gut microbial composition of Korea was the same with other countries although there are some proportional changes that thought to be related to diet.

Furthermore, Kemperman et al. [11] investigated the effects of black tea and red wine grape extract polyphenols on gut microbial diversity by NGS and compared to PCR-DGGE in an *in-vitro* fermenter model. Pyro-sequencing results indicate that there is a shift in *Firmicutes:Bacteroidetes* ratio for both samples containing either black tea or red wine grape extract, concluding that different polyphenol complex have different impacts on gut microbiota. DGGE was able to analyze microbial community composition as well, in a simpler and more cost effective way, however NGS method gives more profound results for microbial community profiling.

2.4. INTERACTION OF POLYPHENOLS AND GUT MICROBIOTA

Polyphenols are one of the most studied metabolites for the analysis of the effects on gut microbiota [11, 13, 14]. Polyphenols are secondary metabolites of edible plants. Food products containing polyphenols are fruits, tea, coffee, wine, chocolate, vegetables, cereals etc. [13, 64]. The effects of polyphenols on human gut microbiota have become one of the researches of interest in recent years due to their health benefits and wide range of contents.

There are two mechanisms of interaction between polyphenols and human gut microbiota: (i) Human gut microbiota is able to catalyze reactions for degradation and conversion of polyphenols so that the formed metabolites are more easily absorbed from intestine, (ii) Formed phenolic metabolites can affect the composition of gut microbiota acting as growth stimulator and/or inhibitor [6, 9].

Many studies indicate that the intestinal microbiota plays an important role in the metabolism of polyphenols, which is done by biotransformation of polyphenols followed by decomposition of compounds with the help of intestinal bacteria forming aglycones [11, 13, 14, 65]. Although aglycon form of polyphenols are absorbed from small intestine, most of the polyphenols are in the form of esters, glycosides or polymers in nature and are not able to be absorbed from small intestine [65]. Esterification, glycosylation and polymerization combined to molecular weight are parameters affecting intestinal absorption of polyphenols [66]. Esterification may occur in catechins by gallic acid and in caffeic acid by quinic acid [66]. Researches indicate that recovery of esterified catechins in urine was 10- fold lower than non- esterified cathecins. Lower absorbtion and recovery was also observed for esterified caffeic acid. Most phenolics except catechins and proanthocyanidins are found in glycosylated forms [66]. While it is known that glycosylated polyphenls such as rhamnosides of quercetin have lower absorption rates in intestine, some studies indicate that glycosylated polyphenols such as quercetin glycosides have higher absorption rate than deglycosylated form. Some of the polyphenols are present in polymerized form in nature. Previous studies stated that high molecular weight and polymerization of proanthocyanidins and tea theaflavins resulted lower absorption in

intestine and recovery in urine. Therefore, these substances need intestinal microflora to be hydrolyzed, deglycosylated or cleaved by enzymes to be absorbed [65].

According to studies, gut microbial groups are known to transform those polyphenols by secretion of specific enzyme. For instance, *Eubacterium rectale* and *Bacteroides fragilis* are known to hydrolyze isoflavones, flavonols, anthocyanins, ellagitannins and lignans by glycosidase activity [7, 9]. Moreover, *Lactobacillus* and *Clostridium* species are capable of demethylase activity for demethylation of flavonols, flavan-3-ols, anthocyanins and lignans. Gut bacteria are also capable of dihydroxylation and fission activities of different phenolic compounds [7, 9].
Although the mechanism is not yet well known, gut bacteria such as *C. coccoides-E. rectale* group, *Bacteroides* and *Streptococcus* species, *Lactobacillus* and *Enterococcus* species are known to metabolize phenolic compounds, especially flavonoids such as quercetin, catechin and epicatechin by transforming them into smaller metabolites for ease their absorption [7, 9, 13, 14].

Microbial bioconversion and metabolism of polyphenols are performed as gut bacteria might use polyphenols or their degradation products as energy source for growth. This property of polyphenols make them potential prebiotics on related bacterial groups [13, 14, 24, 31, 44]. However antimicrobial properties of polyphenols are also stated in the studies. For instance, quercetin and other flavonols inhibited the growth of *Escherichia coli* by inhibiting the ATPase activity. Quercetin is also known to cause cell membrane disruption and diminished cell motility [7, 67]. Bacterial growth inhibition by cell membrane disruption as well as membrane transport inhibition have been also noted in the study of tea polyphenols [68].

2.5. TEA POLYPHENOLS

Tea, produced from leaves of *Camellia sinensis*, is one of the polyphenol-rich sources. Polyphenols in tea are dominated by flavonoids such as epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG), and theaflavins (THF) as well as phenolic acids such as gallic acid, alkaloids such as methylxanthines, non-proteic amino acids such as gamma-aminobutyric acid (GABA) and

polyamines [69, 70]. However, compositions of plant nutrients change among regions due to the differences in soil and water as well as among manufacturing process affecting intestinal microbiota of individuals differently.

During manufacturing, variation in processing of teas leads to changes in composition of different types of teas. Teas such as green, black, oolong, white, yellow and pu-erh are manufactured by different rates of oxidative processing leading to oxidation and partial polymerization of flavan-3-ols to theaflavins and thearubigins [70, 71]. Green tea which is the minimally processed one is therefore dominated by catechins. In contrast, the most oxidized tea which is black tea is dominated by theaflavins and thearubigins making 60-70% of total phenolic content [68, 70]. Table 2.3 indicates the differences in phenolic contents between green tea and black tea.

Table 2.3. Comparison of phenolic contents of green tea and black tea [70].

	Green tea	**Black tea**
EGCG	184.0 mg	22.0 mg
EGC	40.0 mg	19.0 mg
EC	20.0 mg	5.0 mg
Flavonol derivatives	9.0 mg	8.0 mg
Theaflavins	0.2 mg	14.4 mg
Thearubigins	2.6 mg	192.7 mg

2.6. ANALYSIS OF EFFECTS OF HUMAN GUT MICROBIOTA ON TURKISH BLACK TEA POLYPHENOLS

Turkish black tea is one of the most consumed beverages in Turkey. Though there are studies regarding the effect of black tea on microbial composition of gut, studies concerning the relation of Turkish black tea with human gut microbiota of a Turkish individual is not yet well studied. Metabolism of Turkish black tea polyphenols (BTP) by human gut microbiota was examined in the study of Özcan [72]. In this study, pH controlled (pH 6,8) *in-vitro* batch fermenters (135 mL) were used to mimic the intestinal conditions in the presence or absence of Turkish black tea extract (1000 mg/L) with fecal

samples collected freshly from one healthy male volunteer. Tea extract without fecal sample was used as a control to observe whether degradation of polyphenols occur spontaneously or by gut microbiota. The fermenters were run at 37°C under flowing nitrogen with slow agitation for 48 h. During fermentation, samples were collected at 7 time points (0, 4, 8, 10, 24, 30, 48 h) from every reactor for analysis of degradation compounds. While profile of polyphenols (catechins, gallic acid, and theaflavins) of fermenter cultures were analyzed using HPLC-PDA, degradation products in fermenter cultures were determined by GC-MS analysis. Plate counting was performed to analyze the bacterial composition (total anaerobes, total aerobes, total coliforms, *Lactobacillus* spp., *Clostridia* group, *Enterobacteria* group, *and Staphyloccus aureus)* in fermenter samples collected at time 0 and 48 h.

HPLC-PDA results showed that in black tea added fecal samples; total catechins, gallic acid and theaflavins were degraded after around 8 to 10 h of fermentation and remained fully degraded till the end of 48 h. However, in control samples degradation rates were much slower and the rate was around 40 to 60% even at the end of fermentation. GC-MS analysis of degradation products showed that while pyrocatechol and pyrogallol were initially not present in fermenter samples, they formed after 4 h fermentation, reaching their highest concentrations after 24 h followed by decrease of pyrogallol till the end of fermentation. 4-hydroxyphenylacetic acid and 3-(3-hydroxyphenyl) propionic acid concentrations increased in black tea added fecal cultures but remained absent in fecal samples lacking tea extract till the of fermentation. The results of bacterial community analysis did not reveal conclusive effects as plate counts were done only with samples collected in the beginning (0 h) and at the end (48 h) of fermentation. This study showed that colonic microbiota had a potency to transform and increase digestion of Turkish BTP by degrading into smaller and more easily absorbed polyphenol compounds [58].

2.7. AIM OF THE STUDY

The aim of this study was to evaluate possible influence of Turkish black tea polyphenols on the colonic microflora of a Turkish individual in in-vitro batch fermenter fecal cultures by using real-time PCR & FISH for bacterial quantification and bacterial composition analysis. By this research, microbial community shifts in gut of the Turkish individual,

affected by Turkish black tea polyphenols were analyzed. The results were compared with the previous work to conclude whether black tea polyphenol metabolites had interaction with gut microbiota, or not.

3. MATERIALS AND METHODS

3.1. SAMPLES

Samples were provided from the previous work of Özcan [72], which were from pH controlled (pH 6.8) *in-vitro* batch fermenters carried out with freshly collected fecal samples of a Turkish individual in basal medium under anaerobic conditions at 37°C for 48 h in the presence (1000 mg/L) (R1) and absence of black tea extract (R2). Fermentation was performed in triplicate (F1, F2 & F3) with fecal material obtained from the same person at different times. Samples collected from fermenters at 7 time points (0, 4, 8, 10, 24, 30, 48 h) were kept at -80^{o}C until analysis.

3.2. QUANTIFICATION OF BACTERIAL GROUPS BY REAL-TIME POLYMERASE CHAIN REACTION (PCR)

3.2.1. Bacterial Strains and Culture Conditions

Reference strains; *Atopobium parvulum* ATCC 33793**,** *Bacteroides fragilis* ATCC 25285, *Bifidobacterium bifidum* ATCC 11863, *Clostridium coccoides* ATCC 29236, *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 25922 and *Lactobacillus acidophilus* ATCC 4356, used for preparation of standard curves were cultivated according to bacterial culture guide of ATCC. Briefly, lyophilized cells were suspended in 0.5 mL of appropriate growth media and then 0.25 mL of suspensions were added into 3 mL of appropriate broth. Cultures were grown at 37^{o}C under appropriate conditions as indicated at Table 3.1.

Table 3.1. Growth media and culturing conditions for bacterial strains used in Q-PCR as standards

Reference strains	Growth media	Culturing conditions
Atopobium parvulum ATCC 33793	Gifu anaerobic medium (GAM) agar/GAM broth (Hyserve, Germany)	Anaerobic, 48 h
Bacteroides fragilis ATCC 25285	GAM agar/GAM broth	Anaerobic, 48 h

Bifidobacterium bifidum ATCC 11863	Brain Hearth Infusion agar (GBL, Turkey)/Reinforced *Clostridium* broth (Oxoid, UK)	Anaerobic, 24 h
Clostridium coccoides ATCC 29236	GAM agar/GAM broth	Anaerobic, 48 h
Enterococcus faecalis ATCC 19433	Tryptic soy agar (TSA) / Tryptic soy broth (TSB) (LabM, UK)	Aerobic, 24 h
Escherichia coli ATCC 25922	TSA/ TSB	Aerobic, 24 h
Lactobacillus acidophilus ATCC 4356	MRS agar/ MRS broth (LabM, UK)	Aerobic with 5% CO_2, 72 h

The purities of the cultures were checked by transferring several drops of culture on agar media. After second activation of cultures by transferring 1 mL of activated culture on 5 mL fresh broth media, DNAs from each culture were extracted with QIAamp DNA mini kit (Qiagen, Germany). Stock cultures were prepared by adding 100% glycerol into broth cultures with a final 20 % glycerol concentration, dividing cultures into cryo-vials and storing at -80^{o}C until further usage.

3.2.2. DNA Extraction From Bacterial Cultures and Fermenter Samples

DNA extraction from reference bacterial cultures stated above was performed by using QIAamp DNA Mini Kit (Giagen, Germany), following manufacturer's instructions. On the other hand, DNA extraction from fermenter samples was performed by using QIAamp DNA stool mini kit (Qiagen, Germany) with slight modifications. Briefly, fermenter samples (100-400 µL) stored at -80^{o}C were transferred into 2 mL microcentrifuge tubes containing 4-5 glass beads and washed 2 times by suspending them in 1 mL phosphate buffered saline (PBS) (Gibco, USA), spinning down at full speed for 5 min afterwards. Silica beads (0.3 g of 0.1 mm diameter and 0.1 g of 0.5 mm diameter) and 1.4 mL ASL buffer were added onto pellets and vortexed until the sample was completely homogenized. Then, the suspensions were incubated at 95° C for 5 min, vortexed briefly and bead beated with benchtop homogenizer (MP Bio, USA) for 3 cycles with the speed of 7 for 1 min each. Samples were centrifuged at full speed for 1 min, 1.2 mL of the supernatant was transferred into new 2 mL microcentrifuge tubes and kit procedure was followed. Isolated DNAs from both bacterial culture and fermenter samples were suspended in 50 µL AE buffer and run on 1.5 % agarose gel to check the qualities. The concentrations of DNA samples were determined by absorbance at 260 nm with NanoDrop

spectrophotometer (Thermo Scientific, USA) and were stored at -20^{o}C until further analysis.

3.2.3. Standard Curve Preparation

In order to create standard curves (cycle threshold value (Ct) vs. gene copy number) for Q-PCR analysis, serial dilutions (10 fold dilutions from 10 ng/µL to 10^{-5} ng/µL) of extracted DNAs from *Atopobium parvulum* ATCC 33793**,** *Bacteroides fragilis* ATCC 25285, *Bifidobacterium bifidum* ATCC 11863, *Clostridium coccoides* ATCC 29236, *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 25922 and *Lactobacillus acidophilus* ATCC 4356 reference strains were used for *Atopobium*, *Bacteroides-Prevotella-Porphyromonas*, *Bifidobacterium*, *Clostridium coccoides*, *Enterococcus*, *Enterobacteriaceae* and *Lactobacillus* group standards, respectively. For universal bacteria quantification, *E. coli* ATCC 25922 was used since experiment conditions for universal bacterial primers were generally arranged according to *E. coli* strains in studies [73, 74].

To insert the values into standard curves, number of gene copies in a known amount of DNA for each bacterial group was calculated as below [67]:

$$\text{Gene copy} = \text{amount of DNA (ng)} \times \frac{6.022 \times 10^{23}}{\text{genome size (base pair/bp)} \times 650\ \text{(g)}} \times \frac{1\ \text{(g)}}{10^{9}\ \text{(ng)}} \qquad (3.1)$$

Genome sizes for each bacteria indicated in formula 3.1 were obtained from NCBI database. The following average genome sizes for each bacterial group were used: 3 Mb for *Atopobium* and *Enterococcus*, 4 Mb for *Bacteroides-Prevotella-Porphyromonas* and *C. coccoides*, 2.3 Mb for *Bifidobacterium*, 4.6 Mb for *Enterobacteriaceae* and 2.9 Mb for *Lactobacillus* group [75, 76]. **650 g** in formula 3.1 states that the average weight of a bp was assumed as 650 Daltons, meaning that one mole of a bp weighs 650 g [74]. It was also assumed that each bacterial cell consisted 1 copy of 16S rDNA gene (ignoring multiple gene copies) [74].

3.2.4. Q-PCR Primer Design and Reaction Conditions

Primer specificities and Q-PCR reaction conditions for each primer set were chosen by performing gradient PCR with each primer set, using appropriate standard DNAs from pure bacterial cultures stated above as targets and running PCR products on 2% agarose gel afterwards. PCR with non-target bacterial DNAs were also performed to check any unspecific binding of primers. Properties of primer sets targeting different bacterial groups of gut microbiota were shown in Table 3.2.

Table 3.2. 16S rDNA gene-targeted group-specific primers

Target bacterial group	**Sequence (5'-3')**	**Amplicon size (bp)**	**Annealing temp. (C^o)**	**References**
Atopobium	**F:** GGGTTGAGAGACCGACC **R:** CGGRGCTTCTTCTGCAGG	190	55	[77]
Bacteroides-Prevotella-Porphyromonas	**F:** GGTGTCGGCTTAAGTGCCAT **R:**CGGA(C/T)GTAAGGGCCGTGC	140	60	[76]
Bifidobacterium	**F:** GGGTGGTAATGCCGGATG **R:**TAAGCGATGGACTTTCACACC	278	60	[78]
Clostridium coccoides	**F:** AAATGACGGTACCTGACTAA **R:**CTTTGAGTTTCATTCTTGCGAA	440	55	[77]
Enterobacteriaceae	**F:**GTTGTAAAGCACTTTCAGTGGTGAGGAAGG **R:**GCCTCAAGGGCACAACCTCCAAG	424	60	[79]
Enterococcus	**F:**CCCTTATTGTTAGTTGCCATCATT **R:** ACTCGTTGTACTTCCCATTGT	144	62	[76]
Lactobacillus	**F:** AGCAGTAGGGAATCTTCCA **R:** CACCGCTACACATGGAG	341	62	[76]
Universal bacteria	**F:** TCCTACGGGAGGCAGCAGT **R:**GGACTACCAGGGTATCTAATCCTGTT	466	60	[80]

Bacterial quantification of fermenter samples by Q-PCR was performed with Rotor-Gene Q real-time PCR cycler (Qiagen). Fermenter samples and standards were run at the same reaction as well as with negative control and no template control (NTC) to avoid any false-positive results. Each reaction mixture (12 µL) consisted of 2 µL DNAse RNAse free ddH_2O (Gibco, USA), 1 µL of 10 µM forward and reverse primers of target bacterial group

each, 6 µL SYBR Green (Applied Biosystems, USA), 0.1 µg/µL BSA (Ambion, USA) and 2 µL extracted fermenter DNA samples at appropriate dilutions. The thermal cycling conditions were as follows: an initial DNA denaturation step at 95^{o}C for 10 min followed by 40 cycles of denaturation at 95^{o}C for 15 seconds and annealing at optimal temperature (Table 3.1) for 60 s. To determine specificity, melting curve analysis was performed by slow heating from 65 to 95^{o}C (1^{o}C per cycle of 5 s) with simultaneous fluorescence collection. All reactions were performed in triplicate.

3.2.3. Data Analysis

In order to obtain data from Q-PCR quantification, standard curves for each bacterial group were sketched by the software program with entered gene copy numbers stated above. A representative standard curve (cycle threshold vs. gene copy number) is given in Figure 3.1.

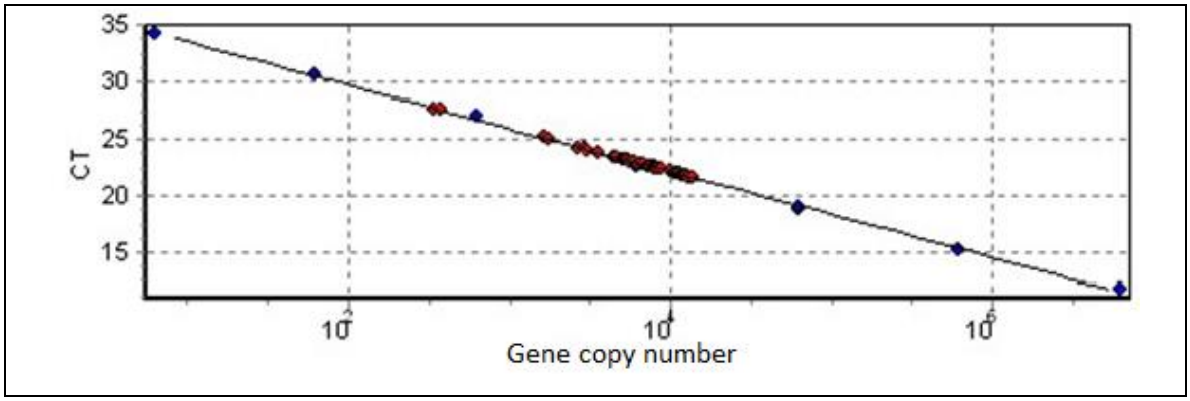

Figure 3.1. Representative Q-PCR standard curve for *Enterococcus* group. Blue dots represent standard samples and red dots represent fermenter samples.

As indicated by formula 3.2, to calculate number of bacterial cells per mL feces, gene copy numbers obtained from standard curves for each bacterial group were multiplied by the volume of extracted DNA from each sample (µL) and then divided by the volume of DNA used in PCR reaction (µL) and the volume of fermenter sample from which DNA was extracted (mL). The equation for calculating number of cells per mL feces is stated below [81]:

$$\text{number of cells/mL} = \frac{\text{gene copy x volume of isolated DNA (µL)}}{\text{volume of DNA per reaction (µL) x volume of the sample (mL)}} \quad (3.2)$$

"Index of specific bacteria (ISB)" was used to determine the changes in bacterial population, by the equation below:

$$\text{ISB} = [(\text{Ns (T1)} - \text{Ns (T0)}) - (\text{Nc (T1)} - \text{Nc (T0)}] \quad (3.3)$$

In formula 3.3, **Ns** indicates the number (log10) of bacteria in fermenter sample treated with black tea extract, **Nc** is the number (log10) of bacteria in control sample, **T1** is a specific time point and **T0** is the 0 h time point [45].

3.3. BACTERIAL COMPOSITION ANALYSIS BY FLUORESCENT *IN-SITU* HYBRIDIZATION (FISH)

3.3.1. Hybridization Protocol

For FISH analysis, oligonucleotide probes labelled with Cy3 dye, targeting 16S rDNA genes were used. The studied bacterial groups were *Bacteroides-Prevotella* group, *Bifidobacterium* group, *Clostridium coccoides-Eubacterium rectale* group & *Lactobacillus−Enterococcus* group and total bacteria. The properties of bacterial probes used in FISH study are indicated in Table 3.3.

The assay was performed with 500µL previously fixed (with 4 % paraformaldehyde) fermenter samples from the study of Özcan [72]. FISH procedure was performed as described by Hidalgo et al. [30] with slight modifications. Briefly, 50 µL of fixed samples stored at −20 °C were washed twice with PBS to reduce background noise and sonicated for 1 min. After diluting appropriately, 20 µL of samples were transferred onto wells of teflon and poly-L-lysine-coated 10-well slides (EMS, USA) and dried on a hotplate (Wisd Laboratory Instruments, Ireland) at hybridization temperature. Dried slides were then dehydrated in 50%, 80%, and 100% (v/v) ethanol series for 3 min each and allowed to dry. For gram positive bacteria, dried cells were treated with 20 µL of lysozyme solution (1 mg/mL in 100 mM Trizma HCl pH 7.2) at room temperature for 15 min to increase cell permeability, before dehydration in ethanol series.

3.3. FISH oligonucleotide probes used in this study [30]

Probe	**Target bacterial group**	**Sequence (5'-3')**	**Temperature (hybridization-wash) (°C)**
Bac 303	*Bacteroides-Prevotella* group	CCAATGTGGGGGACCTT	46-48
Bif 164	*Bifidobacterium* group	CATCCGGCATTACCACCC	50-50
Erec482	*Clostridium coccoides-Eubacterium rectale* group	GCTTCTTAGTCARGTACCG	50-50
Lab 158	*Lactobacillus−Enterococcus* group	GGTATTAGCAYCTGTTTCCA	50-50
EUB 338*	Total bacteria	GCTGCCTCCCGTAGGAGT	46-48
EUB338II*	Total bacteria	GCAGCCACCCGTAGGTGT	46-48
EUB338III*	Total bacteria	GGTCGGTCTCTCAACCC	46-48

*These probes are used together in equimolar concentrations

Five µL of 50 ng/µL target probes and 20 µL of hybridization mixture containing 0.9 M NaCl, 20 mM Tris-HCl and 0.1 % SDS (pH 7.2) were added onto the surface of each well and the slides were left overnight to hybridize at appropriate temperature. After hybridization, slides were transferred to pre-warmed wash buffer containing 0.9 M NaCl, 20 mM Tris-HCl and 50 ng/mL DAPI solution and incubated at hybridization temperature for a further 15 min. Thereafter, slides were dipped into ice-cold distilled water for 2−3 s and 5 µL of antifade solution (DABCO (Sigma, USA)) was added to each well. The slides were covered with coverslips and stored in the dark at 4°C until examination. Hybridized cells were examined under Confocal Microscope (Zeiss, Germany) with 100x magnification. 10 random fields were viewed for image analysis.

3.3.2. Image analysis

The images were analyzed with ImageJ program, using “analyze particles” tool for co-localized images of Cy3 and DAPI labeled cells. Images containing cell aggregates,

considered as over quantification range (3% of total images), were counted manually. The following equation was used to calculate bacterial cells in per mL sample:

$$\text{Cells / mL sample} = \text{N x 2 x 6924.54 x 50 x q} \quad (3.4)$$

In formula 3.3; **N** indicates the average number of counted cells, **2** is the dilution factor during fixation steps, **6924.54** is the magnification constant, **50** is the constant used to work the value back to 1 mL from the amount added onto slides and **q** is the dilution factor [82]. Same as Q-PCR analysis, "Index of specific bacteria (ISB)" (equation 3.3) was used to determine the changes in bacterial population [45].

3.4. STATISTICAL ANALYSIS

To determine the effects of studied factors (time and treatment), two-factor repeated-measures analysis of variance (ANOVA) was used with time and treatment as two factors. Two-sample t-test was used to assess the significant differences between treatments (R1 and R2) at the same time point. To determine significance for reactors of each fermentation experiment, one way analysis of variance (ANOVA) followed by Tukey's test for multiple comparison with time within same treatment samples was conducted. P values of 0.05 and 0.01 were used for the level of significance of the tests. Statistical analyses were performed with IBM SPSS 20.0 version.

4. RESULTS

The effects of BTP on gut microbiota in *in-vitro* fecal fermentations during 48 h were examined by using Q-PCR and FISH analysis to investigate bacterial composition in fermenter samples.

4.1. QUANTIFICATION OF BACTERIAL GROUPS BY Q-PCR ANALYSIS

4.1.1. Evaluation of Results of Q-PCR Analysis for Each Fermentation Experiment

Q-PCR analysis was performed to determine the numbers of bacterial groups of fermenter samples in presence (R1) or absence (R2) of black tea extract in triplicate fermenter cultures (F1, F2, F3) with fecal samples obtained from the same person at different time periods.

In the absence of black tea extract, (F1/R2) (control samples) in the first fermenter, all bacterial groups except *Enterobacteriaceae* group showed stable patterns during 48 h fermentation with less than 1 log cells/mL feces difference (Figure 4.1.a). *Enterobacteriaceae* group counts showed significant decrease in 4^{th} and 10^{th} h till 8.69 log cells/mL feces followed by a significant increase to 9.43 log cells/mL feces in 30^{th} h. While *Lactobacillus* group was found as the most abundant (approx. 10^9 cell/mL feces) bacterial group, *Enterococcus* group was the least abundant (~ 10^6 cell/mL feces) bacterial group.

When black tea extract was added into fermenter (F1/R1), less than 1 log changes were screened in numbers of *Atopobium*, *Bacteroides-Prevotella-Porphyromonas*, *Bifidobacterium*, *C. coccoides* groups and total bacterial count (log cells/mL feces) of F1/R1 samples compared to F1/R2 samples (Figure 4.1.a-b). Different than control samples (F1/R2), a 2 log difference was screened in *Enterobacteriaceae* group in black tea added samples (F1/R1) after 8 h of incubation ($P<0.01$). Furthermore, log cell counts of *Enterococcus* group increased to over 10^6 cells/mL feces in R1 sample in 24, 30 and 48 h, where those log cell counts were under 10^6 cells/mL feces in control (F1/R2) samples. Cell

counts of *Lactobacillus* group also increased to 10^{10} cells/mL feces in R1 samples in all time points, where cell counts were under 10^{10} cells/mL feces in control samples (Figure 4.1.b).

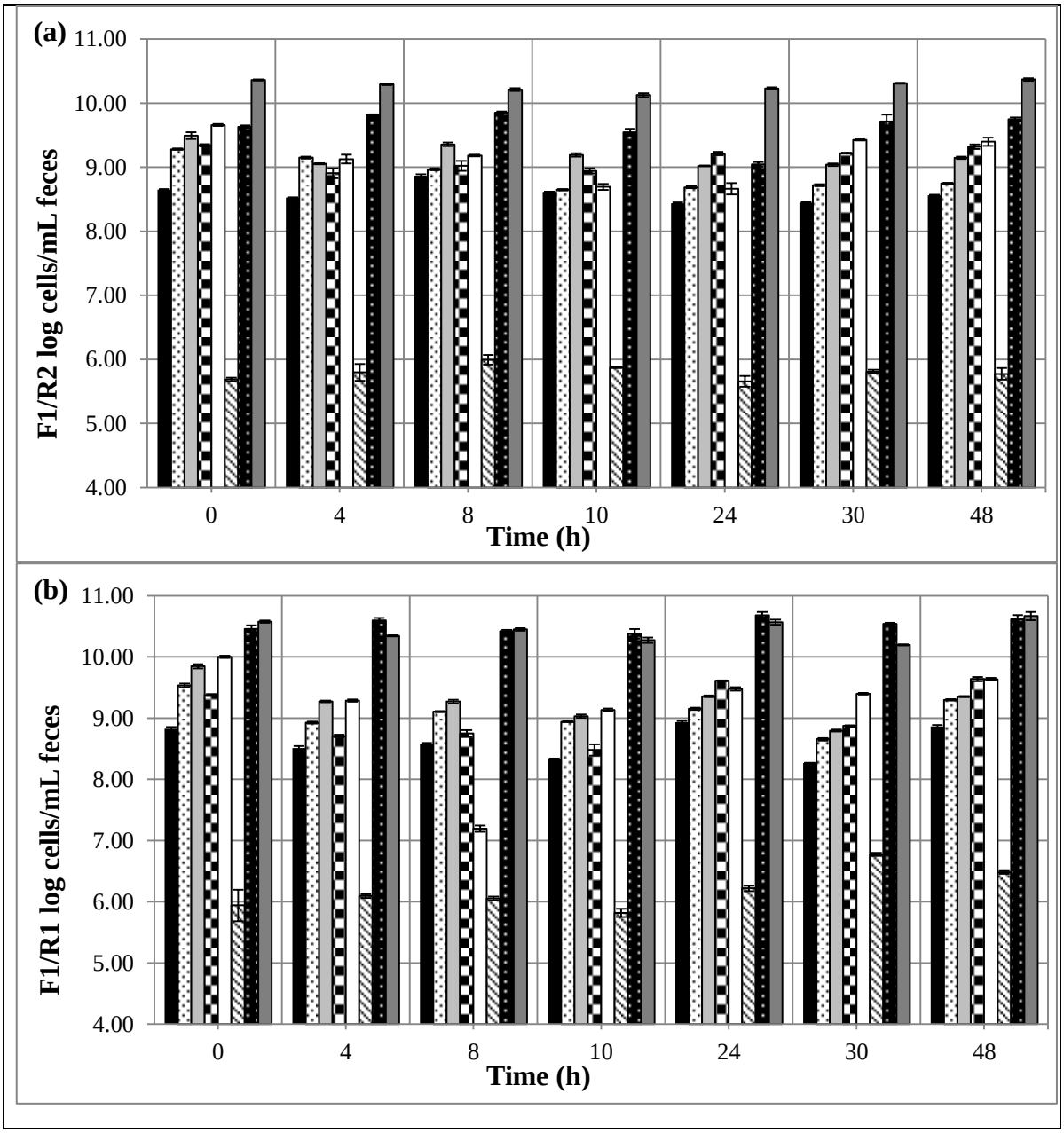

Figure 4.1. Bacterial profile of 48 h fecal fermenter cultures in fermenter 1 (F1) (a) in the absence of black tea extract and (b) in the presence of black tea extract. Columns indicate ■:*Atopobium*, ⬚:*Bacteroides-Prevotella-Porphyromonas*, ▒:*Bifidobacterium*, ▪:*C. coccoides*, □:*Enterobacteriaceae*, ▨:*Enterococcus*, ■:*Lactobacillus* group and ■:total bacteria, respectively. Values are means ± SD of three experimental replicates.

In control samples of second fermenter cultures (F2/R2); *Atopobium*, *Bacteroides-Prevotella-Porphyromonas*, *C. coccoides*, *Lactobacillus* groups and total bacterial count showed slight alterations in log cell numbers during 48 h fermentation with less than 1 log difference (Figure 4.2.a). In *Bifidobacterium* group, there was an increase in 8^{th} h ($P<0.01$) which remained until the end of fermentation. Like F1/R2 culture, *Enterobacteriaceae* and group *Enterococcus* groups showed alternating patterns with significant increase with more than 1 log cells/mL feces till 8^{th} h of fermentation. Moreover, *C. coccoides* group counts declined significantly in 4^{th}, which was followed by an significant increase in 24^{th} h. Similar as F1/R1 culture, *Lactobacillus* group was the most abundant (~ 10^9) bacterial group and *Enterococcus* group was the least abundant bacterial group with less than 10^6 cells/mL feces in most of the time points.

When black tea extract was added into fermenter (F2/R1), minor differences were screened in numbers of *Atopobium*, *Bacteroides-Prevotella-Porphyromonas*, *Bifidobacterium*, *C. coccoides*, *Enterobacteriaceae* groups compared to (F2/R2) samples (Figure 4.2.a-b). Different than the general trend, notable escalation was screened in the numbers of *Atopobium* (8.36 log cells/mL feces), *C. coccoides* (8.91 log cells/mL feces) and *Enterobacteriaceae* groups (9.22 log cells/mL feces) in 8^{th} h time point and a decrease was screened in *Bifidobacterium* group (7.49 log cells/mL feces) in 24^{th} h time point. Different than control samples (F2/R2), *Enterococcus* group counts were generally over 10^6 cells/mL feces in black tea added (F2/R1) sample, where cell counts were under 10^6 cells/mL feces in most of the time points in control samples (F2/R2). The highest bacterial count was monitored in *Lactobacillus* group in 8^{th} h (10^{10} cells/mL feces) with more than 1 log cell/mL feces difference compared to F2/R2. Moreover, significant increment was observed in total bacterial counts after 10 h (Figure 4.2.b).

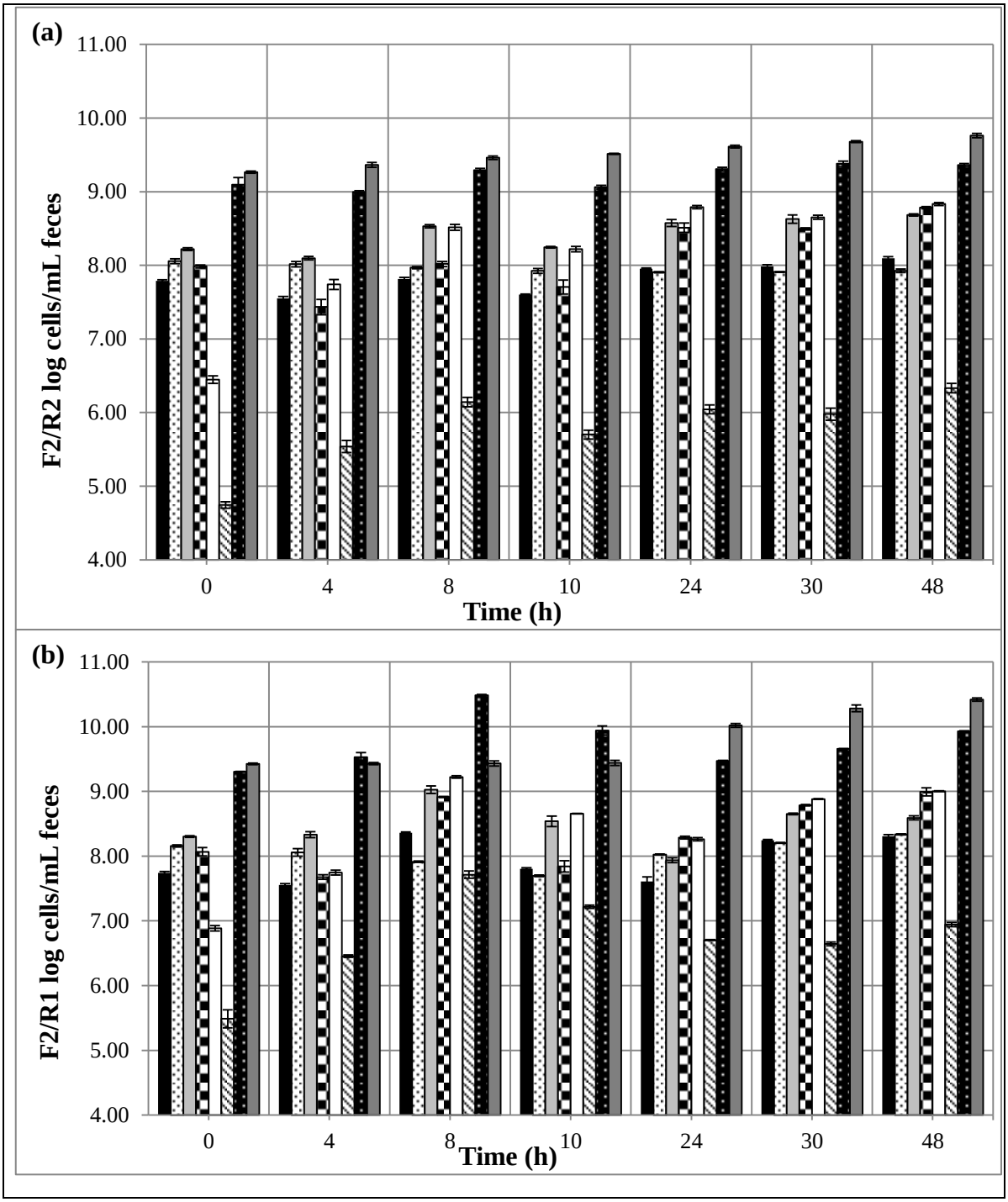

Figure 4.2. Bacterial profile of 48 h fecal fermenter cultures in fermenter 2 (F2) (a) in the absence of black tea extract and (b) in the presence of black tea extract. Columns indicate ■:*Atopobium*, ⬚:*Bacteroides-Prevotella-Porphyromonas*, ▒:*Bifidobacterium*, ▚:*C. coccoides*, □:*Enterobacteriaceae*, ▨:*Enterococcus*, ■:*Lactobacillus* group and ■:total bacteria, respectively. Values are means ± SD of three experimental replicates.

In control sample of the third fermenter (F3/R2), steady patterns were observed in *Atopobium*, *Bacteroides-Prevotella-Porphyromonas*, *Enterococcus*, *Lactobacillus* groups and total bacterial count (Figure 4.3.a). Significant increase in log cell numbers in *Bifidobacterium* and *Enterobacteriaceae* groups was monitored till 8^{th} h. The cell counts of those bacterial groups remained stable till 24^{th} h and then decreased notably at 30^{th} h. Cell numbers of *Bifidobacterium* and *Enterobacteriaceae* groups were then reached to their highest values (10^8) in 48^{th} h. Furthermore, there was a notable increase in 24^{th} and 48^{th} h in *C. coccoides* group cells. Unlike other fermenter samples (F1/R2 & F2/R2), *C. coccoides* group was the most abundant bacterial group in most of the time points in F3/R2 samples.

Cell numbers of bacterial groups in black tea extract added fermenter (F3/R1) were close to cell counts in control samples (F3/R2) except *Lactobacillus* group (Figure 4.3.a-b). Different than the general trend in F3/R1 samples, numbers of *C. coccoides* group and *Enterococcus* group significantly escalated to ~10^9 and ~10^8 cells/mL feces respectively, where the numbers were ~10^8 and 10^7 cells/mL feces in control (F3/R2) samples (Figure 4.3.b). Moreover, variation in cell numbers of *Lactobacillus* group between F3/R1 and F3/R2 was ~1 log cells/mL feces in most of the time points.

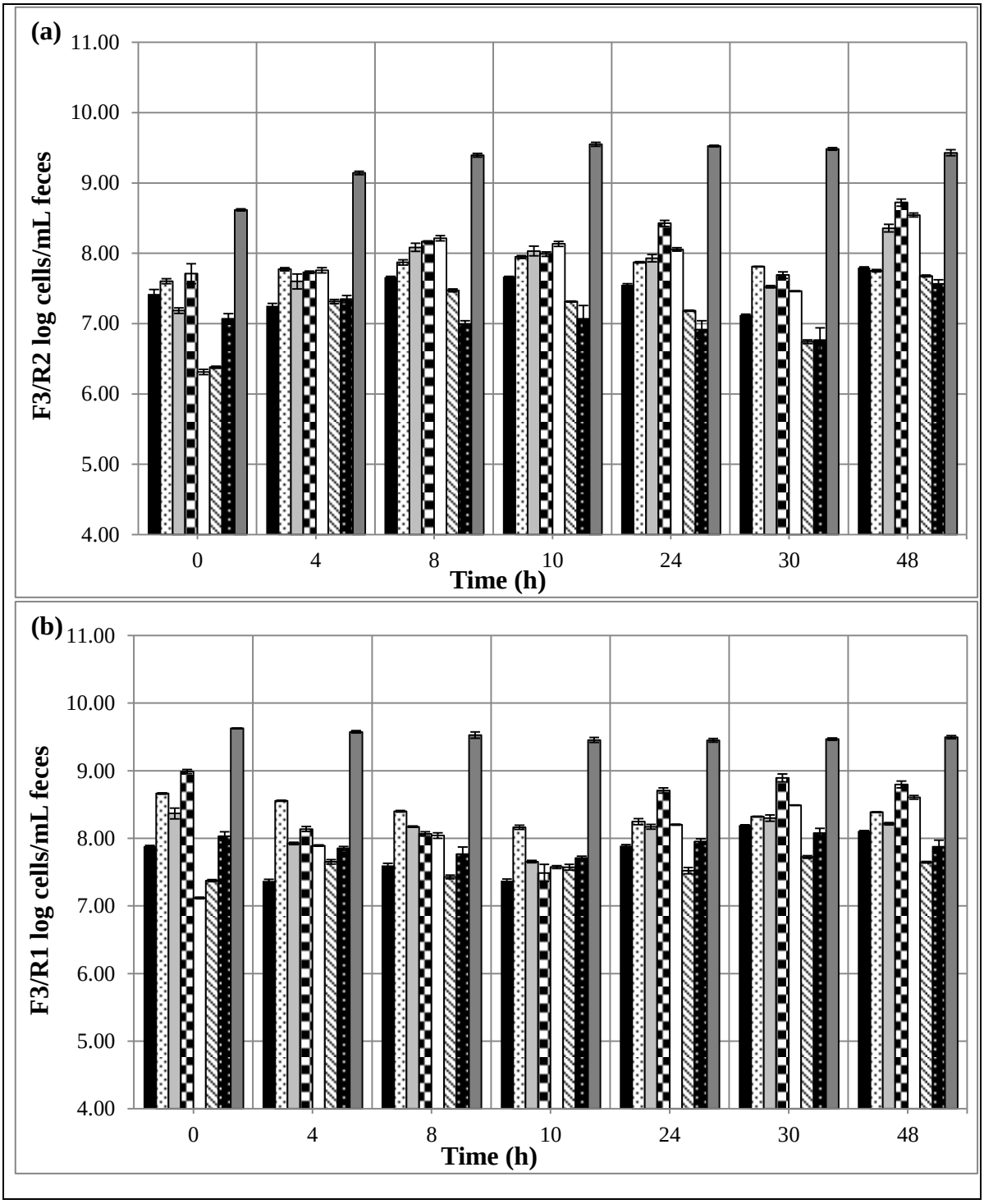

Figure 4.3. Bacterial profile of 48 h fecal fermenter cultures in fermenter 3 (F3) (a) in the absence of black tea extract and (b) in the presence of black tea extract. Columns indicate ■:*Atopobium,* ⬚:*Bacteroides-Prevotella-Porphyromonas,* ▫:*Bifidobacterium,* ◪:*C. coccoides,* □:*Enterobacteriaceae,* ▨:*Enterococcus,* ■:*Lactobacillus* group and ■:total bacteria, respectively. Values are means ± SD of three experimental replicates.

4.1.2. Evaluation of Q-PCR Analysis Results of All Fermenters

The mean values of three fermenter samples for Q-PCR results, calculated as ISB which indicates the changes relative to control were compared to interpret the effects of BTP on modulation of gut microbiota. Data were collected from three fermenters which were run at different times with fecal samples of the same individual obtained right before fermentation.

Among the studied bacterial groups, no significant change was noted in any of the bacterial groups ($P>0.05$) (Figure 4.4). Although the cell numbers of *Lactobacillus* group (8, 10, 24 & 30^{th} h) as well as in 30^{th} h of *Atopobium* and *Enterococcus* groups and 48^{th} h of *Atopobium* and *Bacteroides-Prevotella-Porphyromonas* groups increased in black tea added (R1) samples relative to control (R2) samples, the increments were not significant ($P>0.05$). Slight decrease in black tea added samples (R1) compared to control samples (R2) was screened in numbers of *Atopobium, Bacteroides-Prevotella-Porphyromonas, C. coccoides, Enterobacteriaceae, Enterococcus* groups as well as in total bacterial count in most of the time points, although decrease was not significant ($P>0.05$). The highest reduction in bacterial numbers in R1 sample compared to control (R2) sample was examined in 10^{th} h of *Enterobacteriaceae* group (0.74 log cells/mL feces), however the reduction in the certain time point was not significant ($P>0.05$). Moreover, significant difference was not screened in any of the bacterial group cell numbers at a specific time point.

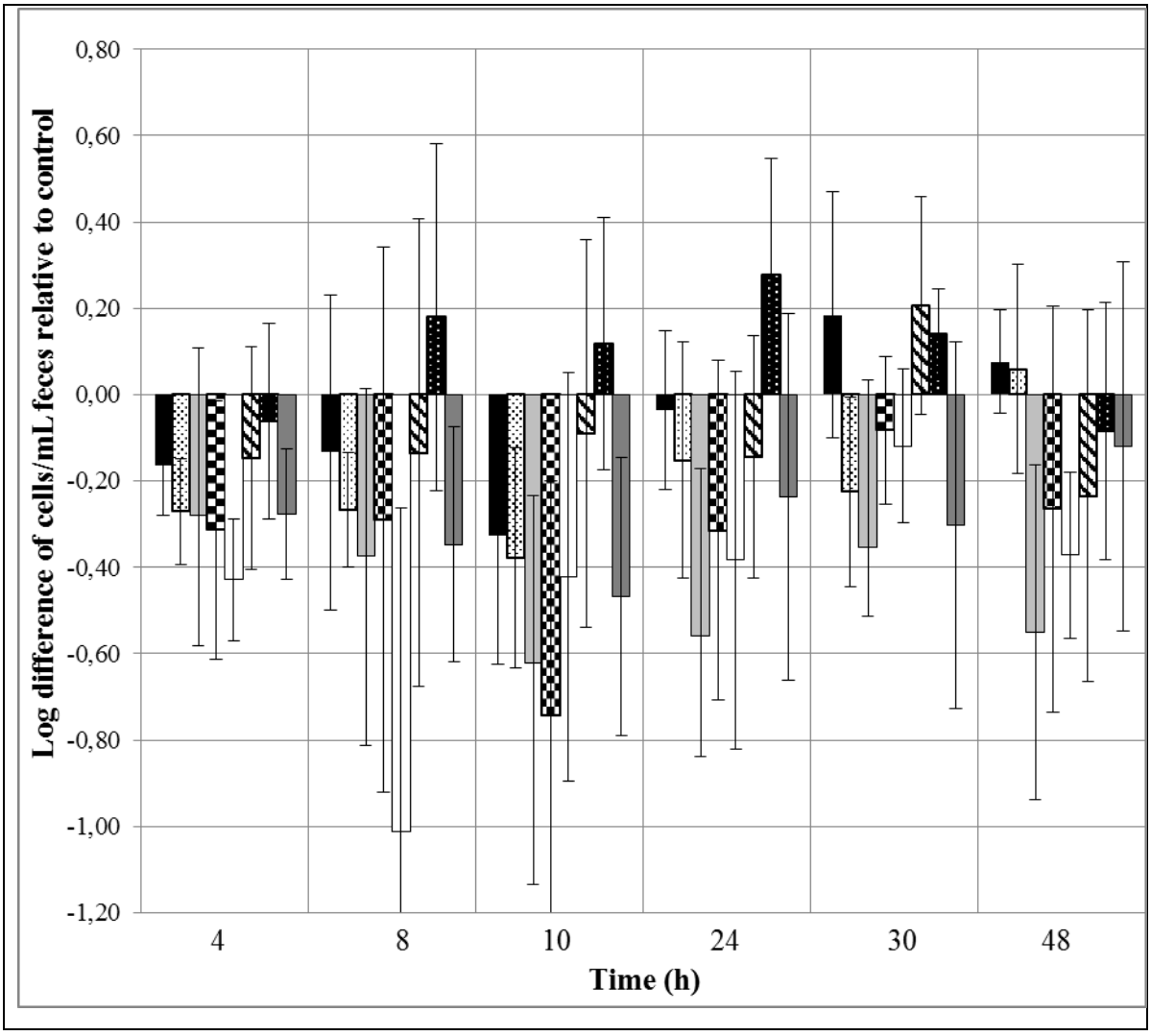

Figure 4.4. Changes in bacterial group numbers during 48 h black tea fermentation in a fecal batch culture analyzed by Q-PCR. Columns indicate ■ :*Atopobium,* ⬚ : *Bacteroides-Prevotella-Porphyromonas,* ▒ :*Bifidobacterium,* ▩ :*C. coccoides,* □ :*Enterobacteriaceae,* ▨ :*Enterococcus,* ■ :*Lactobacillus* group and ■ :total bacteria, respectively. Values are means ± SEM of fermenter replicates (n=3).

4.2. CHANGES IN BACTERIAL COMPOSITION DETERMINED BY FLUORESCENT *IN-SITU* HYBRIDIZATION (FISH)

4.2.1. Evaluation of Results of FISH Analysis for Each Fermentation Experiment

FISH analysis was performed with the same fermenter samples as Q-PCR analysis. Although the samples of three separate fermenters were analyzed by Q-PCR, samples of 2

representative fermenters (F1 & F2) were used for FISH analysis because of the high cost and labor intensiveness of the technique.

In control samples of the first fermenter F1/R2, all bacterial groups and total bacteria numbers showed stable patterns with less than 1 log cells/mL feces difference during 48 h fermentation (Table 4.1.). Notably, *C. coccoides-E. rectale* group and total bacterial counts decreased from 8.90 and 9.61 log cells/mL feces to 8.04 and 9.05log cells/mL feces, respectively in 10 h period ($P<0.01$). Decrease in *C. coccoides-E. rectale* group and total bacterial counts were followed by a significant increase till the end of fermentation. While the cell numbers of *Lactobacillus-Enterococcus* group was generally around 8 log cells/mL feces, cell numbers decreased significantly at 8^{th} (7.42 log cells/mL feces), 24^{th} (7.43 log cells/mL feces) and 48^{th} (7.35 log cells/mL feces) hours ($P<0.01$). Addition of black tea extract, F1/R1, also did not change the bacterial growth pattern over time. *Bifidobacterium* group counts increased from 8.10 to 8.50 log cells/mL feces in 8^{th} h ($P<0.01$) followed by a decrease afterwards till the end of fermentation ($P<0.01$). In *C. coccoides-E. rectale* group, lower counts than the general trend (~9 log cells/mL feces) were detected in 4^{th} (8.34 log cells/mL feces) and 10^{th} (8.20 log cells/mL feces) hours of fermentation ($P<0.01$) The highest difference in cell counts were detected in *Lactobacillus-Enterococcus* group, although the increase was less than 1 log cells/mL feces.

Table 4.1. Bacterial counts of 48 h fecal fermenter cultures in fermenter 1 (F1) in black tea added cultures (F1/R1) and control cultures (F1/R2), analyzed by FISH. Values are means ± SD of log cells/mL feces of experimental duplicates.

Bacterial group	**Sample type**	**Fermentation time (h)**						
		0	**4**	**8**	**10**	**24**	**30**	**48**
Bacteroides -Prevotella	BTA*	8.28±0.01	8.01±0.09	8.05±0.05	7.96±0.01	8.03±0.01	7.99±0.05	8.32±0.02
	Control	8.17±0.06	7.85±0.01	7.85±0.00	7.77±0.02	7.81±0.05	7.81±0.06	7.84±0.02
Bifidobacterium	BTA	8.18±0.02	8.10±0.01	8.50±0.01	8.19±0.03	8.02±0.04	8.34±0.05	8.34±0.03
	Control	8.12±0.05	8.08±0.01	8.40±0.05	8.17±0.02	8.27±0.05	8.330.01	8.33±0.03
C. coccoides-E. rectale	BTA	8.99±0.03	8.34±0.04	8.97±0.04	8.20±0.05	8.59±0.02	9.02±0.01	9.06±0.05
	Control	8.90±0.03	8.29±0.04	8.79±0.00	8.04±0.05	8.25±0.02	8.64±0.01	8.82±0.07
Lactobacillus-Enterococcus	BTA	8.18±0.02	8.22±0.02	8.12±0.02	8.31±0.01	8.18±0.01	8.33±0.01	8.10±0.13
	Control	8.02±0.01	7.83±0.03	7.42±0.02	7.80±0.00	7.43±0.10	7.78±0.01	7.35±0.04
Total bacteria	BTA	9.66±0.04	9.42±0.00	9.68±0.15	9.57±0.02	9.66±0.05	9.70±0.02	9.70±0.09
	Control	9.61±0.03	9.16±0.20	9.27±0.02	9.05±0.07	9.39±0.07	9.46±0.02	9.49±0.08

*** BTA=Black tea addition**

The FISH analysis result of second fermenter without addition of black tea extract (F2/R2) gave similar results for all bacterial groups and total bacteria counts. *C. coccoides-E. rectale* group numbers decreased significantly in 10 h period ($P<0.01$) which was followed by a significant increase till the end of fermentation. While the cell numbers of *Lactobacillus-Enterococcus* group flowed around 8 log cells/mL feces, cell numbers decreased significantly at 8^{th} (7.41 log cells/mL feces), 24^{th} (7.39 log cells/mL feces) and 48^{th} (7.28 log cells/mL feces) hours, similar to F1/R2 results ($P<0.01$). In total bacterial group, significant decrease ($P<0.01$) was observed in 4^{th} h compared to initial cell number. Total bacterial cells then increased significantly till the end of fermentation.

Table 4.2. Bacterial counts of 48 h fecal fermenter cultures in fermenter 2 (F2) in black tea added cultures (F2/R1) and control cultures (F2/R2), analyzed by FISH. Values are means ± SD of log cells/mL feces of experimental duplicates.

Bacterial group	**Sample type**	**Fermentation time (h)**						
		0	**4**	**8**	**10**	**24**	**30**	**48**
Bacteroides-Prevotella	BTA*	8.25±0.01	7.91±0.00	8.04±0.01	7.84±0.01	8.03±0.01	8.08±0.01	8.34±0.02
	Control	8.11±0.01	7.83±0.01	7.82±0.02	7.76±0.02	7.79±0.05	7.80±0.02	7.89±0.02
Bifidobacterium	BTA	8.19±0.01	8.11±0.04	8.46±0.00	8.14±0.01	7.96±0.01	8.29±0.02	8.39±0.08
	Control	8.02±0.00	8.00±0.02	8.39±0.04	8.05±0.07	8.10±0.02	8.15±0.06	8.26±0.02
C. coccoides – E. rectale	BTA	9.00±0.01	8.17±0.02	9.01±0.01	8.16±0.07	8.63±0.01	8.99±0.01	9.01±0.02
	Control	8.82±0.03	8.35±0.02	8.36±0.05	7.96±0.01	8.27±0.03	8.54±0.07	8.65±0.03
Lactobacillus-Enterococcus	BTA	8.13±0.04	8.16±0.04	8.08±0.05	8.29±0.03	8.16±0.01	8.30±0.00	8.01±0.03
	Control	8.00±0.12	7.75±0.01	7.41±0.01	7.74±0.06	7.39±0.04	7.75±0.03	7.28±0.08
Total bacteria	BTA	9.64±0.03	9.28±0.03	9.53±0.01	9.49±0.02	9.59±0.01	9.36±0.03	9.42±0.01
	Control	9.54±0.03	8.65±0.01	8.69±0.01	8.67±0.01	8.84±0.01	9.16±0.09	9.30±0.02

*** BTA=Black tea addition**

General pattern of bacterial groups in black tea added fermenter (F2/R1) was similar to that of control samples (F2/R2). Similar to F1/R1 samples, significantly lower counts than the general flow were observed in 4^{th} (8.17 log cells/mL feces) and 10^{th} h (8.16) of fermentation in *C. coccoides-E. rectale* group. Notably, *Bifidobacterium* group counts increased significantly till 8^{th} h which was followed by a significant decrease till 30^{th} h. Complementary to F1/R1 results, the highest difference in bacterial numbers were detected in *Lactobacillus-Enterococcus* group, although the difference between time points was less than 1 log cells/mL feces.

Total bacterial count displayed values between 10^9-10^{10} cells/mL feces. Cell count values of bacterial groups; *Bacteroides-Prevotella*, *Bifidobacterium*, *C. coccoides-E. rectale*, *Lactobacillus-Enterococcus*, varied between 10^7-10^9 cells/mL feces with *C. coccoides-E. rectale* group counts being the highest. The cell counts obtained from each fermenter replicates (n=2) were close to each other.

Representative images of FISH analysis are shown in Figures 4.5-4.9.

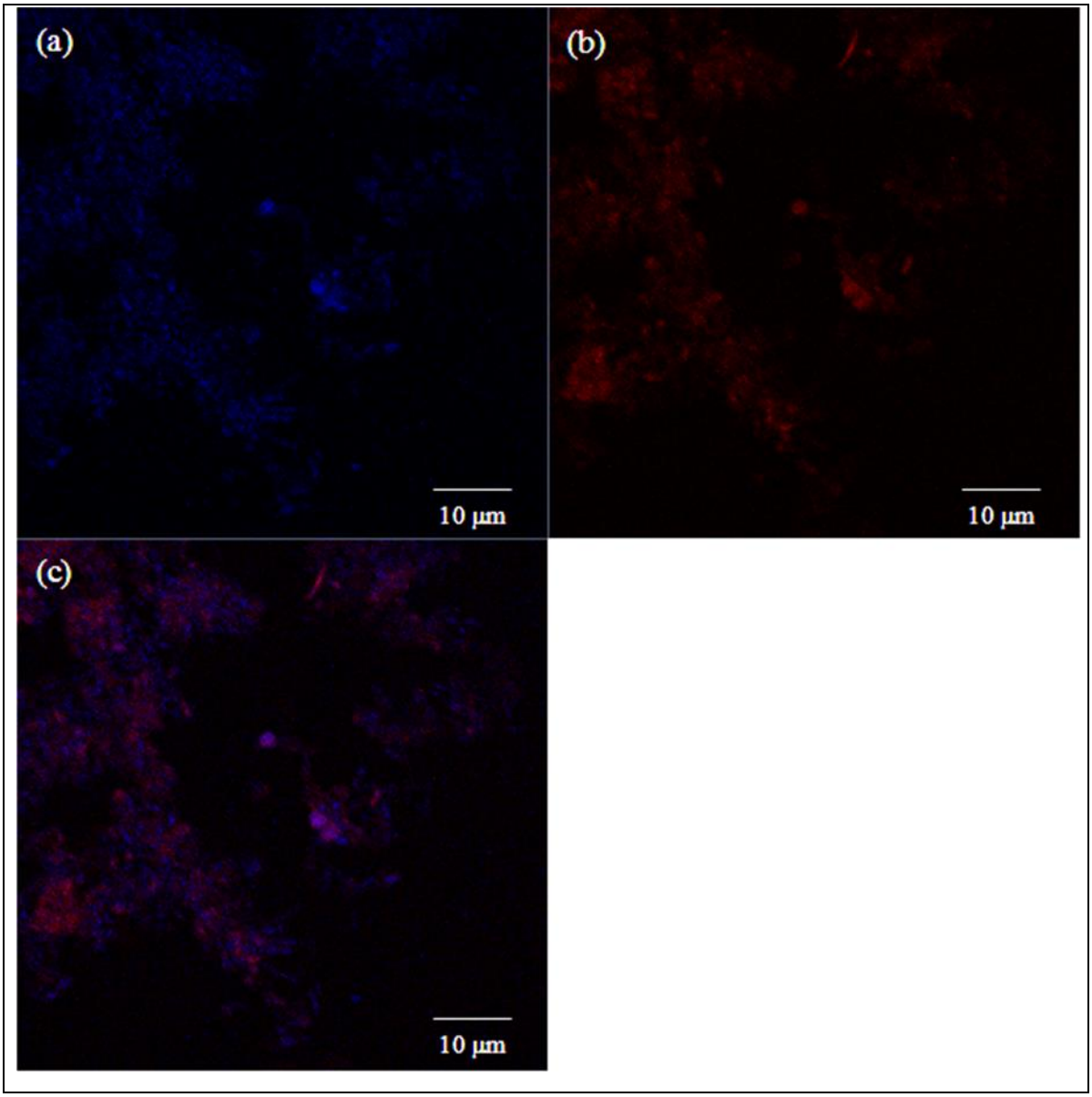

Figure 4.5. Confocal microscope image of *Bacteroides-Prevotella* spp (Bac 303) of fermentation samples at 100x magnification a: DAPI image, b: Cy3 image, c: Merged image of DAPI and Cy3

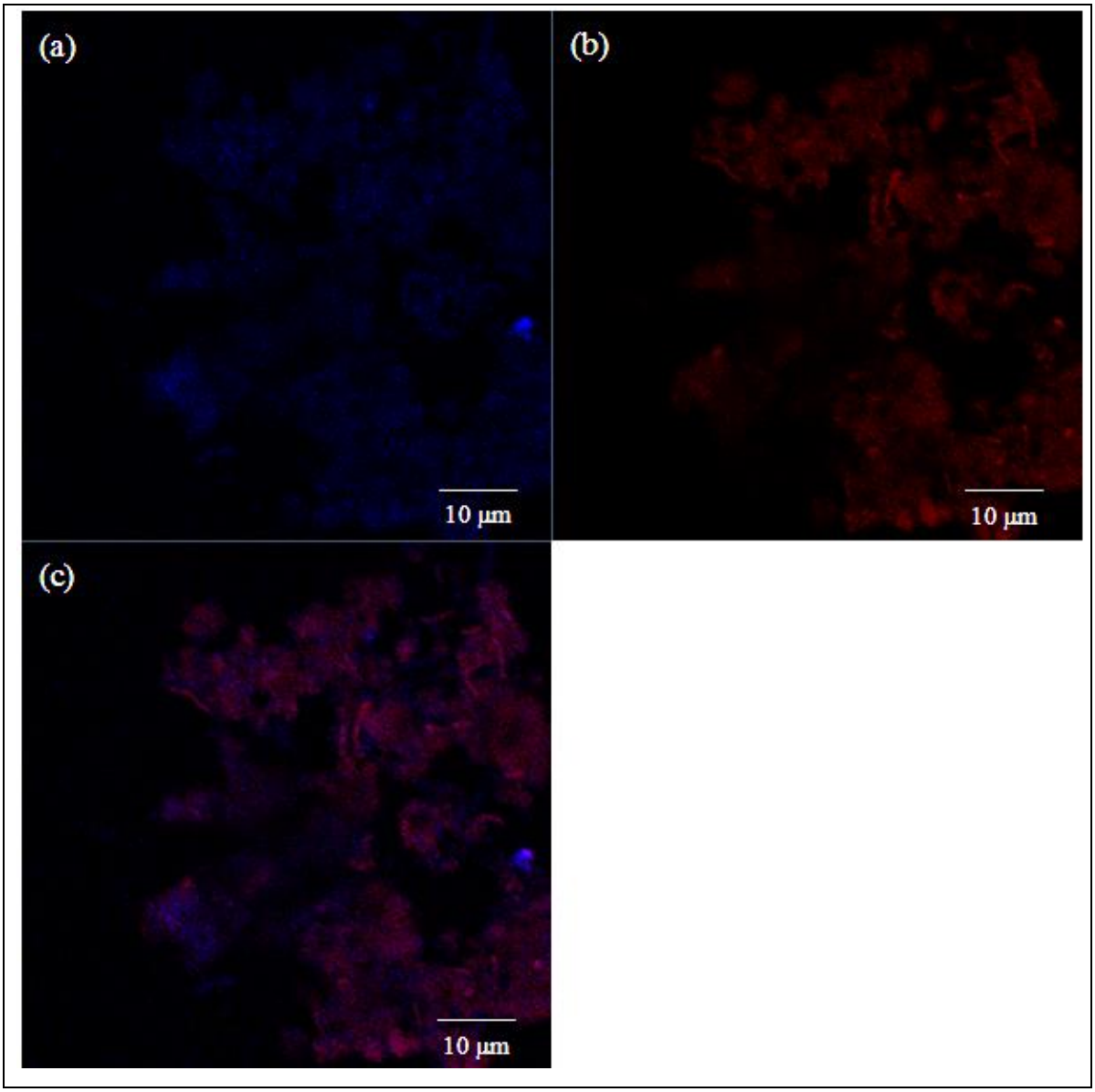

Figure 4.6. Confocal microscope image of *Bifidobacterium* spp (Bif 164) of fermentation samples at 100x magnification a: DAPI image, b: Cy3 image, c: Merged image of DAPI and Cy3

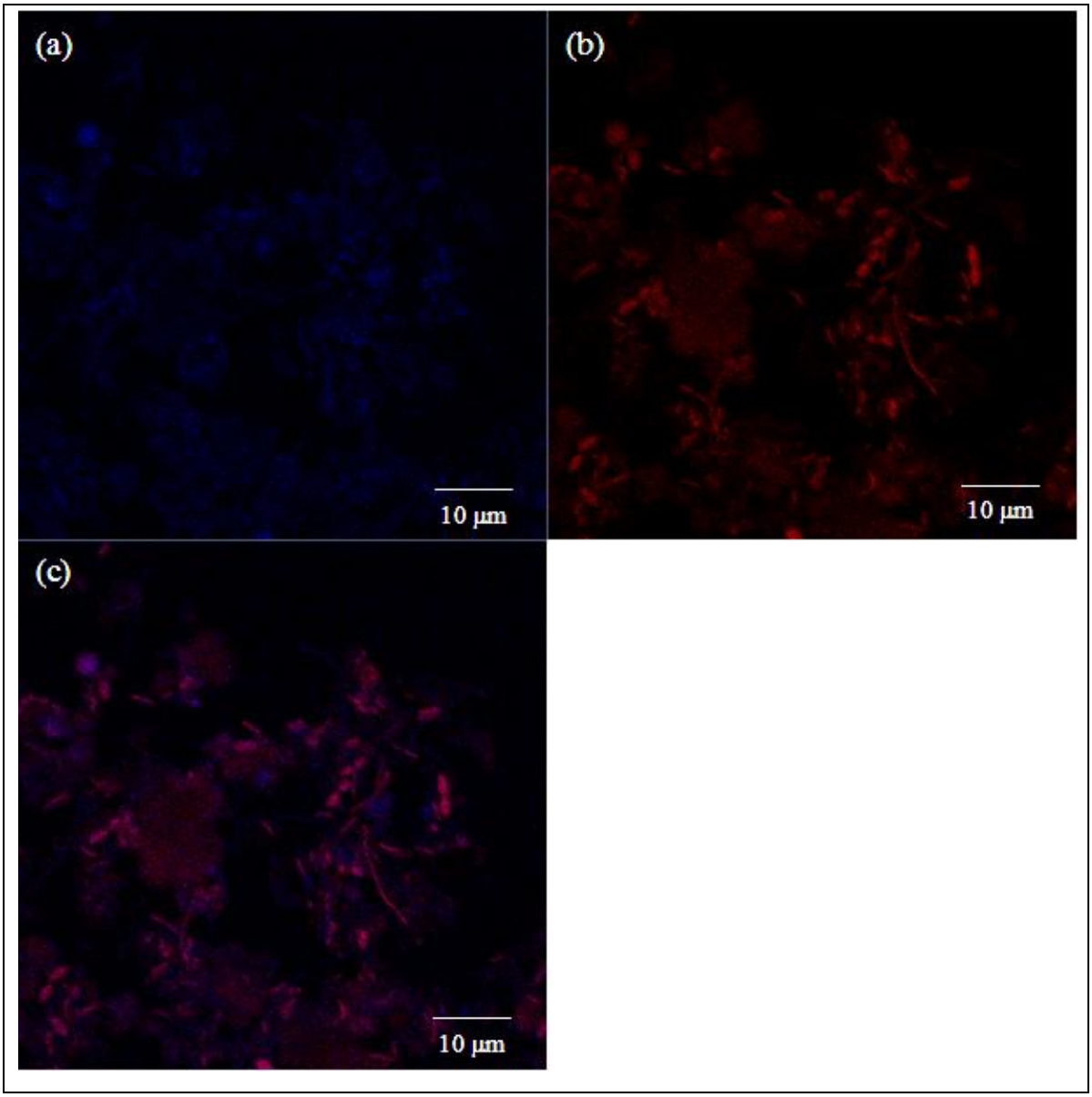

Figure 4.7. Confocal microscope image of *C. coccoides-E. rectale* group (Erec 482) of fermentation samples at 100x magnification a: DAPI image, b: Cy3 image, c: Merged image of DAPI and Cy3

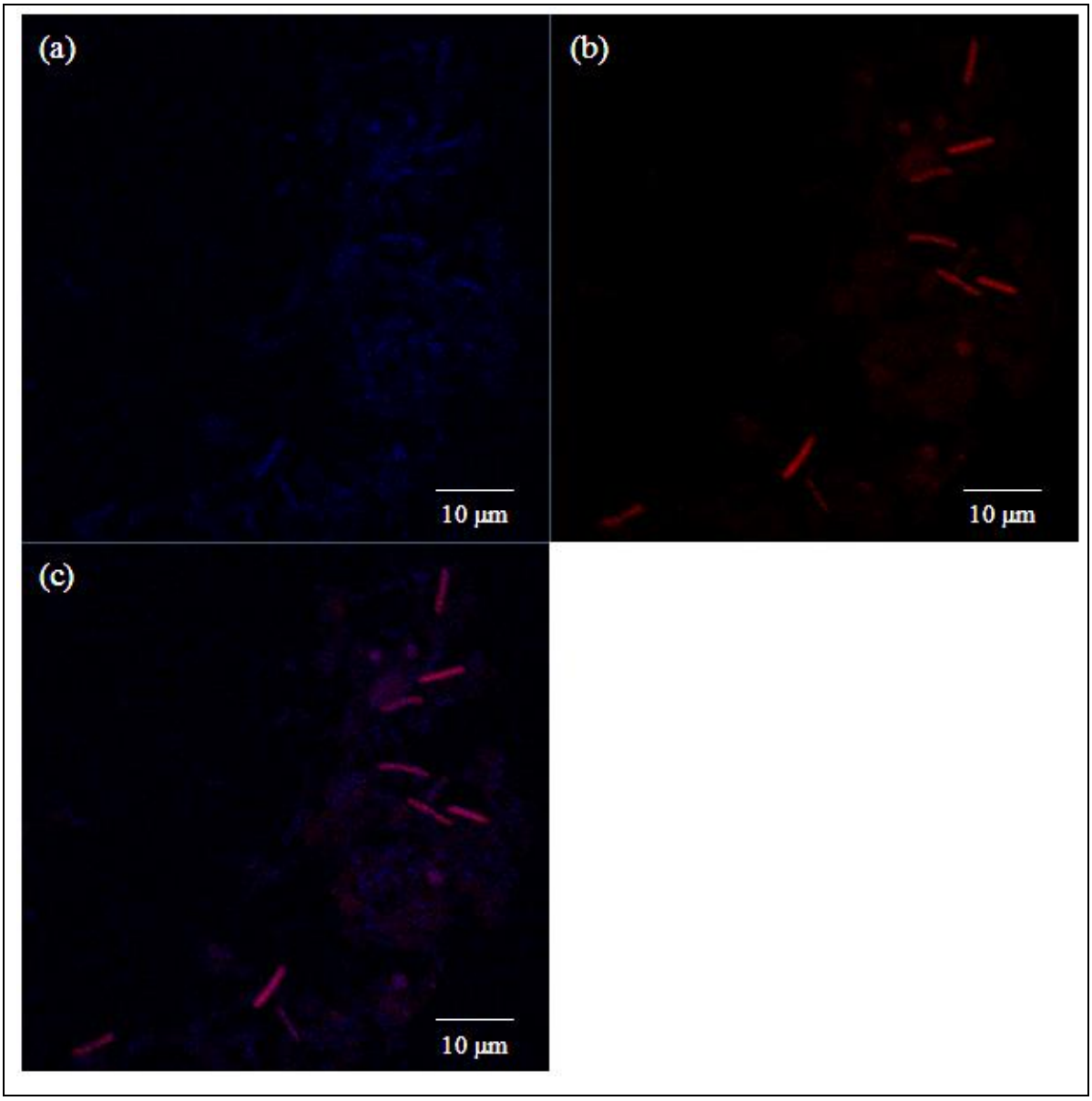

Figure 4.8. Confocal microscope image of *Lactobacillus−Enterococcus* spp (Lab 158) of fermentation samples at 100x magnification a: DAPI image, b: Cy3 image, c: Merged image of DAPI and Cy3

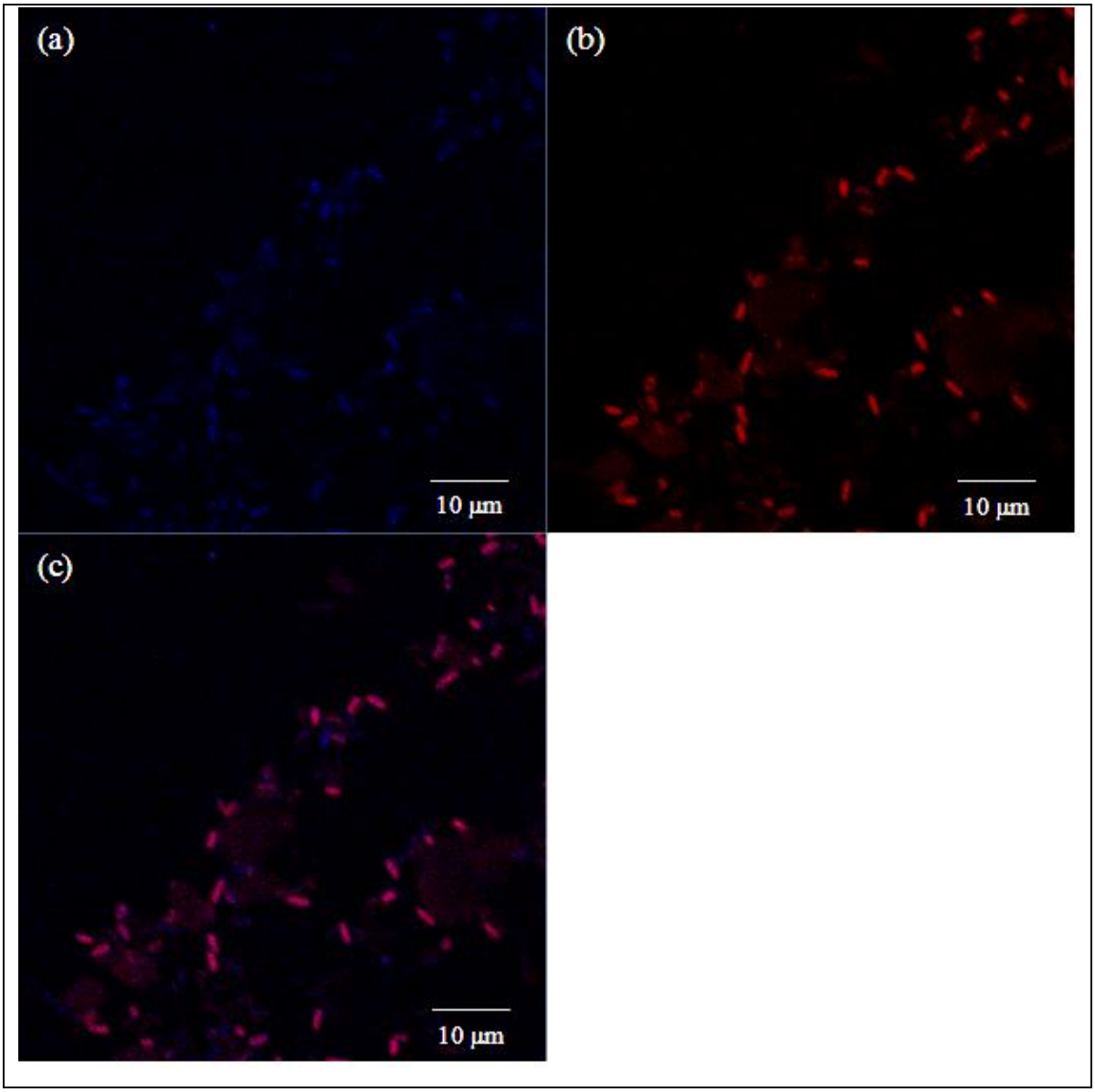

Figure 4.9. Confocal microscope image of total bacteria (EUB 338, EUB 338II, EUB 338III) of fermentation samples at 100x magnification a: DAPI image, b: Cy3 image, c: Merged image of DAPI and Cy3

4.2.2. Evaluation of FISH Analysis Results of All Fermenters

The mean values of fermenter samples for FISH results, calculated as ISB which indicates the changes relative to control were compared to interpret the effects of BTP on modulation of gut microbiota. Data were collected from two fermenters which were run at

different times with fecal samples of the same individual obtained right before fermentation.

FISH results showed that there was an increase in growth of *Bacteroides-Prevotella, C. coccoides-E. rectale, Lactobacillus-Enterococcus* groups and total bacterial count, and a decrease in growth of *Bifidobacterium* group in the presence of black tea compared to control in 48 h fecal batch fermentation. However, the results were significant only for *C. coccoides-E. rectale* and *Lactobacillus-Enterococcus* groups.

As indicated in figure 4.10., when R1 values were compared to R2 values at the same time point, significant increase was screened in all time points of *Lactobacillus-Enterococcus* group counts. Moreover, *Bacteroides-Prevotella* group cells showed a significant increase in 8^{th} & 48^{th} h and *C. coccoides-E. rectale* group cells showed a significant increase in 30^{th} h in R1 samples relative to control samples. Decline of bacterial population of *Bifidobacterium* group in R1 samples relative to control samples was observed to be significant in 24^{th} hour.

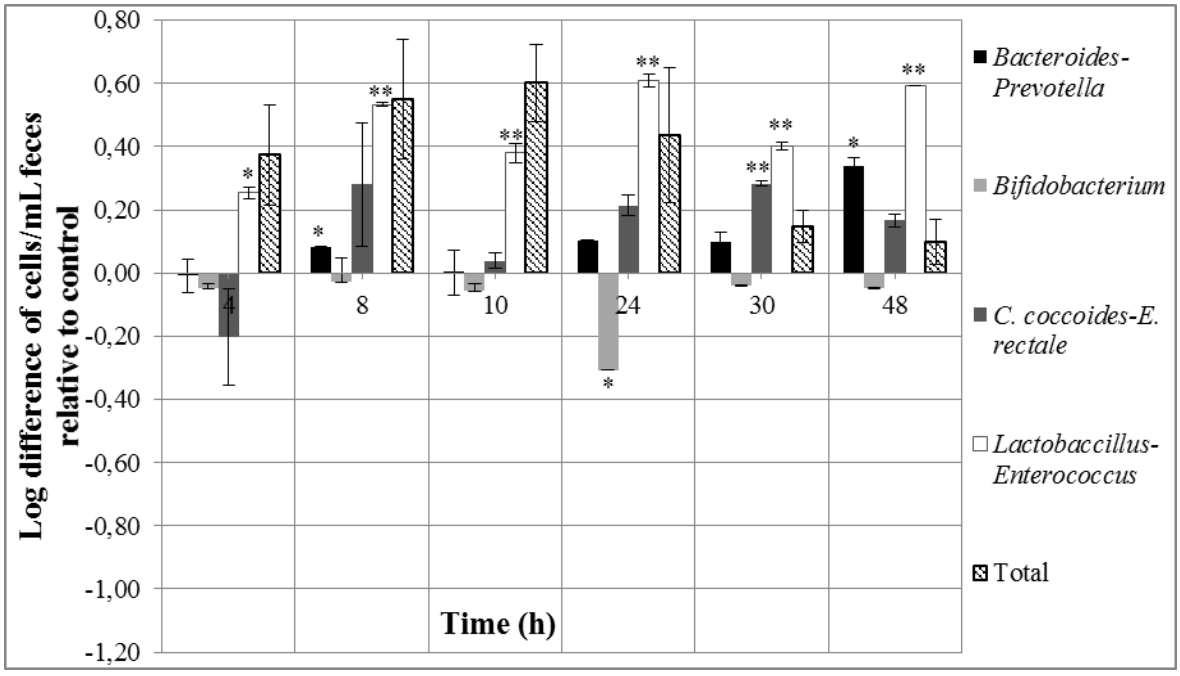

Figure 4.10. Changes in bacterial group numbers during 48 h black tea fermentation in a fecal batch culture analyzed by FISH. Values are means ± SEM of fermenter replicates (n=2). Mean value was significantly different from that of the control: *P<0.05, **P<0.01.

5. DISCUSSION

Black tea is one of the most consumed beverages and is the significant part of polyphenol intake worldwide. Recent studies about relations between tea polyphenols and gut microbiota focus on two-way interactions between phenolic compounds of tea and gut microbial population [9, 17, 56-58, 83]. Tea polyphenols (TP) need to undergo a wide range of bioconversions to be transformed into smaller compounds (aglycones) for easier absorption from intestinal barrier and bioconversion of tea polyphenols are conducted by several enzymatic reactions such as demethylation, isomerization, fission of certain molecule groups, deglycosylation and hydrolysis [9, 11, 13, 14, 65]. Some colonic bacteria have important roles on metabolism of phenolic compounds by releasing specific enzymes, that human are not able to secrete [2]. Meanwhile, formation of phenolic metabolites may alter specific bacterial composition levels, possibly linked to several beneficial health effects such as reducing cardiovascular risk, antimicrobial and anti-inflammatory properties and antidiarrheal effects [6, 9, 68]. Due to support of tea polyphenols on the growth of some intestinal bacterial groups, potential prebiotic effect of tea polyphenols especially with bifidogenic and lactogenic effects were studied in literature [6, 13, 14, 31, 44, 45].

In the present study, the effects of Turkish BTPs on gut microbiota of a Turkish individual was determined by using Q-PCR and FISH analysis of samples obtained from *in-vitro* fermentation systems by addition of water extracted BTP (1000 mg/L). The amount of black tea extract used at these conditions represent the way of black tea consumption and daily phenolic intake for humans [11, 72]. The phenolic content of the extract, used in the present study determined by Özcan [72], consisted of flavanols such as catechin (C) (14.32 mg/g dry extract), epicatechin (EC) (5.18 mg/g dry extract), epigallocatechin gallate (EGCG) (6.17 mg/g dry extract), epicatechin gallate (ECG) (1.14 mg/g dry extract), and gallocatechin gallate (GCG) (1.83 mg/g dry extract); phenolic acids such as gallic acid (GA) (11.36 mg/g dry extract), and theaflavins (TF) (2.17 % area).

The results obtained from Q-PCR and FISH analysis were calculated as index of specific bacteria (ISB) indicating the differences in bacterial numbers in presence of black tea

relative to control. While the mean values of three fermenter samples gathered by using Q-PCR analysis did not show any significant differences between the populations of bacterial groups in black tea added and control fermenter samples (Figure 4.4), the numbers of *C. coccoides-E. rectale* (30 h) and *Lactobacillus-Enterococcus* groups in black tea supplemented cultures increased significantly ($P<0.01$) relative to control cultures of the same samples in FISH analysis (Figure 4.10). Although FISH analysis results showed that there was a significant increase in the growth of *Bacteroides-Prevotella* group at 8^{th} and 48^{th} h of incubation in black tea added samples compared to control samples, the change in the numbers were not significant at other fermentation points ($P>0.05$). On the other hand, *Bifidobacterium* group showed slight decrease in the presence of black tea extract, however, the results were not significant ($P>0.05$) except a significant decrease at 24^{th} h ($P<0.05$) in FISH analysis. In both analyses, no significant differences were observed in total bacterial counts ($P>0.05$) with the addition of black tea extract.

Addition of black tea extract stimulated the growth of *Lactobacillus* and *Enterococcus* groups according to results of both Q-PCR and FISH analysis. Although the results of Q-PCR in black tea extract added samples showed slight stimulation in the numbers of *Lactobacillus* group after 8, 10, 24 & 30 h incubation and only after 30 h incubation for *Enterococcus* group ($P>0.05$), the increase in *Lactobacillus-Enterococcus* groups according to FISH analysis was significant at all time points (4, 8, 10, 24, 30 & 48 h) compared to the numbers in control samples ($P<0.05$).

The obtained data was also correlated with the results of Özcan [72] in which the metabolites of the same fermenter samples were analyzed by HPLC and GC. In the study of Özcan, the results of HPLC analysis of the same samples showed that the concentrations of C, EGCG, EC, ECG, GA, and TF were significantly degraded after 8, 10 and 24 h of incubation compared to control samples, suggesting that these polyphenols might be utilized by fecal bacteria present in fermenter.

Previous studies showed that the effect of polyphenols on gut microbiota was highly variable among individuals. The reason of this variability was stated as the large inter-individual variation in gut bacterial composition, causing variations of gut microbial groups in response to a polyphenol intervention [11, 13, 31, 56, 84]. Besides inter-

individual differences in gut bacterial composition, composition & concentration and exposure time of polyphenols have been considered as parameters for variable effects of polyphenols on gut microbiota [9, 11, 13, 24, 68, 84].

The stimulation of *Lactobacillus-Enterococcus* group in the presence of phenolic compounds was reported in many *in-vitro* fermenter gut microbiota studies in literature [13, 14, 46]. EGCG, GCG, EGCG3"Me isolated from oolong tea samples stimulated the growth of *Lactobacillus-Enterococcus* group analyzed by FISH analysis after 24 h of fecal fermentation suggesting that oolong tea polyphenols and their metabolites might have showed prebiotic-like activity on *Lactobacillus-Enterococcus* group, by reaching to colon and possibly affecting the growth [14, 44, 85]. Similar results were also reported in the studies of Cueva et al. [13] and Bialonska et al. [46], where gut microbiota analyses were conducted by FISH. Grape seed flavan-3-ol monomer (GSE-M) and oligomer (GSE-O) fractions (600 mg/L each) were degraded completely by gut microbiota after 10-24 h of fermentation parallel to increase in the growth of *Lactobacillus-Enterococcus* group in i*n-vitro* batch cultures compared to control samples suggesting that grape seed extracts of different flavan-3-ol profile might have a potency to stimulate the growth of *Lactobacillus-Enterococcus* group [13]. Also, i*n-vitro* batch culture fermentation of pomegranate by-product (1.5 mL) lead to higher numbers of *Lactobacillus-Enterococcus* group compared to control samples proposing that stimulation in the growth of *Lactobacillus-Enterococcus* group in the presence of pomegranate by-products might be due to decomposition of pomegranate metabolites by specific strains of *Lactobacillus* species [46].

Stimulation of *Lactobacillus-Enterococcus* group was observed in *in-vivo* human and animal intervention studies as well [20, 23, 86, 87]. Polyphenols from red wine (50 mg/kg), green tea (300 mg), grape pomace concentrate (60 g/kg) and the extracts (7.2 g/kg) of grape seed and blackcurrant (13.4 mg/kg) enhanced the growth of *Lactobacillus-Enterococcus* group compared to placebo treatment in rat, pig and human feces, broiler chick cecal digesta and rat cecal digesta, respectively. All studies suggested that polyphenols might have positive effects on *Lactobacillus-Enterococcus* growth as polyphenol metabolites formed by enzymes, such as β-glucosidase, secreted by *Lactobacillus* and *Enterococcus* groups might act as growth precursors for *Lactobacillus* and *Enterococcus* groups.

On the other hand, addition of red wine polyphenols (0.6 mg/mL and 272 mL/day, respectively) in *in-vitro* fecal batch fermentation [31] and *in-vivo* human intervention [24] studies lead no significant results on the growth of *Lactobacillus* group compared to control fermenter culture and placebo treatment according to results of FISH and Q-PCR analysis, respectively.

Like *Lactobacillus-Enterococcus* group, addition of black tea extract stimulated the growth of *C. coccoides-E. rectale* group according to results of FISH analysis, although Q-PCR analysis did not reveal any significant result in the numbers of *C. coccoides* group in this study. The increase in *C. coccoides-E. rectale* group obtained by FISH analysis was significant at 30 h compared to the numbers in control samples ($P<0.01$). Since *E. rectale* groups was analyzed in addition to *C. coccoides* group in FISH, where only *C. coccoides* group was analyzed in Q-PCR, differences in results between FISH and Q-PCR might be due to the significant increase in *E. rectale* group. Also, in Q-PCR analysis the primer design is an important parameter that has to be taken into account since it might affect primer specificity and hybridization efficiency, resulting biases in quantitative assessments of bacterial groups [33, 36].

Studies suggested that polyphenols might have an ability to influence the growth of specific gut bacteria by metabolic transformations of phenolics by the enzymes of gut bacteria. *Clostridium and E. rectale* groups are known to display enzymatic activities for hydrolysis, demethylation and fission of different polyphenol compounds such as C and EC [9, 45, 88]. So, degradation products of C, EGCG, EC, ECG and TF during fermentation [72] might be the reason of the stimulated numbers of *Clostridium and E. rectale* groups in BTP added fermenters in our study suggesting that BTP might act as precursors on the growth these groups.

Similar to our results, addition of (+)-catechin (1000 mg/L) stimulated the growth of *C. coccoides-E. rectale* group after analysis of fecal batch cultures (17 h) by FISH analysis parallel to degradation of (+)-catechin at the same time period resulting that (+)-catechin might act as a precursor on the growth of *C. coccoides-E. rectale* group [45]. Comparable results were reported in *in-vivo* human intervention study of Queipo-Ortuno et al. [24] and animal study of Viveros et al. [23], where the effects of 797.86 mg/dose red wine

polyphenols (containing C, EC and GA) and 60 g/kg grape seed-grape pomace concentrate (containing 48.7 extractable polyphenols and 26.6 hydrolysable polyphenols) were analyzed by Q-PCR and plate counting methods, respectively.

However, controversial results are also present in literature both in *in-vitro* and *in-vivo* human and animal intervention studies [11, 14, 20, 23, 44, 46, 47, 85]. While polyphenols from black tea (1000 mg/day containing C, GA and TF) [11], oolong tea (EGCG, GCG, EGCG3"Me) [14], cocoa (494 mg/day containing flavanols) [44], red wine (50 mg/kg containing C and EC) [86] and the extracts of red wine grape extract (1000 mg/day containing C) [11], blackcurrant extract (30 g/day) [20], grape seed (60 g/kg containing (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-O-gallate) [23] inhibited the growth of *C. coccoides* and *C. coccoides-E. rectale* groups compared to control treatments according to results of Q-PCR, FISH and plate counting analysis. FISH analyses revealed that polyphenols from black tea [47] and pomegranate by-product (1.5 mL) [46] had no effects on the growth of *C. coccoides* and *C. coccoides-E. rectale* groups. The reasons of controversial results might be due to the differences in polyphenol composition and concentration, and inter-individual differences in gut bacterial composition of the studies [9, 11, 13, 24].

Different than *Lactobacillus-Enterococcus* and *C. coccoides-E. rectale* groups, the numbers of *Bifidobacterium* group decreased in black tea added fermenter samples relative to control both in Q-PCR and FISH analysis results, however the decrease in numbers were not significant ($P>0.05$).

Although *Bifidobacterium* species are generally known to metabolize short chain fatty acids by the activity of glycosyl hydrolases such as β-glycosidase [89], recently the capacity of *Bifidobacterium* species to metabolize polyphenols have been studied by many researchers in *in-vitro* and *in-vivo* human and animal intervention gut microbiota studies [11, 14, 20, 24, 30, 44, 47]. While the stimulated effects of polyphenols from different sources on the growth of *Bifidobacterium* group in concentration dependent manner have been reported [14, 20, 24, 30, 44, 45, 46], Q-PCR analysis of *in-vitro* continuous fermentation (SHIME) of fecal cultures with black tea extract or red wine grape extract (1000 mg/day each, containing C, GA and TF) showed that in the presence of both

extracts, *Bifidobacterium* group were significantly inhibited compared to non-treated samples suggesting that BTP as well as red wine grape extract might have showed inhibitive action to *Bifidobacterium* group [11]. Moreover, plate counting results of GSE (337 mg/g), GSE-M (414 mg/g), and GSE-O (279 mg/g) fractions showed inhibitive effects on certain *Bifidobacterium* species [90]. Concentration dependent antimicrobial activities of polyphenols have been stated in many studies with different mechanisms of action [9, 68, 71, 84, 91, 92]. TP can disturb cell membrane function, inhibit glucose cell transport or lead iron deficiency in environment by forming polyphenol-metal ion complexes. Especially, high amount of gallate derived flavan-3-ols such as ECG, as gallolated polyphenols have been observed to have higher antimicrobial activity than non-gallolated ones [91, 92]. So, the decrease in the number of *Bifidobacterium* group in the presence of black tea extract in our study might also be related with the inhibitory activity of phenolic compounds.

The mean values of fermenter samples did not conclude any significant change in any of the time points of *Bacteroides, Prevotella* and *Porphyromonas* group from Q-PCR analysis in the presence of BTP compared to control ($P>0.05$), while *Bacteroides-Prevotella* group in FISH analysis showed significant increase in 8 h and 48 h of fermentation when black tea extract was added ($P<0.05$).

Even though it is mostly known that in human gut, the growth of *Bacteroides* group is stimulated in protein and fat-high diet and the growth of *Prevotella* group is stimulated in carbohydrate-high diet [8], there are also studies indicating that *Bacteroides* and *Prevotella* groups are capable of metabolizing polyphenols by several enzymatic reactions [9, 88]. In the study of Queipo-Ortuno [24], red wine polyphenols stimulated the growth of *Bacteroides-Prevotella* group in *in-vivo* human intervention study where gut microbiota analysis was conducted by Q-PCR suggesting that red wine polyphenols might act as prebiotics on the growth of *Bacteroides-Prevotella* group [24]. Controversially, polyphenols of oolong tea [14] and Yunnan Chinese tea extract [84], red wine grape extract [11], blackcurrant extract [20], pomegranate by-product [46] as well as gallic acid [30] inhibited the growth of *Bacteroides* and *Prevotella* groups compared to control in gut microbiota studies suggesting that polyphenols may inhibit potential harmful or pathogen

bacteria such as *Bacteroides* and *Prevotella* group, but the mechanism has not been well studied.

On the other hand, several *in-vitro* and *in-vivo* human and animal intervention gut microbiota studies also concluded that phenolic compounds from different sources [13, 31, 44, 47, 56, 85] did not have any significant effects on *Bacteroides* and *Prevotella* groups compared to control groups. The variation between results might be due to the proportion of polyphenols or time of exposure as the studied amounts of polyphenols or exposure time might not have been enough to make significant changes in the numbers of *Bacteroides* and *Prevotella* groups [31, 56].

In this study, the growth of *Atopobium* and *Enterobacteriaceae* groups did not change by black tea exposure during 48 h fecal fermentation compared to control, according to Q-PCR analysis. There is limited knowledge about the effects of phenolic compounds on the growth of *Atopobium* and *Enterobacteriaceae* groups since those bacterial groups are not yet well studied. According to an *in-vivo* human intervention study, 2-weeks of black tea consumption, with unknown amount of phenolic compounds, lead no changes in the growth of *Atopobium* group as well as *Enterobacteriaceae* group compared to placebo treatments, where gut microbiota analysis was conducted by FISH [47]. Another *in-vivo* human intervention gut microbiota study performed by multiplex PCR concluded that 12 weeks of green tea consumption, in which daily consumption of EGCG was more than 0.56 g and C was more than 1.35 g, lead no changes in the growth of *Actinobacteria* group and *Proteobacteria* group which are the higher taxa of *Atopobium* group and *Enterobacteriaceae* group, respectively [56]. On the other hand, stimulation of *Atopobium* group with 1000 mg/L of gallic acid supplementation was observed by FISH analysis in 24 h of fecal batch fermentation culture compared to control culture suggesting that gallic acid might act positively on the growth of *Atopobium* group [30]. Although it was known that *Actinobacteria* group can ferment catechin-type polyphenols, the role of *Atopobium* group in the gut and interaction of *Atopobium* group species with polyphenols is not clear [30, 70].

Finally, both of our Q-PCR and FISH results revealed that BTP exposure did not show any significant effects on total bacterial count in any of the time points during 48 h

fermentation. Except *in-vitro* studies of Hidalgo et al. [30] and Bialonska et al. [46], where gallic acid and polyphenols of pomegranate by-product stimulated the growth of total bacteria compared to control; other *in-vitro* studies are in agreement with our results [11, 13, 14, 31]. The stability in the numbers of total bacterial group might be explained by a possible energy balance in gut microbial composition, as overall gut microbial composition is generally known to be quite stable despite changes in numbers of certain bacterial groups [56].

6. CONCLUSION

In this study, the effects of BTP on human intestinal microflora was investigated in *in-vitro* batch fecal fermenter cultures. Q-PCR and FISH were performed to quantify the bacterial groups and analyze bacterial composition in fermenter cultures. While literature data mostly confirm our results, some inconsistencies are also present between literature and obtained results. Differences of our results from the present studies might be due to polyphenol composition, plant sources of polyphenols and exposure time of gut bacterial population to polyphenol compounds. Also, inter-individual variations of gut microbial composition might result in differences of gut microbial groups in response to a polyphenol exposure [11, 13, 14, 31, 56].

Results of the study indicated that BTP might have a potential to modulate gut microbiota. Although Q-PCR results did not show any significant effects in the presence of black tea compared to control, FISH analysis revealed that BTP increased the numbers of *Lactobacillus* & *Enterococcus* and *E. rectale-C. coccoides* groups in the presence of BTP compared to control.

Similar data were obtained when Q-PCR and FISH results were compared, suggesting that that both Q-PCR and FISH are applicable methods to enumerate gut microbial population. However, variations in bacterial numbers were observed in some of the bacterial groups when Q-PCR analysis was compared to FISH analysis. This variation might be due to biases in Q-PCR analysis where amplification of environmental DNA including DNAs of dead bacterial cells lead to over-estimation of Q-PCR results. On the other hand, the variation may be due to biases in FISH analysis, since FISH probes target rDNA in the cells and small cells or DNA might lower the hybridization efficiency, leading underestimated data. Moreover, variation of fluorescent signals between bacterial groups due to different environmental conditions and light intensities of different bacterial probes in FISH analysis might make the results more flexible. Storage conditions and storage time for fixed cells for FISH analysis also should have been taken into consideration as those parameters might affect stable cell numbers and probe affinities of cells in FISH analysis, leading biased results.

As a conclusion, this study provided valuable information about the impacts of Turkish BTP on bacterial groups of a Turkish individual in *in-vitro* batch fermentation cultures. However, mechanisms of black tea polyphenols and their degradation products on modulation of gut microbiota is still not clear and needs to be investigated in future studies such as *in-vivo* human intervention studies followed by metagenomic and metabolomic analyses. Unlike our study where fermentation was performed with fecal samples obtained from single person, further studies should be performed with fecal samples obtained from larger numbers of volunteers.

REFERENCES

1. A. W. Walker, S. H. Duncan, P. Louis and H. J. Flint. Phylogeny, Culturing, and Metagenomics of the Human Gut Microbiota. *Trends in Microbiology*, 22:267-274, 2014.

2. G. R. Gibson and M. B. Roberfroid. *Colonic Microbiota, Nutrition and Health*, Kluwer Academic, Dordecht, 1999.

3. S. E. Power, P. W. O'Toole, C. Stanton, R. P. Ross and G. F. Fitzgerald. Intestinal Microbiota, Diet and Health. *British Journal of Nutrition*, 111:387-402, 2014.

4. S. Macfarlane and G. T. Macfarlane. Bacterial Diversity in the Human Gut. *Advances in Applied Microbiology*, 54:261-289, 2004.

5. A. N. Payne, A. Zihler, C. Chassard and C. Lacroix. Advances and Perspectives in in Vitro Human Gut Fermentation Modeling. *Trends in Biotechnology*, 30:17-25, 2012.

6. D. Hervert-Hernández and I. Goñi. Dietary Polyphenols and Human Gut Microbiota: A Review. *Food Reviews International*, 27:154-169, 2011.

7. J. M. Laparra and Y. Sanz. Interactions of Gut Microbiota with Functional Food Components and Nutraceuticals. *Pharmacological Research*, 61:219-225, 2010.

8. C. De Filippo, D. Cavalieri, M. Di Paola, M. Ramazzotti, J. B. Poullet, S. Massart, S. Collini, G. Pieraccini and P. Lionetti. Impact of Diet in Shaping Gut Microbiota Revealed by a Comparative Study in Children from Europe and Rural Africa. *Proceedings of the National Academy of Science USA*, 107:14691-14696, 2010.

9. M. V. Selma, J. C. Espin and F. A. Tomas-Barberan. Interaction between Phenolics and Gut Microbiota: Role in Human Health. *Journal of Agricultural and Food Chemistrty*, 57:6485-6501, 2009.

10. S. Maccaferri, B. Vitali, A. Klinder, S. Kolida, M. Ndagijimana, L. Laghi, F. Calanni, P. Brigidi, G. R. Gibson and A. Costabile. Rifaximin Modulates the Colonic Microbiota of Patients with Crohn's Disease: An in vitro Approach Using a Continuous Culture Colonic Model System. *Journal of Antimicrobial Chemotherapy*, 65:2556-2565, 2010.

11. R. A. Kemperman, G. Gross, S. Mondot, S. Possemiers, M. Marzorati, T. Van de Wiele, J. Doré and E. E. Vaughan. Impact of Polyphenols from Black Tea and Red Wine/Grape Juice on a Gut Model Microbiome. *Food Research International*, 53:659-669, 2013.

12. E. Likotrafiti, K. M. Tuohy, G. R. Gibson and R. A. Rastall. An in Vitro Study of the Effect of Probiotics, Prebiotics and Synbiotics on the Elderly Faecal Microbiota. *Anaerobe*, 27:50-55, 2014.

13. C. Cueva, F. Sanchez-Patan, M. Monagas, G. E. Walton, G. R. Gibson, P. J. Martin-Alvarez, B. Bartolome and M. V. Moreno-Arribas. In Vitro Fermentation of Grape Seed Flavan-3-Ol Fractions by Human Faecal Microbiota: Changes in Microbial Groups and Phenolic Metabolites. *FEMS Microbiology Ecology*, 83:792-805, 2013.

14. X. Zhang, X. Zhu, Y. Sun, B. Hu, Y. Sun, S. Jabbar and X. Zeng. Fermentation in Vitro of Egcg, Gcg and Egcg3"Me Isolated from Oolong Tea by Human Intestinal Microbiota. *Food Research International*, 54:1589-1595, 2013.

15. J. Vermeiren, P. Van den Abbeele, D. Laukens, L. K. Vigsnaes, M. De Vos, N. Boon and T. Van de Wiele. Decreased Colonization of Fecal Clostridium Coccoides/Eubacterium Rectale Species from Ulcerative Colitis Patients in an In-Vitro Dynamic Gut Model with Mucin Environment. *FEMS Microbiology Ecology*, 79:685-696, 2012.

16. M. Aguirre, A. Eck, M. E. Koenen, P. H. Savelkoul, A. E. Budding and K. Venema. Diet Drives Quick Changes in the Metabolic Activity and Composition of Human Gut Microbiota in a Validated in Vitro Gut Model. *Research in Microbiology*, 167:114-125, 2016.

17. C. Cueva, A. Jiménez-Girón, I. Muñoz-González, A. Esteban-Fernández, I. Gil-Sánchez, M. Dueñas, P. J. Martín-Álvarez, M. A. Pozo-Bayón, B. Bartolomé and M. V. Moreno-Arribas. Application of a New Dynamic Gastrointestinal Simulator (Simgi) to Study the Impact of Red Wine in Colonic Metabolism. *Food Research International*, 72:149-159, 2015.

18. J. Kabeerdoss, R. S. Devi, R. R. Mary and B. S. Ramakrishna. Faecal Microbiota Composition in Vegetarians: Comparison with Omnivores in a Cohort of Young Women in Southern India. *British Journal of Nutrition*, 108:953-957, 2012.

19. M. Massot-Cladera, T. Perez-Berezo, A. Franch, M. Castell and F. J. Perez-Cano. Cocoa Modulatory Effect on Rat Faecal Microbiota and Colonic Crosstalk. *Archives Biochemistry and Biophysics*, 527:105-112, 2012.

20. A.-L. Molan, Z. Liu and M. Kruger. The Ability of Blackcurrant Extracts to Positively Modulate Key Markers of Gastrointestinal Function in Rats. *World Journal of Microbiology and Biotechnology*, 26:1735-1743, 2010.

21. A. H. Smith and R. I. Mackie. Effect of Condensed Tannins on Bacterial Diversity and Metabolic Activity in the Rat Gastrointestinal Tract. *Applied and Environmental Microbiology*, 70:1104-1115, 2004.

22. Y. Y. Choy, P. Quifer-Rada, D. M. Holstege, S. A. Frese, C. C. Calvert, D. A. Mills, R. M. Lamuela-Raventos and A. L. Waterhouse. Phenolic Metabolites and Substantial Microbiome Changes in Pig Feces by Ingesting Grape Seed Proanthocyanidins. *Food and Function*, 5:2298-2308, 2014.

23. A. Viveros, S. Chamorro, M. Pizarro, I. Arija, C. Centeno and A. Brenes. Effects of Dietary Polyphenol-Rich Grape Products on Intestinal Microflora and Gut Morphology in Broiler Chicks. *Poultry Science*, 90:566-578, 2011.

24. M. I. Queipo-Ortuno, M. Boto-Ordonez, M. Murri, J. M. Gomez-Zumaquero, M. Clemente-Postigo, R. Estruch, F. Cardona Diaz, C. Andres-Lacueva and F. J. Tinahones.

Influence of Red Wine Polyphenols and Ethanol on the Gut Microbiota Ecology and Biochemical Biomarkers. *American Journal of Clinical Nutrition*, 95:1323-1334, 2012.

25. J. Zimmer, B. Lange, J. S. Frick, H. Sauer, K. Zimmermann, A. Schwiertz, K. Rusch, S. Klosterhalfen and P. Enck. A Vegan or Vegetarian Diet Substantially Alters the Human Colonic Faecal Microbiota. *European Journal of Clinical Nutrition*, 66:53-60, 2012.

26. A. Scalbert and G. Williamson. Dietary Intake and Bioavailability of Polyphenols. *The Journal of Nutrition*, 130:2073-2085, 2000.

27. S. Roowi, A. Stalmach, W. Mullen, M. E. Lean, C. A. Edwards and A. Crozier. Green Tea Flavan-3-Ols: Colonic Degradation and Urinary Excretion of Catabolites by Humans. *Journal of Agricultural and Food Chemistrty*, 56:1296-1304, 2010.

28. J. J. van der Hooft, R. C. de Vos, V. Mihaleva, R. J. Bino, L. Ridder, N. de Roo, D. M. Jacobs, J. P. van Duynhoven and J. Vervoort. Structural Elucidation and Quantification of Phenolic Conjugates Present in Human Urine after Tea Intake. *Analytical Chemistry*, 84:7263-7271, 2012.

29. A. M. Aura, I. Mattila, T. Hyotylainen, P. Gopalacharyulu, V. Cheynier, J. M. Souquet, M. Bes, C. Le Bourvellec, S. Guyot and M. Oresic. Characterization of Microbial Metabolism of Syrah Grape Products in an in Vitro Colon Model Using Targeted and Non-Targeted Analytical Approaches. *European Journal of Nutrition*, 52:833-846, 2013.

30. M. Hidalgo, M. J. Oruna-Concha, S. Kolida, G. E. Walton, S. Kallithraka, J. P. Spencer and S. de Pascual-Teresa. Metabolism of Anthocyanins by Human Gut Microflora and Their Influence on Gut Bacterial Growth. *Journal of Agricultural and Food Chemistrty*, 60:3882-3890, 2012.

31. F. Sanchez-Patan, C. Cueva, M. Monagas, G. E. Walton, G. R. Gibson, J. E. Quintanilla-Lopez, R. Lebron-Aguilar, P. J. Martin-Alvarez, M. V. Moreno-Arribas and B. Bartolome. In Vitro Fermentation of a Red Wine Extract by Human Gut Microbiota:

Changes in Microbial Groups and Formation of Phenolic Metabolites. *Journal of Agricultural and Food Chemistrty*, 60:2136-2147, 2012.

32. G. Xie, A. Zhao, L. Zhao, T. Chen, H. Chen, X. Qi, X. Zheng, Y. Ni, Y. Cheng, K. Lan, C. Yao, M. Qiu and W. Jia. Metabolic Fate of Tea Polyphenols in Humans. *Journal of Proteome Research*, 11:3449-3457, 2012.

33. M. H. Fraher, P. W. O'Toole and E. M. Quigley. Techniques Used to Characterize the Gut Microbiota: A Guide for the Clinician. *Nature Reviews Gastroenterology Hepatology*, 9:312-322, 2012.

34. P. Namsolleck, R. Thiel, P. Lawson, K. Holmstrøm, M. Rajilic, E. E. Vaughan, L. Rigottier-Gois, M. D. Collins, W. M. de Vos and M. Blaut. Molecular Methods for the Analysis of Gut Microbiota. *Microbial Ecology in Health and Disease*, 16:71-85, 2004.

35. K. Sivieri, M. L. Morales, S. M. Saad, M. A. Adorno, I. K. Sakamoto and E. A. Rossi. Prebiotic Effect of Fructooligosaccharide in the Simulator of the Human Intestinal Microbial Ecosystem (Shime(R) Model). *Journal of Medical Food*, 17:894-901, 2014.

36. G. Rastogi and R. K. Sani. Molecular Techniques to Assess Microbial Community Structure, Function, and Dynamics in the Environment. In: I. Ahmad, F. Ahmad and J. Pichtel, editors, *Microbes and Microbial Technology*, pages 28-57. Springer New York, New York, 2011.

37. J. Gong and C. Yang. Advances in the Methods for Studying Gut Microbiota and Their Relevance to the Research of Dietary Fiber Functions. *Food Research International*, 48:916-929, 2012.

38. F. Cecchini, L. Iacumin, M. Fontanot, G. Comi and M. Manzano. Identification of the Unculturable Bacteria Candidatus Arthromitus in the Intestinal Content of Trouts Using Dot Blot and Southern Blot Techniques. *Veterinary Microbiology*, 156:389-394, 2012.

39. A. Sghir, G. Gramet, A. Suau, V. Rochet, P. Pochart and J. Dore. Quantification of Bacterial Groups within Human Fecal Flora by Oligonucleotide Probe Hybridization. *Applied and Environmental Microbiology*, 66:2263-2266, 2000.

40. W. Tottey, J. Denonfoux, F. Jaziri, N. Parisot, M. Missaoui, D. Hill, G. Borrel, E. Peyretaillade, M. Alric, H. M. Harris, I. B. Jeffery, M. J. Claesson, P. W. O'Toole, P. Peyret and J. F. Brugere. The Human Gut Chip "Hugchip", an Explorative Phylogenetic Microarray for Determining Gut Microbiome Diversity at Family Level. *PLoS One*, 8:1-12, 2013.

41. O. Paliy and R. Agans. Application of Phylogenetic Microarrays to Interrogation of Human Microbiota. *FEMS Microbiology Ecology*, 79:2-11, 2012.

42. O. Paliy, H. Kenche, F. Abernathy and S. Michail. High-Throughput Quantitative Analysis of the Human Intestinal Microbiota with a Phylogenetic Microarray. *Applied and Environmental Microbiology*, 75:3572-3579, 2009.

43. M. Rajilic-Stojanovic, H. G. Heilig, D. Molenaar, K. Kajander, A. Surakka, H. Smidt and W. M. de Vos. Development and Application of the Human Intestinal Tract Chip, a Phylogenetic Microarray: Analysis of Universally Conserved Phylotypes in the Abundant Microbiota of Young and Elderly Adults. *Environmental Microbiology*, 11:1736-1751, 2009.

44. X. Tzounis, A. Rodriguez-Mateos, J. Vulevic, G. R. Gibson, C. Kwik-Uribe and J. P. Spencer. Prebiotic Evaluation of Cocoa-Derived Flavanols in Healthy Humans by Using a Randomized, Controlled, Double-Blind, Crossover Intervention Study. *American Journal of Clinical Nutrition*, 93:62-72, 2011.

45. X. Tzounis, J. Vulevic, G. G. Kuhnle, T. George, J. Leonczak, G. R. Gibson, C. Kwik-Uribe and J. P. Spencer. Flavanol Monomer-Induced Changes to the Human Faecal Microflora. *British Journal of Nutrition*, 99:782-792, 2008.

46. D. Bialonska, P. Ramnani, S. G. Kasimsetty, K. R. Muntha, G. R. Gibson and D. Ferreira. The Influence of Pomegranate by-Product and Punicalagins on Selected Groups of Human Intestinal Microbiota. *International Journal of Food Microbiology*, 140:175-182, 2010.

47. V. Mai, H. A. Katki, H. Harmsen, D. Gallaher, A. Schatzkin, D. J. Baer and B. Clevidence. Effects of a Controlled Diet and Black Tea Drinking on the Fecal Microfloracomposition and the Fecal Bile Acid Profile of Human Volunteers in a Double-Blinded Randomized Feeding Study. *The Journal of Nutrition*, 134:473-478, 2004.

48. A. Partty, R. Luoto, M. Kalliomaki, S. Salminen and E. Isolauri. Effects of Early Prebiotic and Probiotic Supplementation on Development of Gut Microbiota and Fussing and Crying in Preterm Infants: A Randomized, Double-Blind, Placebo-Controlled Trial. *Journal of Pediatrics*, 163:1272-1277, 2013.

49. S. H. Duncan, K. P. Scott, A. G. Ramsay, H. J. M. Harmsen, G. W. Welling, C. S. Stewart and H. J. Flint. Effects of Alternative Dietary Substrates on Competition between Human Colonic Bacteria in an Anaerobic Fermentor System. *Applied and Environmental Microbiology*, 69:1136-1142, 2003.

50. L. Cocolin, V. Alessandria, P. Dolci, R. Gorra and K. Rantsiou. Culture Independent Methods to Assess the Diversity and Dynamics of Microbiota During Food Fermentation. *International Journal of Food Microbiology*, 167:29-43, 2013.

51. C. H. Nakatsu, A. Armstrong, A. P. Clavijo, B. R. Martin, S. Barnes and C. M. Weaver. Fecal Bacterial Community Changes Associated with Isoflavone Metabolites in Postmenopausal Women after Soy Bar Consumption. *PLoS One*, 9:1-11, 2014.

52. K. Terpend, S. Possemiers, D. Daguet and M. Marzorati. Arabinogalactan and Fructo-Oligosaccharides Have a Different Fermentation Profile in the Simulator of the Human Intestinal Microbial Ecosystem (Shime (R)). *Environmental Microbiology Reports*, 5:595-603, 2013.

53. L. B. Bindels, A. M. Neyrinck, N. Salazar, B. Taminiau, C. Druart, G. G. Muccioli, E. Francois, C. Blecker, A. Richel, G. Daube, J. Mahillon, C. G. de los Reyes-Gavilan, P. D. Cani and N. M. Delzenne. Non Digestible Oligosaccharides Modulate the Gut Microbiota to Control the Development of Leukemia and Associated Cachexia in Mice. *PLoS One*, 10:1-16, 2015.

54. T. V. de Wiele, N. Boon, S. Possemiers, H. Jacobs and W. Verstraete. Prebiotic Effects of Chicory Inulin in the Simulator of the Human Intestinal Microbial Ecosystem. *FEMS Microbiology Ecology*, 51:143-153, 2004.

55. S. G. Parkar, D. Rosendale, G. Paturi, T. D. Herath, H. Stoklosinski, J. E. Phipps, D. Hedderley and J. Ansell. In Vitro Utilization of Gold and Green Kiwifruit Oligosaccharides by Human Gut Microbial Populations. *Plant Foods for Human Nutrition*, 67:200-207, 2012.

56. P. L. Janssens, J. Penders, R. Hursel, A. E. Budding, P. H. Savelkoul and M. S. Westerterp-Plantenga. Long-Term Green Tea Supplementation Does Not Change the Human Gut Microbiota. *PLoS One*, 11:1-15, 2016.

57. A. Chaplin, P. Parra, F. Serra and A. Palou. Conjugated Linoleic Acid Supplementation under a High-Fat Diet Modulates Stomach Protein Expression and Intestinal Microbiota in Adult Mice. *PLoS One*, 10:1-13, 2015.

58. L. Chen, W. C. S. Tai and W. L. W. Hsiao. Dietary Saponins from Four Popular Herbal Tea Exert Prebiotic-Like Effects on Gut Microbiota in C57bl/6 Mice. *Journal of Functional Foods*, 17:892-902, 2015.

59. Y. Y. Lam, C. W. Ha, J. M. Hoffmann, J. Oscarsson, A. Dinudom, T. J. Mather, D. I. Cook, N. H. Hunt, I. D. Caterson, A. J. Holmes and L. H. Storlien. Effects of Dietary Fat Profile on Gut Permeability and Microbiota and Their Relationships with Metabolic Changes in Mice. *Obesity (Silver Spring)*, 23:1429-1439, 2015.

60. M. A. Sappok, O. Perez Gutierrez, H. Smidt, W. F. Pellikaan, M. W. Verstegen, G. Bosch and W. H. Hendriks. Adaptation of Faecal Microbiota in Sows after Diet Changes and Consequences for in Vitro Fermentation Capacity. *Animal*, 9:1453-1464, 2015.

61. D. B. Seo, H. W. Jeong, D. Cho, B. J. Lee, J. H. Lee, J. Y. Choi, I. H. Bae and S. J. Lee. Fermented Green Tea Extract Alleviates Obesity and Related Complications and Alters Gut Microbiota Composition in Diet-Induced Obese Mice. *Journal of Medical Food*, 18:549-556, 2015.

62. M. J. Claesson, Q. Wang, O. O'Sullivan, R. Greene-Diniz, J. R. Cole, R. P. Ross and P. W. O'Toole. Comparison of Two Next-Generation Sequencing Technologies for Resolving Highly Complex Microbiota Composition Using Tandem Variable 16s Rrna Gene Regions. *Nucleic Acids Research*, 38:1-13, 2010.

63. Y. Nam, M. Jung, R. S. W., M. Kim and J. Bae. Comparative Analysis of Korean Human Gut Microbiota by Barcode Pyrosequencing. *PLoS One*, 6:1-15, 2011.

64. C. Cueva, M. V. Moreno-Arribas, P. J. Martin-Alvarez, G. Bills, M. F. Vicente, A. Basilio, C. L. Rivas, T. Requena, J. M. Rodriguez and B. Bartolome. Antimicrobial Activity of Phenolic Acids against Commensal, Probiotic and Pathogenic Bacteria. *Research in Microbiology*, 161:372-382, 2010.

65. C. Manach, A. Scalbert, C. Morand, C. Remesy and L. Jimenez. Polyphenols Food Sources and Bioavailability. *American Journal of Clinical Nutrition*, 79:727-747, 2004.

66. A. Scalbert, C. Morand, C. Manach and C. Remesy. Absorption and Metabolism of Polyphenols in the Gut and Impact on Health. *Biomedicine and Pharmacotherapy*, 56:276-282, 2002.

67. A. Staroscik. Calculator for Determining the Number of Copies of a Template, http://cels.uri.edu/gsc/cndna.html [retrieved 19 September 2016].

68. J. van Duynhoven, E. E. Vaughan, F. van Dorsten, V. Gomez-Roldan, R. de Vos, J. Vervoort, J. J. van der Hooft, L. Roger, R. Draijer and D. M. Jacobs. Interactions of Black Tea Polyphenols with Human Gut Microbiota: Implications for Gut and Cardiovascular Health. *American Journal of Clinical Nutrition*, 98:1631S-1641S, 2013.

69. K. Gao, A. Xu, C. Krul, K. Venema, Y. Liu, Y. Niu, J. Lu, L. Bensoussan, N. P. Seeram, D. Heber and S. M. Henning. Of the Major Phenolic Acids Formed During Human Microbial Fermentation of Tea, Citrus, and Soy Flavonoid Supplements, Only 3,4-Dihydroxyphenylacetic Acid Has Antiproliferative Activity. *The Journal of Nutrition*, 136:52-57, 2006.

70. M. da Silva Pinto. Tea: A New Perspective on Health Benefits. *Food Research International*, 53:558-567, 2013.

71. N. Khan and H. Mukhtar. Tea Polyphenols for Health Promotion. *Life Sciences*, 81:519-533, 2007.

72. E. Özcan. Metabolism of Turkish Black Tea Polyphenols by Human Gt Microbiota (Master's Thesis), Yeditepe University. 2014.

73. S. J. Ott, M. Musfeldt, U. Ullmann, J. Hampe and S. Schreiber. Quantification of Intestinal Bacterial Populations by Real-Time Pcr with a Univeral Primer Set and Minor Groove Binder Robes: A Global Aproach to the Enteric Flora. *Journal of Clinical Microbiology*, 42:2566-2572, 2004.

74. S. R. Lyons, A. I. Griffen and E. J. Leys. Quantitative Real-Time Pcr for Porphyromonas Gingivalis and Total Bacteria. *Journal of Clinical Microbiology*, 38:2362-2365, 2000.

75. A. Santacruz, M. C. Collado, L. Garcia-Valdes, M. T. Segura, J. A. Martin-Lagos, T. Anjos, M. Marti-Romero, R. M. Lopez, J. Florido, C. Campoy and Y. Sanz. Gut Microbiota Composition Is Associated with Body Weight, Weight Gain and Biochemical Parameters in Pregnant Women. *British Journal of Nutrition,* 104:83-92, 2010.

76. T. Rinttila, A. Kassinen, E. Malinen, L. Krogius and A. Palva. Development of an Extensive Set of 16s Rdna-Targeted Primers for Quantification of Pathogenic and Indigenous Bacteria in Faecal Samples by Real-Time Pcr. *Journal of Applied Microbiology*, 97:1166-1177, 2004.

77. T. Matsuki, K. Watanabe, J. Fujimoto, T. Takada and R. Tanaka. Use of 16s Rrna Gene-Targeted Group-Specific Primers for Real-Time Pcr Analysis of Predominant Bacteria in Human Feces. *Applied and Environmntal Microbiology*, 70:7220-7228, 2004.

78. T. Ringel-Kulka, J. Cheng, Y. Ringel, J. Salojarvi, I. Carroll, A. Palva, M. de Vos and R. Satokari. Intestinal Microbiota in Healthy U.S. Young Children and Adults-a High Throughput Microarray Analysis. *PLoS One*, 8:1-10, 2013.

79. A. Martinon, U. P. Cronin and M. G. Wilkinson. Comparison of in-House and Commercial Real-Time Pcr Systems for the Detection of Enterobacteriaceae and Their Evaluation within an Interlaboratory Study Using Infant Formula Samples. *Food Analytical Methods*, 4:485–496, 2011.

80. M. A. Nadkarni, F. E. Martin, N. A. Jacques and N. Hunter. Determination of Bacterial Load by Real-Time Pcr Using a Broad-Range (Universal) Probe and Primers Set. *Microbiology*, 148:257-266, 2002.

81. K. M. Ritalahti, B. K. Amos, Y. Sung, Q. Wu, S. S. Koenigsberg and F. E. Loffler. Quantitative Pcr Targeting 16s Rrna and Reductive Dehalogenase Genes Simultaneously Monitors Multiple Dehalococcoides Strains. *Applied and Environmental Microbiology*, 72:2765-2774, 2006.

82. L. Hoyles and A. L. McCartney. What Do We Mean When We Refer to Bacteroidetes Populations in the Human Gastrointestinal Microbiota? *FEMS Microbiological Letters*, 299:175-183, 2009.

83. N. L. Cluny, L. K. Eller, C. M. Keenan, R. A. Reimer and K. A. Sharkey. Interactive Effects of Oligofructose and Obesity Predisposition on Gut Hormones and Microbiota in Diet-Induced Obese Rats. *Obesity (Silver Spring)*, 23:769-778, 2015.

84. R. A. Kemperman, S. Bolca, L. C. Roger and E. E. Vaughan. Novel Approaches for Analysing Gut Microbes and Dietary Polyphenols: Challenges and Opportunities. *Microbiology*, 156:3224-3231, 2010.

85. H. C. Lee, A. M. Jenner, C. S. Low and Y. K. Lee. Effect of Tea Phenolics and Their Aromatic Fecal Bacterial Metabolites on Intestinal Microbiota. *Research in Microbiology*, 157:876-884, 2006.

86. P. Dolara, C. Luceri, C. de Filippo, A. P. Femia, L. Giovannelli, G. Caderni, C. Cecchini, S. Silvi, C. Orpianesi and A. Cresci. Red Wine Polyphenols Influence Carcinogenesis, Intestinal Microflora, Oxidative Damage and Gene Expression Profiles of Colonic Mucosa in F344 Rats. *Fundamental and Molecular Mechanisms of Mutagenesis*, 591:237-246, 2005.

87. K. Goto, S. Kanaya, T. Ishigami and Y. Hara. The Effects of Tea Catechins on Fecal Conditions of Elderly Residents in a Long-Term Care Facility. *Journal of Nutritional Science and Vitaminology*, 45:135-141, 1999.

88. L. Marin, E. M. Miguelez, C. J. Villar and F. Lombo. Bioavailability of Dietary Polyphenols and Gut Microbiota Metabolism: Antimicrobial Properties. *BioMed Research International*, 2015:2014.

89. A. O'Callaghan and D. van Sinderen. Bifidobacteria and Their Role as Members of the Human Gut Microbiota. *Frontiers in Microbiology*, 7:2016.

90. R. Tabasco, F. Sanchez-Patan, M. Monagas, B. Bartolomé, M. V. Moreno-Arribas, C. Pelaez and T. Requena. Effect of Grape Polyphenols on Lactic Acid Bacteria and Bifidobacteria Growth: Resistance and Metabolism. *Food Microbiology*, 28:1345-1352, 2011.

91. N. Caturla, E. Vera-Samper, J. Villalain, C. R. Mateo and V. Micol. The Relationship between the Antioxidant and the Antibacterial Properties of Galloylated Catechins and the Structure of Phospholipid Model Membranes. *Free Radical Biology and Medicine*, 34:648-662, 2003.

92. A. Takagaki and F. Nanjo. Metabolism of (-)-Epigallocatechin Gallate by Rat Intestinal Flora. *Journal of Agricultural and Food Chemistry*, 58:1313-1321, 2010.