METABOLISM OF TURKISH BLACK TEA PHENOLICS BY HUMAN GUT MICROBIOTA

by
Ezgi Özcan

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APPROVED BY:

Assoc. Prof. F. Yeşim EKİNCİ
(Supervisor) .....

Asst. Prof. Özlem Güçlü ÜSTÜNDAĞ
(Co-supervisor) .....

Asst. Prof. May KORACHI .....

Asst. Prof. Ali Özhan AYTEKİN .....

Asst. Prof. Derya Kahveci .....

DATE OF APPROVAL: …./…./….

To all kids who remind me journey to hope

Berkin Elvan and my niece Duru...

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ABSTRACT

METABOLISM OF TURKISH BLACK TEA PHENOLICS BY HUMAN GUT MICROBIOTA

Human gut microbiota has diverse microbial community with diverse and intense metabolic activity on non-digested dietary substances, easing the absorption. Phenolics, kind of dietary compounds with exceptional nutritive and health related value, are expose to colonic metabolism and/or release their local or systemic effects on human body. In this study, gut microbiota dependent metabolism of Turkish black tea phenolics were investigated in a pH-controlled fermentor system using fecal inocula from a Turkish healthy male. Before being set up, brewing conditions and inoculation amount of black tea extract were optimized and initially performed in static cultures. Cultures were grown in synthetic medium at 37°C under flowing nitrogen with (R1) or without (R2) addition of black tea extract (3125 mg/L) for 48 h. The activities of microbiota on phenolic metabolism were monitored by degradation of catechins using HPLC-PDA and production of their metabolites using GC-MS. For microbial profile, plate counting and FISH analysis were performed. EC, EGCG, ECG and theaflavins were significantly and completely metabolized during 24 h period relative to control (R3) ($p<0.05$), leading to generation of pyrocatechol, pyrogallol, 4-hydroxyphenylacetic acid, 3-(3-hydroxyphenyl) propionic acid with a significant correlation ($p<0.05$) and trace amount of GA were observed at the end of incubation, yielding the pyrogallol and pyrocatechol ($p<0.05$). Moreover, the uncontrolled pH in static cultures led to faster degradation of phenolics. The bacterial plate counting analysis revealed that no significant differences were observed in black tea inoculated cultures compared to control ($p>0.05$). In FISH analysis, the background noise obstructed the quantification of bacteria in the images, so the analysis should be repeated to obtain reproducible accuracy.

ÖZET

SİYAH ÇAY FENOLİKLERİNİN İNSAN KALIN BAĞIRSAK MİKROFLORASI İLE ETKİLEŞİMİ

Kalın bağırsak geniş ve çesitli mikrobiyel topluluğa sahip olup, mikrobiyel faaliyeti sebebiyle besinlerin sindirilmesinde büyük rol oynayan bir yapıdır. Fenolikler besinler yoluyla vücuda alındıktan sonra emilmeden kalın bağırsağa ulaşarak buradaki mikroorganizmalar tarafından parçalanıp emilebilen ve sağlığa ve mikrobiyel topluluğa yararlılıkları ve yüksek besin değeriyle ile bilinen maddelerdir. Bu çalışmada, Türk siyah çayı fenolikleri üzerinde bağırsak aktivitesini incelemek için, sağlıklı bir bireyden elde edilen dışkı ile bağırsağın distal bölümünün taklit edildiği pH kontrollü fermentör sistemi kurulmuştur. Bu sistemi desteklemek adına, siyah çayın demleme ve inokülasyon koşulları statik fermentör sisteminde önceden optimize edilmiştir. Fermantasyon sistemi 48 saat boyunca anaerobik ortamda siyah çay özütü (3125 mg/L) eklenerek (R1) veya eklenmeden (R2) çalıştırılmıştır. Fenoliklerin yapılarındaki degişikler HPLC ve açığa çıkan ürünler GC-MS yöntemi ile izlenmiştir. Ayrıca, bakteri profili, fermantasyon boyunca hem koloni sayımı hem de FISH yöntemi kullanılarak incelenmiştir. Pirokateşol, pirogallol, 4-hidroksifenilasetik asit ve 3-(3-hidroksifenil) propionik asit üretilmesi ile ilişkili olarak ($p<0.05$), EC, EGCG, ECG ve tiflavinler 24 saat içinde tamamen yıkılırken ve kontrole göre anlamlı bir azalım gösterirken ($p<0.05$), inkübasyonun sonunda bir miktar GA gözlenmiş ve bu süreçte pirokateşol, pirogallol üretmiştir ($p<0.05$). Ayrıca, statik kültürlerde pH'nın kontrol edilmemesi, kateşinlerin daha hızlı yıkılmasına neden olmuştur. Koloni sayımı yöntemi ile 48 saat içinde kontrole (R3) göre bir farklılık gözlenmemiştir ($p>0.05$) ve FISH analizinde, görüntülerdeki arkaplanda kirliliğinin sayımı etkilemesi üzerine deneyin daha sonraki çalışmalar için tekrarlanması gerekmektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

ANOVA	Analysis of variance
Bac 303	*Bacteroides* spp
BHI	Brain Heart Infusion
Bif 164	*Bifidobacterium* spp
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
C	Catechin
CBG	Cytosolic β-glucosidase
CFU	Colony forming unit
CG	catechin gallate
COMT	catechol-O-methyltransferases
CV	Coefficient of variance
DGGE	Denaturating Gradient Gel Electrophoresis
EC	Epicatechin
ECD	Electrochemical Detector
ECG	Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
Erec482	*Eubacterium rectale−Clostridium coccoides* group
EUB 338	Total bacteria
EUB338II	Total bacteria
EUB338III	Total bacteria
FISH	Fluorescence in situ hybridization
FOS	Fructooligosaccharides
GA	Gallic acid
GAE	Gallic acid equivalent
GC	Gallocatechin

GCG	Gallocatechin gallate
GC-MS	Gas Chromatography Mass Spectrometry
GIT	Gastrointestinal Tract
HITchip	Human Intestinal Tract Chip
HMA	Human Microbiota Associated Animals
HMP	Human Microbiome Project
HPLC	High performance liquid chromatography
Lab 158	*Lactobacillus−Enterococcus* spp
LC-MS	Liquid Chromatography Mass Spectrometry
LLE	Liquid-liquid Extraction
LPH	Lactase phlorhizin-hydrolase
MetaHIT	Metagenomics of the Human Intestinal Tract
MS	Mass Spectrophotometry
MSA	Mannitol Salt Agar
NMR	Nuclear magnetic resonance
NSP	Non-starch polysaccharides
PAD	Phenolic acid decarboxylase
PBS	Phospate buffer saline
PC	Phenolic Compounds
PCR	Polymerase Chain Reaction
PDA	Photodiode array
RCM	Reinforced Clostridium Medium
RS	Resistant Starch
SCFA	Short Chain Fatty Acid
SHIME	Simulator of Human Intestinal Microbial Ecosystem
SPE	Solid Phase Extraction
SULT	Phenol sulfotransferases
TF	Theaflavins
TIM	Gastrointestinal Model
TMCS	Trimethylchlorosilane

TP	Total Phenolic
TR	Thearubings
UDPGT	UDP glucuronosyl transferase
VRBG	Violet Red Bile Glucose

1. INTRODUCTION

Human gut microbiota comprises approximately 10^{12} bacteria/g of colonic content with varying metabolic activity depending on dietary and genetic and environmental differences. The gut microbiota produces enzymes to degrade the complex substrates. Dietary substances such as carbohydrate and non-carbohydrate compounds including phenolics that escape digestion by host enzymes are the substrates for the intestinal microbiota. Phenolics are abundantly present in diet. The gut microflora may cause changes in the chemical structures of phenolic compounds, and produce microbial metabolites and have an influence on the bioavailability and bioactivity. Black tea is one of the most consumed beverages in Turkey with an exceptionally high phenolic content containing representative phenolic compounds such as catechins, gallic acid, theaflavins (TF) and thearubigins (TR).

Many *in vivo* and *in vitro* studies have been conducted to investigate the interaction of phenolics and gut microbiota. *In vitro* batch and continuous culture fermentation systems, where the fecal inoculums were used to represent the activity of microbiota, have mostly been used as gut model systems due to some limitations in *in vivo* studies. The batch culture systems are mostly used to get basic information on gut microbial fermentation profiles.

Although there are many studies on the metabolism of black tea phenolics by human gut microbiota and their influence on gut microbiota, the interaction of gut microbiota and black tea phenolics in the Turkish context has not been investigated. This study provides basic information about the gut microbiota activity in which fecal slurry was collected from a male volunteer living in Turkey. Therefore, in this study, the interaction of black tea phenolics and gut microbiota was investigated in an *in vitro* fermentation and the analysis of degradation of precursor phenolics and production of their microbial metabolites were performed using HPLC and GC-MS, respectively. Moreover, the effect of black tea relative to control on gut microbiota was assessed by selective plate counting and fluorescent *in situ* hybridization (FISH).

2. LITERATURE REVIEW

2.1. HUMAN GUT MICROBIOTA

Human gut is a special digestive organ with its metabolic capacity and diversity in biochemical transformation [1]. The simple biological functions of human gut are absorption, secretion and storage of water and waste materials [2]. In adults, the large intestine is approximately 1.5 m long with 500 ml volume and 200 g of solid content which half consists of bacterial cells [1]. The movement of colon with around 48-70 h transit time for adults is very slow when compared to digestive system through the stomach and small intestine (4–6 h) [3]. This slow metabolic movement of human colon provides large area for colonization of microbiota and efficient digestion of substrates. Therefore, the majority of microorganisms in human body reside in human colon.

The human colon consists of three main sections: proximal, transverse and distal. In proximal part, the saccharolytic fermentation mainly takes place due to the availability of intense fermentable carbohydrates and as a result of this; short chain fatty acid (SCFA) production is exceptionally intense. This section has an acidic pH (5–6), and rapid bacterial growth. In transverse colon, the substrate availability decreases which results in slower bacterial growth and more neutral environment. On the other hand, in distal colon where pH is close to neutral, while carbohydrate availability decreases, proteolytic fermentation become very important resulting in the production of SCFA, in addition to ammonia, amines, phenols, thiols and indoles as well, and bacterial population remain static. Figure 2.1 shows the sections of human colon with their physiologic conditions and metabolic actions.

The daily amount that reaches the colon from small intestine is about 1.5 kg majority being water [1]. The daily substrate availability in the healthy human colon is about 20–60 g carbohydrates and 5–20 g protein [4]. The primer metabolic function of human microbiota is to break down non-digested and non-absorbed dietary substances that escape from small

intestine to their metabolites, on the other hand, it has a role in the synthesis of vitamins (B, K) and proteins, and metabolism of bile acids, sterols and xenobiotics. The metabolic activity of microflora is largely influenced by the amount and type of substrate available to the organisms from food residues and by colonic residence times (transit time) [5].

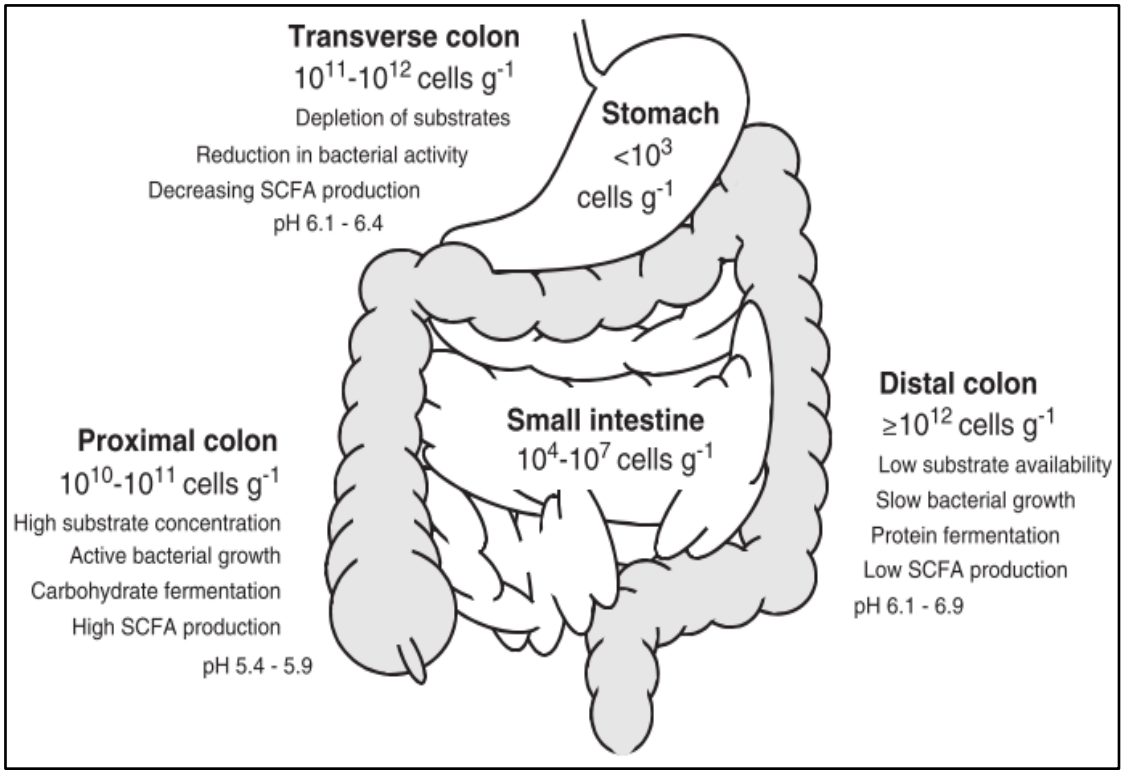


Figure 2.1. Schematic representation of human intestine system: the amount of bacterial cells per gram of intestinal contents, nutrient availability and typical bacterial metabolic activity in different parts of healthy individuals [6].

The human gut consists of bacterial and archaeal species up to 10^{12} cells per gram [4], [6], [7]. The evolution of bacterial residency in human gut starts immediately after birth and it develops depending on environmental factors such as diet, and drug intake. For example, in newborn infant, *Bifidobacterium* population dominates microbial ecology due to bifidogenic effects of breast milk, however, more diverse microbiota evolves in children and adults [8]. To assess human gut microbial diversity, culture based techniques are employed, but most

recently molecular techniques on both organism level and gene level have been developed [9–12]. Approximately 800 species with nine bacterial (*Actinobacteria, Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Verrucomicrobia, Cyanobacteria*) and one archaeal phyla are estimated to exist in the human colon [13], [14]. *Bacteroidetes* (including *Bacteroides*) *and Firmicutes* (including the group of species such as *Clostridia* and lactic acid bacteria) are more commonly dominant and make up to 90 % of intestinal microbiota, followed by *Actinobacteria* (including *Bifidobacterium* spp.) and *Proteobacteria* and the others are present to a lesser extent [11], [15]. Whereas the genera *Bacteroides, Bifidobacterium, Eubacterium, Clostridium*, and *Ruminococcus* are predominant in human colon, facultative anaerobes such as *Enterobacter, Enterococcus, Escherichia Klebsiella, Lactobacillus,* are among the subdominant genera. However, the species and strain levels can change between individuals [9], [15]. This diversity and the individuality of these communities can be explained by diet, host genotype and microbial interactions in humans [4], [10], [15], [16]. The bacterial diversity in the colon derives from multiple carbon and energy sources available for the fermentation and host dependent regulations such as substrate availability and colonic transit time. For instance, the colonic microbiota of African children was dominated by *Bacteriodetes* (73 %) with abundance of the genus *Prevotella* and *Xylanibacter* compared to European children whose was dominated by 51 % *Firmicutes* and 27 % *Bacteriodetes* with absence of the genus *Prevotella* and *Xylanibacter* [17]. *Prevotella* and *Xylanibacter* are well-known to have bacterial genes for hydrolysis of.cellulose and xylan

2.1.1. Functions of Human Gut Microbiota

Besides its simple biological functions, the human gut may have influence on host health and nutrition due to the metabolic functions of its microflora [18]. Metabolic activity is defined as extracting energy from indigestible dietary components and leads to the production of SCFA, vitamins and amino acids. The gut microbiota composition and its metabolic activity is largely affected by both endogenous (intestinal mucines, enzymes, hormones, mucosa cells) and exogenous substrates such as diet which serve as energy and carbon source for gut microbiota [18], [19]. Dietary substances that escape digestion by host enzymes are the

substrates for the intestinal microbiota. The main substances are particularly carbohydrates including resistant starch (RS), non-starch polysaccharides (NSP), plant cell wall polysaccharides and non-digestible oligosaccharides (such as fructooligosaccharide (FOS) or inulin) [4], [5], [18]–[20]. Moreover, non-carbohydrate compounds such as resistant protein, lignin, and other compounds associated with nutritional importance such as phytochemicals including phenolics and carotenoids are also considered as non-digested dietary compounds and can reach the colon without absorption [19].

The gut microbiota comprises many genes that encode enzymes to degrade the complex substrates [12], [19], [21]. The enzymes include carbohydrate-active enzymes, which were identified by metagenomic sequencing [22], hydrolases, reductases and decarboxylases which are responsible for hydrolyzing the glycosides, amides, esters, lactones and also can fulfill ring cleavage, reduction and decarboxylation reactions [23].

Due to their metabolic functionality, the gut microbiota can be divided into two kind of species that exert either harmful or beneficial effects on the human host [2]. The metabolites of microbiota that are from substrates available in the colon are responsible for these harmful and beneficial effects. Gut fermentation is a complicated process due to its diversity and metabolic capability. Generally, a metabolite produced by one individual species can be provided as a growth substrate for another. The production of SCFA such as butyrate, acetate and propionate from carbohydrate metabolism support immunological response of human cells and participate in the defense mechanism against pathogens. When butyrate is the major substrate for colonocytes, acetate and propionate circulate in portal vein and eventually utilized by the liver (propionate) or peripheral tissues, predominantly muscles (acetate) [24], [25]. The microbial metabolism of plant cell wall polysaccharides can cause to release and transformation of phenolic compounds that can have potent anti-inflammatory and antioxidant activity [8]. Unlike carbohydrate fermentation, the protein fermentation end products (e.g. ammonia, amines and phenolic compounds) may be toxic to the host [26]. Protein fermentation occurred excessively, in the distal colon, has been linked to diseases such as colon cancer [27]. While the genera *Bifidobacterium* and *Lactobacillus* are primarily capable

of carbohydrate fermentation, other groups, such as *Bacteroides* and *Clostridia* can be able to ferment both proteolytic and amino acid [2], [4], [28].

As mentioned earlier, the human colon harbors both harmful and pathogenic bacteria. The diversity and abundance of these bacteria are highly dependent on the diet. Moreover, the end products because of the fermentation of dietary substances are exceptionally important and effective on human gut health. This importance gives rise to interest on functional food concept related to nutritive dietary substances with beneficial health effects on gut microbiota. Moreover, the importance of identifying the specific bacterial species responsible for specific metabolism of dietary substances and of determinining the enzymes and reactions involved in this metabolism is being recognized.

2.2. PHENOLIC COMPOUNDS

2.2.1. Classification of Phenolic Compounds

Phenolic compounds (PC) are secondary metabolites produced in plants under stress conditions [29]. They are abundantly found in fruits, vegetables and beverages such as tea, coffee, and wine. Phenolics are reported to be biologically active compounds, having antioxidant capacity related to the prevention of degenerative diseases such as cancer, cardiovascular and neurodegenerative diseases [30], [31], [32].

They are a diverse group of compounds with more than 8000 structures identified in plants, which are naturally found conjugated to sugars and organic acids [33], [34], [35]. They are classified into different groups (such as flavonoids and non-flavonoids) according to the amount and organization of their carbon atoms attached to the phenol ring. In addition to monomeric phenolics, dimeric (proanthocyanidins, theaflavins) and polymeric (thearubigins, tannins) structures are also present [33].

Favonoids share a basic structure consisting of two benzene rings (ring A and B) linked through a three-carbon chain which forms a heterocyclic pyrone C ring [33], [36], [37] (Figure 2.2). Flavonoids are divided into 6 subclasses, flavonols, flavones, isoflavones, flavanones, flavanol and anthocyanidins, depending on the oxidation position of the central pyrone ring as shown in Table 2.1 [33], [34].

In contrast, non-flavonoid phenolics include a more heterogeneous group of compounds, ranging from the simple phenolic acids, such as gallic acid and hydroxycinnamic acids,acetophenones, stilbenes, phenylacetic acids, phenylpropionic acids, xhantones, naphtoquinones, coumarins to more complex compounds such as lignans and tannins [34].

2.2.2. Tea Phenolics

Tea (*Camellia sinensis*) leaves are a good source of phenolic compounds, which may constitute up to 30% of the dry leaf weight [38]. The main phenolics present in fresh leaves are monomeric flavanols such as (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-O-gallate (EGCG) and (-)-epicatechin-3-O-gallate (ECG) with gallocatechingallate (GCG), gallocatechin (GC), catechingallate (CG), and catechin(C), present in smaller quantities (Table 2.1, Figure 2.3). Tea leaves are also a source of gallic acid containing up to 4.5 g/kg fresh wt [39]. Moreover, flavonols such as quercetin, myricitin, kaempferol and their glycosides are also present in tea leaves [40].

However, there are some differences in the phenolic composition of fresh tea leaves, green tea and black tea. Green tea generally contains most of the phenolics present in fresh tea leaves, but in black tea production, destruction of about 90 % of catechins takes place due to enzymatic oxidative reactions by polyphenol oxidase and peroxidase, leading to the formation of dimeric (eg. theaflavins (TF)) and polymeric phenolic compounds (eg. thearubigins (TR)) [34]. In green tea production, limited oxidation may also occur. In the study of Del Rio et al [41], while green tea infusions contains considerable amounts of catechins (4572 mg/L) and no TF were identified, the catechin content is reduced in black tea infusions (101 mg/L) and

TF content was found as 224 mg/L. Similarly, while total catechin content in water extracts of commercially available green teas varied in the range of 0.44-10 % wt/wt in US, that content in water extracts of black tea varied 0.54-6.95 % wt/wt and whereas green tea did not contain theaflavins, black tea extracts contained up to 0.9 % wt/wt theaflavins [42]. The total TR, TF and catechin contents of black teas from different regions were in the range of 9.5-12.2 %, 1.15-1.54 %, 0.68-3.65 % (wt/wt) of black tea leaves, respectively [43], moreover, the contents in seven grades of Turkish black tea leaves were in the range of 5.92-6.83 % and 0.14-0.42 %, 1.83-2.25 % wt/wt, respectively [44].

Theaflavins contain a benzotropolone core produced by the oxidation of two catechins, one with a *vic*-trihydroxyphenyl (pyrogallol) moiety (epigallocatechin and its gallate), and the other with an *ortho*-dihydroxyphenyl (catechol) structure (epicatechin and its gallate) [40]. The enzymatic formation of theaflavins was studied by many researchers [45], [46]. Briefly, *in vitro* oxidation of EC and EGC in presence of polyphenol oxidase explained that theaflavin was formed when EC-quinone formed by the oxidation of EC reacted with the B ring of EGC followed by further oxidation and decarboxylation, which was reviewed in detail by Tanaka et al [46]. The epi form catechins are involved in this reaction resulting in the formation of different TF. Four major types of TFs have been identified in black tea, and they are theaflavin (EC + EGC), theaflavin-3-gallate (ECG+EGC), theaflavin-3'-gallate (EC+ EGCG), and theaflavin-3,3'-digallate (ECG + EGCG) (Figure 2.4).

Unlike TF, the chemical structures of TR, which are much more poorly characterized compounds, are not very well known (Figure 2.5). They have been classified into groups according to their solubility properties (SI, SIa, SII) and chromatographic behavior. SI type TR is soluble in ethyl acetate, whereas SIa and SII types are more soluble in aqueous phase and diethyl ether [47]. The TR content in black tea has been investigated considering their solubility properties [47]–[50]. According to Bailey and Nursten [51], TR can be divided into three groups based on their reverse phase high performance liquid chromatography (HPLC) behavior. Group I covered TR excluded from HPLC column, group II included resolved TR, and group III is TR remaining unresolved and eluted as a “hump” in the chromatogram. In *in*

vitro eznymatic models, polyphenol oxidase produced chromatically resolved TRs, peroxidase caused unresolved TRs [52].

2.2.2.1. Chemical Stability of Tea Phenolics

The stability of tea phenolics (gallic acid, catechins, theaflavins) has been mainly studied by researchers interested in the chemical changes in phenolics during processing and manufacturing of tea and tea drinks, because these compounds are used as indicator of good quality in tea products [43], [53]. In addition to the loss of catechins, degradation leads to the production of negative sensory quality such as off color and the loss of health effects, which restricts the application of catechins in food (including beverages, functional foods, dietary supplements) and pharmaceutical industry [54], [55], [56]. Moreover, since the changes in their structure may influence their bioactivity, stability of tea phenolics under physiological conditions (pH, temperature and oxygen availability) is also important to understand their bioavailability and health benefits in human body [57].

Tea phenolics (gallic acid, catechins and theaflavins) can undergo chemical changes through epimerization and degradation reactions (oxidation and polymerization) with temperature and pH being the most important rate-determining parameters [58]–[60] along with oxygen availability and the presence of metal ions and other ingredients [56], [61]–[63].

Degradation reactions such as oxidation and polymerization occur simultaneously with epimerization reactions with the dominant reaction being determined by temperature, time or pH of the solution. While total catechin content has been used as a measure of catechin degradation during stability studies, concentration of catechins and their respective epimers have been studied to quantify the epimerization reactions taking place. Epimerization reactions result in a decrease in the concentration of the epimerized catechin with an accompanying increase in its respective epimer. A further decrease in the concentration of the produced epimer with time (or a plateu) and/or the % of unaccounted for catechin content is indicative of other degradation reactions taking place.

Alkaline conditions (pH>5) and high temperatures (80-120°C) result in higher epimerization of epi to non epi form of catechins such as EGCG to GCG [58], [62], [64] and also degradation of both theaflavins and catechins in a time dependent manner [63], [65], [66]. Differences in the stability behavior of individual catechins and individual theaflavins, and between catechins and theaflavins were also observed and had been attributed to structural differences.

Complete degradation of EGCG and EGC in sodium phosphate buffer was observed at pH 7.4 after 3 h whereas EC remained unchanged and ECG only decreased by 20% [63]. Likewise, EGC was almost completely degraded after 3 h at pH 7.4 and EGCG was completely destructed at the end of 6 h incubation whereas concentration of ECG and EC decreased by 20 and 5% after 3h, respectively [65]. The lower stability of EGCG and EGC compared to EC and ECG was attributed to the three vicinal hydroxyl groups at positions 3', 4' and 5' in EGCG and EGC, which make them more susceptible to destruction than the two vicinal hydroxyl groups at positions 3' and 4' in ECG and EC [67]. In a mixture of containing four theaflavins (theaflavin (TF1), theaflavin-3-gallate (TF2A), theaflavin-3' -gallate (TF2B), and theaflavin-3,3' -digallate (TF3)), in sodium phosphate buffer at pH 7.4 after 3 h, degradation of TF2B, which was 5% at 3 h, increased rapidly, whereas TF3 degradation rate which led to a 40 % loss in 3 h, decreased after that (lowest) [65]. Incubation of catechins and theaflavin standards in sodium phosphate buffer at pH 7.4 for 6 h led to 65% and 95% degradation of catechins and TF, respectively [65], indicating lower stability of TF which had been attributed to their higher chemical reactivity [60]. Catechins have less steric hindrance and relative inertness whereas theaflavins has higher reactivity, increased number of hydroxyl groups and steric hindrance surrounding the benzotropolone core.

Oxidation rate of phenolics was determined by their structure such that that of catechins with pyrogallol moiety on the B ring (EGC, EGCG) was higher than that of catechol catechins (catechin, EC) [61]. The effect of the gallate group (eg. in ECG) was however dependent on the pH. While a difference was not observed in oxidation rate of catechins (EC and EGC) and their gallate derivatives (ECG and EGCG) under weak basic conditions (pH 7 to 9.5), in

strong basic solutions (pH>10), the oxidation rate of ECG increased significantly indicating oxidation of the gallate group on the C ring [68]. No significant difference was observed between oxidazibility of green and black tea. While oxidation of theaflavins has not been as widely studied yet the oxidazibility of black tea and the high reactivity of theaflavins towards radicals suggest high oxidazibility for theaflavins [61]. Since the presence of oxygen will lead to oxidation, O_2 concentration may affect the stability of catechins. To study the effect of O_2 on the stability of EGCG, EGCG was incubated in phosphate buffer (pH 7.4) at 37°C for 6 h with and without N_2 flushing. When the medium was flushed with N_2 gas for 24 h before EGCG was added, 95% of EGCG remained in buffer after 6 h and no dimers were detected, meaning no autoxidation occurred but GCG was formed, indicating epimerization reaction. On the contrary, 90% EGCG was degraded after 2 h when no nitrogen was flushed and EGCG underwent auto-oxidation, dimers were produced [56]. In an *in vitro* fecal fermentation system under anaerobic conditions, oxidation would be eliminated due to in availability of oxygen. Metal ions may also affect stability of catechins by accelerating oxidation reactions [62], [68], [69].

Therefore, information on the mechanism of phenolic degradation in different systems and the factors that affect it is fundamental to preserve stability of catechins during analysis, processing, in food product formulations. This information also provides a valuable foundation upon which comparison between simultaneous degradation and microbial degradation in biological systems can be made and the health-related effects under human physiological conditions can be understood.

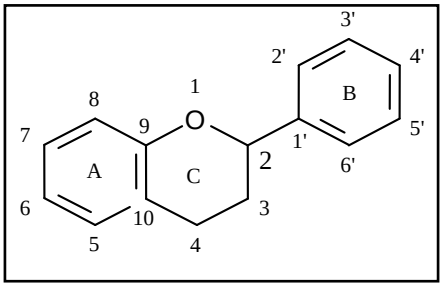


Figure 2.2. Basic structure of flavonoids

Table 2.1. Classification of phenolic compounds according to their chemical structure

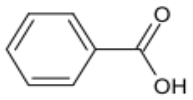
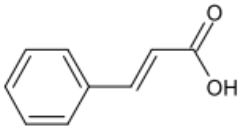
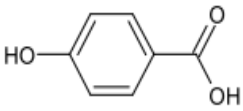
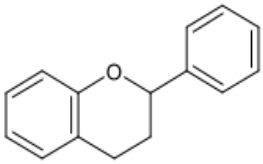
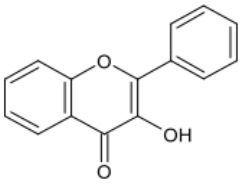
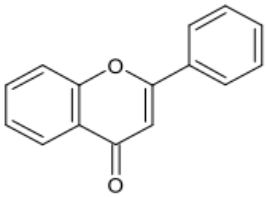
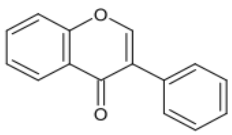
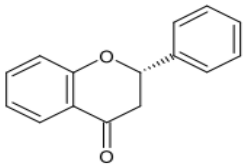
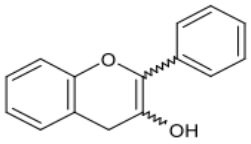
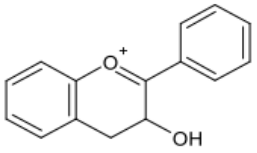
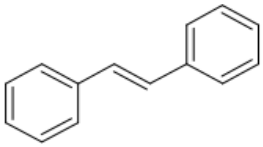
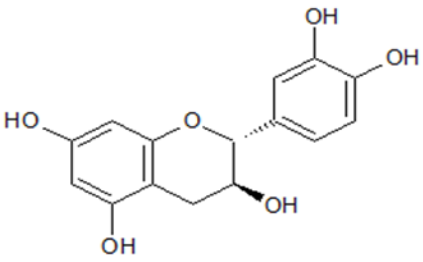
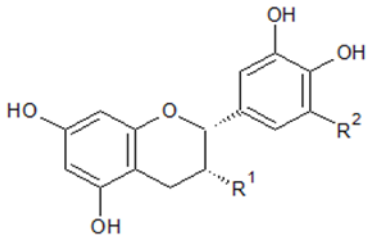
Class	Examples	Basic Structure
Phenolic acids		
Hydroxycinnamic acids	p-coumaric, o-coumaric, caffeic, ferulic, sinapic acid	
Hydroxybenzoic acids	gallic, syringic, p-hydroxybenzoic, protecatehuic and vanillic acid	
Flavonoids		
Flavonols	quercetin, kaempferol, myricetin	
Flavones	apigenin, luteolin	
Isoflavones	genistein, daidzein	

Table 2.1. Classification of phenolic compounds according to their chemical structure (continued)

Flavanones	naringenin, hesperidin	
Flavanol		
monomers	Catechin(C), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG)	
Dimers	proanthocyanidins, procyanidin	
Anthocyanidins	cyanidin, malvidin	
Stilbenes	resveratrol	



(+)catechin



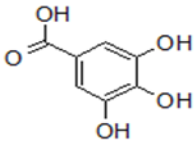
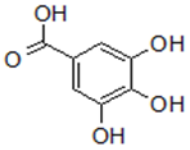
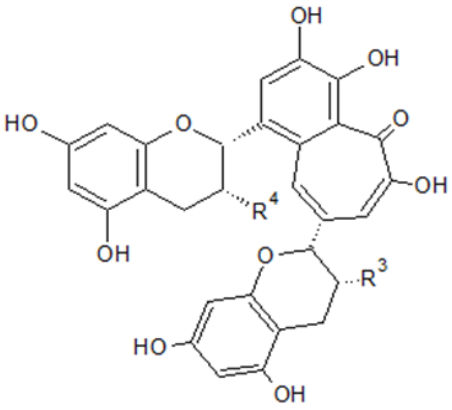
Compound name	R^1	R^2
Epicatechin (EC)	OH	-
Epicatechin gallate (ECG)		-
Epigallocatechin (EGC)	OH	OH
Epigallocatechin gallate (EGCG)		OH

Figure 2.3. Chemical structure of catechins



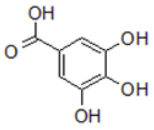
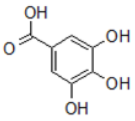
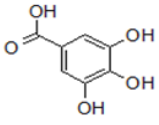
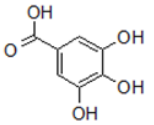
Compound name	R^3	R^4
Theaflavin (TF1)	OH	OH
Theaflavin-3-gallate (TF2)	OH	
Theaflavin-3'-gallate (TF2B)		OH
Theaflavin-3,3'-digallate (TF3)		

Figure 2.4. Chemical structure of Theaflavins

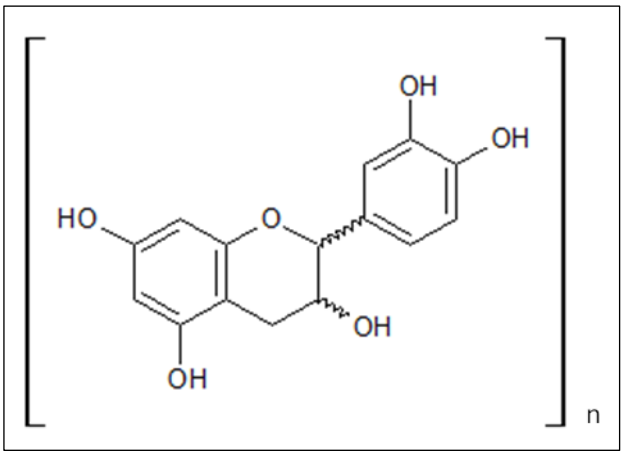


Figure 2.5. Chemical Structure of Thearubigins

2.3. THE INTERACTION BETWEEN PHENOLIC COMPOUNDS AND HUMAN GUT MICROFLORA

The bioavailability and potential bioactivity of phenolic compounds are largely influenced by the metabolic activity of the gut microbiota. The interaction mechanism between phenolic compounds and human gut has two pathways: 1) Human gut microbiota is able to secrete enzymes that catalyze many reactions to ease the absorption of phenolic compounds, thus modulating their health effects on host 2) Phenolic compounds can modulate the microbial composition acting as growth stimulator and/or inhibitor. However, a few species of gut microbiota involved in the metabolism of phenolic compounds have been identified and knowledge on the responsible mechanisms is limited.

2.3.1. Metabolic Pathway of Phenolic Compounds in Human Body

Phenolic compounds are naturally present in various chemical forms in foods. They are mostly in the form of glycosides, esters and polymers that are not digested in their native form [31],[35]. These substances can be subjected to some enzymatic reactions such as hydrolysis and deconjugation in the gastrointestinal tract (GIT). Deconjugation is the separation of either the glycoside or glucuronoside moiety from the phenolic ring, resulting in aglycone

production. When phenolic compounds are hydrolyzed within the epithelium of intestine by the action of cytosolic β-glucosidase enzyme (CBG), aglycones are formed and absorbed by active transportation. Alternatively, aglycones can also be formed by the action of membrane-bound lactase phlorhizin-hydrolase (LPH) in the borders of epithelium and can be absorbed by passive diffusion through epithelium. The activities of these enzymes are dependent on type of substrate. For example, LPH catalyzes the cleavage of wide range of glucosyl moeity from phenolic ring, such as quercetin-3-O-glucoside, which is not hydrolyzed by CBG [70].

However, most of phenolic compounds cannot be absorbed in the small intestine and pass through directly to the colon where they are subjected to colonic enzymatic activity. In the colon, microflora can secrete many enzymes (α-rhamnosidase, β-glucosidase, and β-glucuronidase) for deconjugation of phenolic compounds, which are not produced by human genes.

In the colon, many flavonoids and conjugated moieties are subjected to ring cleavage reactions to form simple phenolic compounds (aglycones) from A and B ring and decarboxylation and/or dehydroxylation reactions occur. Aglycones undergo ring fission by microbial enzymes giving rise to production of aromatic acids, well absorbed through the colonic barrier. These aromatic acids include phenylvaleric, phenylpropionic, phenylacetic and benzoic acids and hydroxycinnamates. For example, in the colon, flavonols are transformed first by C-ring cleavage and then dehydroxylated, resulting in phenylacetic acid, benzoic acids from the B ring and phloroglucinol, propionic acids produced from the A ring [23], [71], [72].

Once microbial deconjugates are absorbed and prior to passage through the blood stream, they are subjected to post-conjugation reactions (methylation, glucuronidation, sulfation) by the action of enzymes; COMT (catechol-O-methyltransferases), UDPGT (UDP glucuronosyl transferase) and SULT (Phenol sulfotransferases), which transform phenolic compounds into phase II metabolites either in the intestine or in the liver [73], [74], [75]. For example, while quercetin glucuronides are formed in gut mucosa [76], catechins are extensively methylated in

the liver [77]. Post-conjugation also known as phase II metabolism is a common detoxification process to restrict aglycones' potential toxic effects and since conjugation increases hydrophilic properties of phenolics, their biliary and urinary excretions are assisted. Furthermore, conjugated forms formed in liver are also secreted to the colon with bile (Figure 2.6) and deconjugated more than once by microbial enzymes and absorbed.

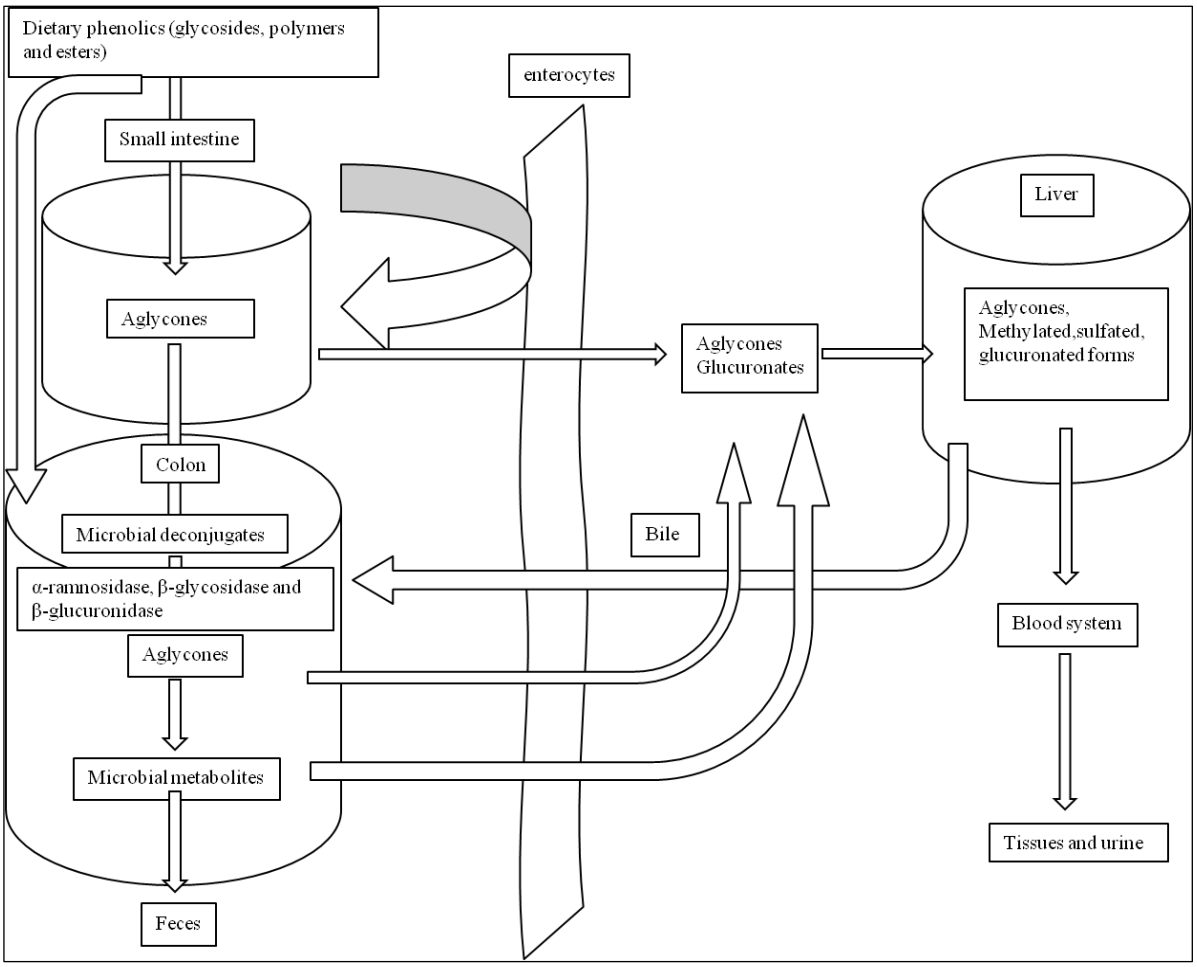


Figure 2.6. Schematic representation of metabolic pathway of dietary phenolics

2.3.2. Role of Gut Microbiota in Phenolic Metabolism

A considerable amount of non-digested phenolic compounds remains in the human gut. The human microflora results in alterations in the chemical structure of phenolic compounds. This process can modify their absorption through the gut and biological activity. Human gut microbiota have the capability to increase digestion of phenolics by breaking them down into more easily absorbed phenolic compounds, which could easily release their anti-oxidative effect for human gut health [78].

Colonic microbiota can generally hydrolyze glycosides, glucuronides, sulfates, amides, esters, and lactones by the help of enzymes such as α-rhamnosidase, β-glucuronidase, β-glucosidase, sulfatase, and esterases. Moreover, there are other reactions catalyzed by gut microbial enzymes such as aromatic ring-cleavage, dehydroxylation (dehydroxylase), decarboxylation (decarboxylase), reductions (reductases, hydrogenases), demethylation (demethylase) and isomerization (isomerase) [71], [78].

Recent knowledge on the gut microbiota and their enzymes involved in phenolic metabolism is restricted to a few model studies. To date, some of the specific gut microbes and microbial enzymes involved in phenolic metabolism have been identified based on selective enrichment of fecal inocula which was recently reviewed by Selma et al [23].

For example, lactic acid bacteria possess many phenolic acid decarboxylase (PAD) and reductase enzymes for degradation of phenolic compounds in foods [79], [80]. Moreover, lactic acid bacteria in the human gut are also capable of encoding those genes. In many dietary fiber rich foods, phenolic acids such as hydroxycinnamic acids (ferulic acid, sinapic acid and p-coumaric acid) are found in their ester forms bonded to the polymeric non-starch polysaccharides in plant cell wall which are not absorbed by human enzymes and can be deesterified from dietary fibers by the help of ferulic acid esterases produced by lactobacillus species in human gut prior to absorption [81]. *L. acidophilus* strains, which are also part of human gut microbiota, were examined for the capacity to produce ferulic acid esterase and

found to be able to transform ferulic acid to p-coumaric acids and probably caffeic acids from bound phenolics to polymeric non-starch polysaccharides [82]. *L. johnsonii,* another lactic acid bacteria found in GIT of many mammals including humans [83], produce enzymes having an ability to degrade common dietary phenolics (ferulic, caffeic, and p-coumaric acid) as a mode of self-protection [84], [85]. In the study of Tabasco et al [86], it was reported that some strains of *L. plantarum, L. delbrueckii* subsp. *bulgaricus* and *L. casei* were resistant to the antimicrobial effects of a rich source of flavan-3-ols (grape seeds extract). Particularly, *L. plantarum* not only catabolized flavan-3-ols by the activity of galloyl esterase, decarboxylase, and benzyl alcohol dehydrogenase activities but also produced other enzymatic activities giving rise to the production of a new unidentified metabolite. Identification of this new metabolite was performed by incubation of *L. plantarum* with monomeric flavan-3-ols and dimeric A- and B-type procyanidins individually [87]. The results of this study showed that *L. plantarum* strain was able to degrade (–) epicatechin-3-O-gallate by cleaving the heterocyclic ring of monomeric flavan-3-ols and giving rise to production of 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol, and caused a remarkable changes in the phenolic profile of the cranberry extract mainly due to the catabolism of hydroxycinnamic and hydroxybenzoic acids but not A- or B-type dimeric procyanidins [87].

In addition to functions of lactic acid bacteria, *Clostridium* and *Eubacterium* are the main groups that have been known to metabolize flavonoids such as isoflavone (daidzein), flavonol (quercetin and kaempferol), flavanone (naringenin and isoxanthumol), and flavan-3-ol (catechin and epicatechin) [19], [23], [88]. Specific bacterial strains can be responsible for the degradation of specific phenolic compounds. For example, quercetin-3-O-glucoside is transformed to 3,4-dihydroxyphenylacetic acid, acetate and butyrate by the human colonic microorganism, *Eubacterium ramulus* [89]. Unlike metabolism of quercetin-3-O-glucoside by *Eubacterium ramulus*, the sugar moeity of quercetin-3-O-rhamnoglucoside and quercetin-3-O-rhamnoside are readily hydrolyzed and formed quercetin by organisms such as *Bacteroides distasonis* (α-rhamnosidase and β-glucosidase), *Bacteroides uniformis* (β-glucosidase) and *Bacteroides ovatus* (β-glucosidase) [90]. *Enterococcus casseliflavus* metabolizes the sugar moiety of quercetin-3-O-glucoside to give formate, acetate and lactate but not aglycone

(quercetin). Moreover, the microbial community differences between individuals lead to different degradation pathways for the same phenolic compounds. For instance, monomeric, dimeric and trimeric flavanols in grape seed extracts and red wine extracts were shown to be degraded with notable inter-individual differences in *in vitro* batch culture fermentations [91], [92]. Furthermore, the chemical structure of phenolics in foods could be effective on the specific metabolism mechanism. The differences in formation rate of equilibrates influenced by the original chemical structure of flavanol precursor [93]. Different grape seed extract fractions (containing flavanol monomers/oligomers) were degraded to different extents by different colonic microbiota [92]. While the fermentation of grape seed extract containing 70% monomers and 28% procyanidins by gut microbiota lead to the formation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone with the equilibrium of 4-hydroxy-5-(3′,4′-dihydroxy-phenyl)-valeric acid and the metabolite 3-(3,4-dihydroxyphenyl)-propionic acid, the conversion of 3-(3,4-dihydroxyphenyl)-propionic acid to 3-(4-hydroxyphenyl)-propionic acid, and especially to phenylpropionic acid was not observed for grape seed extract containing 21% monomers and 78% procyanidins [92]. It can be implied that different pathways present for the metabolism of monomeric and oligomeric flavanols, compared to previous studies which were done for flavanol precursors in different sources [88], [94], [95]. Moreover, *in vivo* study with rat fed different diets containing monomeric, dimeric and oligomeric flavanols showed variations in the urinary excretion of microbial metabolites between different flavanol sources [96].

To address the differences in metabolic products and catabolism pathyways depending upon the type of colonic microbiota and type of precursor, the metabolic pathway of sugar conjugated flavonols such as quercetin–rhamnoside, quercetin–rutinoside, myricetin, luteolin, naringenin and kaempferol–rutinoside) and two phenolic acids (protocatechuic acid and gallic acid) [97] and catechin, epicatechin, epicatechin gallate, epigallocatechin gallate and procyanidin dimer B2 and alkaloids (theobromine and caffeine) [95] were investigated in *in vitro* fermentation model using rat colonic microbiota and designed animal experiments. In those studies, metabolite profile differences were observed between epicatechin and catechin fermentation with the main fermentation products being 5-(hydroxyphenyl)-γ-valerolactone

and phenylacetic acid, respectively [95]. Fermentation of procyanidin dimer B2 did not form the same metabolic products as epicatechin fermentation [95]. Moreover, two complementary fermentation pathways were suggested for each catechin and the production of metabolites were observed to be dependent upon the activity of microflora [95].

The degradation of (epi)catechins starts with cleavage of the heterocyclic C-ring subsequent formation of hydroxyphenylvalerolactones and hydroxyphenylvaleric acids, which are further transformed to hydroxyphenyl propionic acids [71], [93], [98], [99]. EGCG and EGC lost its gallate group giving rise to epicatechin monomer [95]. *In vitro* studies with human fecal slurries showed that catechin degradation metabolites that are circulating in human body, are produced by the intestinal microbiota [71], [88], [93], [94], [98], [100]. After consumption of green tea considerable quantities of flavan-3-ols pass into human colon where they are subjected to metabolism by the gut microbiota [101]. To investigate the metabolism of the flavan-3-ols entering the human colon, incubation of EC, EGC, and EGCG with fecal slurries in an *in vitro* fermentation were carried out by Roowi et al [102]. In addition to *in vitro* incubation, 0-24 h urinary excretion of phenolic acid content was monitored after intake of either green tea or water by healthy individuals and ileostomists, a proposed pathway involved in the colonic metabolism and urinary excretion of flavan-3-ols present in green tea is illustrated in Figure 2.7 [102].

However, knowledge on specific specied in gut microbiota involved in biotransformation of catechins is limited. Kutschera et al [103] isolated *Eggerthella lenta* and *Flavonifractor plautii* from human gut, which can degrade flavanols by cleaving C-ring of both EC and C followed by the formation of 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)-propan-2-ol. *F. plautii* were able to further convert 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol to 5-(3,4-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3,4-dihydroxyphenyl)-γ-valeric acid. In addition to investigation of conversion of individual flavanol compounds, *in vitro* fermentation studies of black tea extract with fecal slurries by healthy individuals were conducted [104], [105], [106]. Although addition of individual phenolic compounds provides information on specific degradation, the use of black tea extract

is required to understand the matrix effect and to test the degradation of compounds such as theaflavins and thearubigins. In the study of Gross et al [104], the metabolite profiling generated from black tea fermentation showed that a limited number of key metabolites particularly 3-phenylpropionic acid and its hydroxylated derivatives were produced with significant inter-individual variability. In the study of Gao et al [105], the fate of phenolic precursors, especially catechins in black tea resulted in the production of 3-methoxy-4-hydroxyphenylacetic acid and smaller amounts of 4-hydroxyphenyl acetic acid, 3-3-hydroxyphenyl propionic acid, 3,4-dihydroxyphenyl acetic acid and 2,4,6-trihydroxybenzoic acid. Van Dorsten et al [106] also studied gut microbial fermentation of dietary phenolics, including black tea and observed strong 3-phenylpropionic acid production as a result of metabolic reaction of black tea phenolics. As it was understood from these studies, the black tea matrix and the diversity of phenolic precursors lead to the formation of wide range of metabolites.

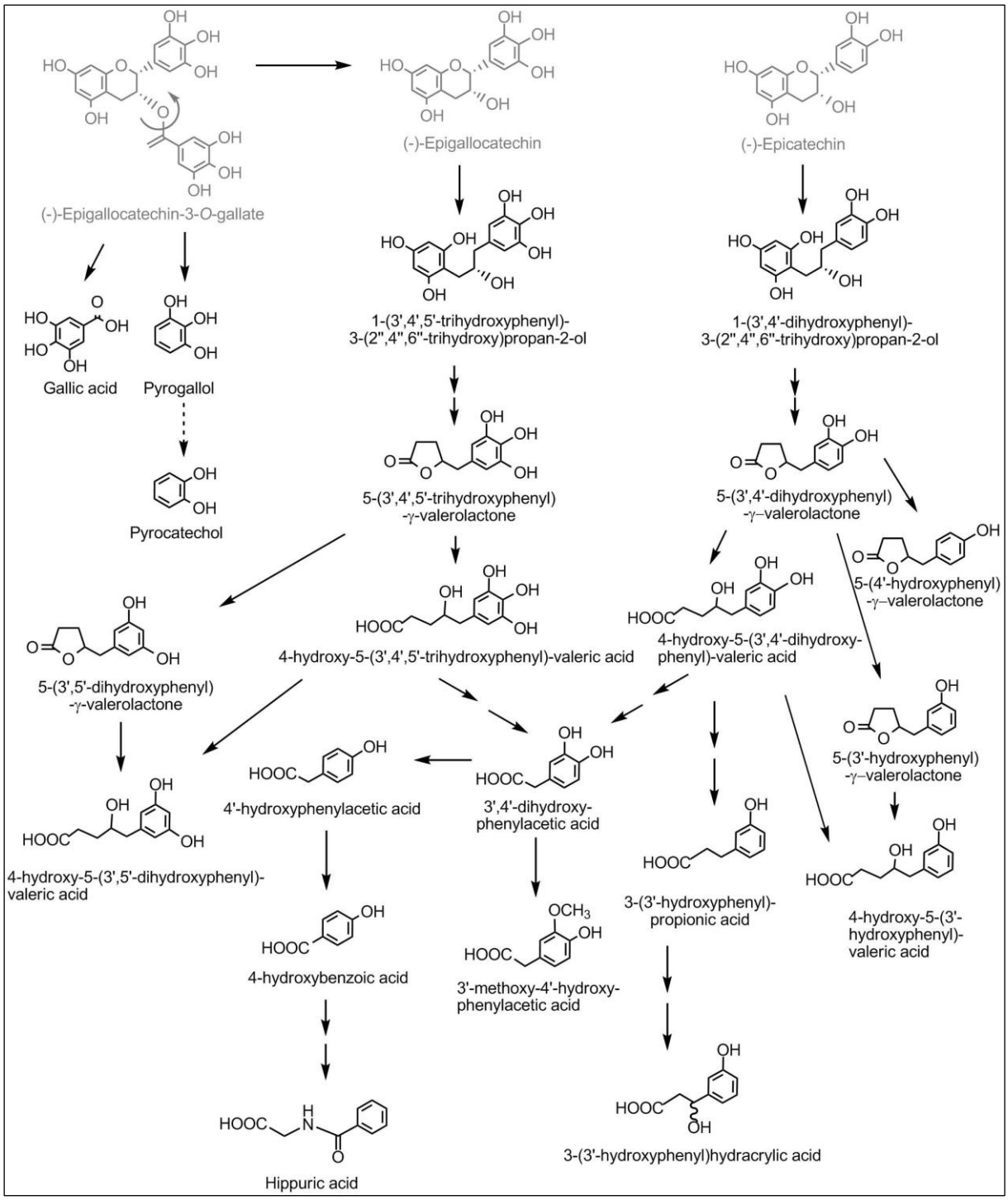


Figure 2.7. Schematic representation of major green tea catechins metabolites in human body [102].

2.3.3. Effects of Phenolics Compounds on Gut Microbiota

Although gut microbiota is largely involved in the phenolic metabolism, in return, phenolic compounds may have an influence on the growth and metabolism of human gut microbiota and modulate the balance of microbial ecosystem in the human colon. The phenolics can serve as substrate for the metabolism of human gut microbiota, as discussed earlier and this process in the gut can lead to the formation of metabolites, therefore, the precursor and/or the metabolite itself may have health related effects such as antimicrobial, antioxidant, anti-inflammatory effect on the human gut microbiota. Moreover, phenolic precursors can act as prebiotics that affect factors of survival for beneficial gut bacteria, mainly lactic acid bacteria and thus, inhibiting the growth of enteric pathogens. Many studies specifically focused on investigation of this interaction on behalf of lactic acid bacteria [107], [108], [109]. For example, tannic acid and grape pomace extract with exceptionally high phenolic content; can act as growth stimulator for *L.acidophilus* by enhancing the nutrient consumption or supplying more energy to cells [107]. The *in vitro* study performed for the effects of flavanol and flavanol rich extracts-mainly catechin on *L. plantarum* showed growth stimulation on cells by affecting transportation system across cell membrane [109].

In vitro [88], [110] and *in vivo* [111] human and animal studies showed that either pure compounds or phenolic rich extracts (monomeric or oligomeric flavan-3-ols from green tea, red wine, grape seed extract, concentrated grape pomace, cocoa drink, tea extract) cause increase in the number of *Lactobacillus* spp. and decrease in the *Enterobacteriaceae*, *Clostridium* and *Bacteroides* groups.

The animal studies are performed using rats to show impact of phenolic rich diet on particular gut microbial population. When rats consumed tannin-rich compounds, *Clostridium leptum* cluster decreased and *Bacteriodes* group increased, significantly [112]. In addition, in the study of Dolara et al [111], red wine phenolics caused a significantly lower level of *Clostridium* spp. and higher numbers of *Lactobacillus* spp. in rats. Also, in the study with rats fed blackcurrant extract powder, a significant decrease was observed in the numbers of

Bacteriodes spp. and *Clostridium* spp. together with a significant increase in the bifidobacteria and lactobacilli [113]. In birds fed with grape pomace concentrate and grape seed extract, it was shown that higher populations of *E.coli, Lactobacillus* spp., *Enterococcus* spp. and *Clostridium* spp. was observed in the ceacal part than the control group [114].

In human intervention studies, changes in the microbial population including *Actinobacteria* and *Clostridium* clusters were observed after the ingestion of tea phenolics [115]. Intervention study on intake of high cocoa flavanol significantly increased the bifidobacteria and lactobacilli populations and also induced a significant decrease in the *C. histolyticum* group [116].

In vitro fermentation studies showed that catechin and EC inhibited the pathogenic and harmful bacteria (*C. perfringens, C. difficile, C. histolyticum, Bacteriodes* spp) and encouraged the growth of potential beneficial bacteria (*Clostridium coccoides-Eubacterium rectale, Bifidobacterium* spp., *Lactobacillus* spp. and *Eschericia coli*) [88], [110]. Moreover, *in vitro* fermentation with fecal inoculation representative of distal part of human gut showed grape seed flavan-3-ol fractions (GSE-monomers and GSE-oligomers) promoted the growth of *Lactobacillus/Enteroccus* and reduced the level of *Clostridium histolyticum* group [92]. Different grape seed fractions revealed different and significant effects on human microbiota in a time dependent manner and the extract containing oligomeric flavanols revealed higher inhibitory activity than those of monomers [117]. Phenolic compounds showed inhibition activity on proteolitic bacteria and decrease in its products which exhibits harmful effects in the intestine and increase in the organic acids and reduction in fecal pH *in vivo* [118], [119].

Moreover, in addition to effects of phenolic precursors, the effects of phenolic metabolites on human gut microbiota were investigated by Lee et al [110]. Growth sensitivity of gut bacteria to tea phenolics and their metabolites varied and growth of certain pathogenic bacteria such as *C. perfringens*, *C. difficile* and *Bacteroides* spp. were significantly suppressed by tea phenolics and their potential metabolites, while *Clostridium* spp., *Bifidobacterium* spp. and *Lactobacillus* spp. were less affected.

Recently, Kemperman et al [120] studied the impacts of black tea phenolic compounds on human gut microbiome in an *in vitro* gut microbial ecosystem (SHIME) and observed the profile of gut microbiota by selective plate counting, quantitative PCR, DGGE, 16S-rRNA sequencing. Pyrosequencing analysis showed a shift in the Firmicutes: Bacteroidetes ratio by stimulating *Klebsiella*, enterococci and *Akkermansia* and reducing bifidobacteria, *B. coccoides, Anaeroglobus* and *Victivallis* in black tea fermentation compared to control group.

2.4. THE ANALYSIS OF INTERACTION BETWEEN PHENOLICS AND GUT MICROBIOTA

Various *in vivo* and *in vitro* approaches have been used for the investigation of intestinal fermentations and for the analysis of interactions between phenolic compounds and gut microbiota [116], [121]. After phenolic effect on microbiota was considered, model studies for intestinal fermentation were also conducted to explain its mechanism [88], [91], [92], [120], [122].

2.4.1. *In vitro* Gut Fermentation Model Systems

In vitro fermentations correspond to an innovative technological platform, which allows the investigation of both being of microbial species and their functions. They are characterized by single or multiple bioreactors with fecal inocula maintained under human gut physiological conditions (temperature, pH, and anaerobiosis). The design and complexity of these systems have been expanded using different fecal inoculation techniques [123], [124], number and type of bioreactors, as well. Choice of the appropriate model system depends on the disadvantages and advantages of each type [6].

Currently available *in vitro* gut model systems include a range from basic batch cultures to continuous culture systems [123], [125], [126], [127], [128]. These models are illustrated in Figure 2.8. They are important because they allow ease of sampling and arrangement of

controlled variables. Selection of parallel fecal fermentations allow to asses the inter-individual variations in metabolism of microbiota [104].

A limitation of *in vitro* gut fermentation model systems is that it does not entirely represent *in vivo* conditions and the use of fecal suspension may not simulate the microbial ecosystem present in the colon where catabolism occur *in vivo*, in fact, although the accumulation of products in fermentation vessel allows ease of collection, observation, identification and quantification metabolites, it does not fully represent the metabolic events that happen *in vivo*. However, the use of *in vitro* model provides information on types of metabolic products and helps to clarify the metabolic pathways involved in the colon and allows to determine the rate of catabolism.

Another limitation of the *in vitro* fermentation model is that the reproducibility and functional stability of human gut microbiota is challenging. Inoculation and colonization of fecal microbiota in these systems is a priority. The quality and freshness of the fecal sample affect the colonization efficiency in simulated gut environment and the process of collection determines the quality of the fecal inocula. The lack of biological replication is another biased concern in these systems. For multistage continuous systems, there are difficulties concerning various sources of error (such as medium preparation and time).to carry out two parallel fermentation systems with the same fecal suspension. Using multiple donors tested in separate fermentation vessels can also assess the replication for the studies to observe inter-individual variations and host specific interactions. However, for continuous systems, it requires much time for the stabilization of fecal microbiota. Alternatively, fecal slurries pooled from different donors can be used to obtain sufficient inocula and the results are extrapolated for the population, however, this process could lead to inter-microbe interactions. Although this system favors the growth of certain microbial population; it is unrepresentative of human microbial ecosystem [6]. Fresh inoculum of fecal sample which are not pooled are generally preferred for simple static batch culture systems in order to generate the short term fermentation [91], [104].

2.4.1.1. Batch Culture Fermentation Gut Model System

Batch fermentation involves incubation of pure or mixed bacterial suspensions with a selective medium for a short period and sampling at regular intervals [129]. These are either closed systems or reactors containing fecal inoculum maintained under anaerobic conditions. The closed single systems generally represent the single part of human gastrointestinal system according to presentation of the degradation profile of selected nutrient compound (Figure 2.8). A commonly used method to study bacterial fermentation is to use selective mediums for the target gut microorganisms and measure the response of dominant bacteria. However, this method is less reliable and do not represent the whole activity of gut microflora and the technique is laborious and subjected to operator mishandling. Therefore, an approach to represent the mutual, symbiotic and competitive activity of gut microbial community was developed by the use of fecal material for the inoculum [123], [125], [126], [129].

Batch culture fermentations are particularly selected for the investigation of production of SCFAs from catabolism of carbohydrates by gut microbiota. They are convenient and cost-effective and allow the use of large number of substrates and fecal material [130], have some advantages for the quick screening of microbial metabolism and the ease of set up and parallel screening. Therefore, they have been widely applied to assess inter-individual variation in phenolic compounds bioconversion [104] or to compare the effects of different food sources [131].

However, microbial growth in these systems is limited to the inoculation density, depletion rate of substrates, and accumulation of metabolic products [6]. They can cause difficulties in microbiological control such as limited growth and accumulation of toxic potential products, thus do not allow the establishment of steady-state conditions and restrict the fermentation period.

Although, these simple, static, batch culture gut models are only adequate for simulating short term conditions in the gut and for parallel screening, for assessment of long-term adaptations of the gut microbial community more complex dynamic models are needed.

2.4.1.2. Continuous Culture Fermentation Gut Model System

Continuous culture fermentation models contain either single or multiple chemostats, permitting long term studies with no substrate depletion and removal of toxic products. They can be used to simulate the intestinal *in vivo* conditions. The optimum conditions of bacterial growth can be determined after steady state conditions are reached. Therefore, these systems are rather time consuming when compared to batch systems.

The human microbiota shows variations in metabolic activity and microbial community in each part of the colon (proximal, transverse and distal) (Figure 2.1). Continuous systems are conducted in terms of these differences, which enable the horizontal colonic process and facilitate nutritional and physicochemical properties of each colon part simulating the environmental conditions such as pH, temperature, anaerobiosis and retention time [125] (Figure 2.8). Most of the studies and results about metabolic function of human microbiota are done using multistage continuous models especially three-stage [125], [127]. Most of the prebiotic (FOS and inulin) and probiotic modulation and interaction with human microflora investigations are performed based on this approach [129], [132].

The techniques for the use of fecal inocula differ among *in vitro* continuous models. Most of the operations are done using liquid fecal suspension. However, this kind of suspension have some limitations due to the free-cell state of the bacterial populations and association with rapid washout of the inoculum, therefore, the immobilization of fecal microbiota in these continuous system are performed [127]. The polysaccharide beads used for immobilization are suspended with fecal microbiota in the first stage of continuous system representing the proximal part of colon. The distribution of bacterial cells throughout the system could be easy because of the active growth of cells and results in high cell density and populations which are close to normal human colon.

The *in vitro* coupled multistage fermentation systems including human digestive system parts (stomach, small intestine, colon and lumen) and simulation of physiological functions are called artificial digestive systems. The human digestive functions of small intestine including

bile secretion, motility, pH and absorption capacity are simulated in TIM-1 [133], whereas TIM-2 includes proximal colon functions with peristaltic mixing, water and metabolic absorption [123]. These models allow mimicking the conditions found *in vivo*. The SHIME (simulator of the human intestinal microbial ecosystem) model consists of the five stage bioreactors in which fermentation maintained in a sequential form. The SHIME model has been used to monitor bioconversion of black tea phenolics and red wine/grape juice extract [106] and to assess the modulation of human gut microbiota by black tea and red wine/grape juice [120].

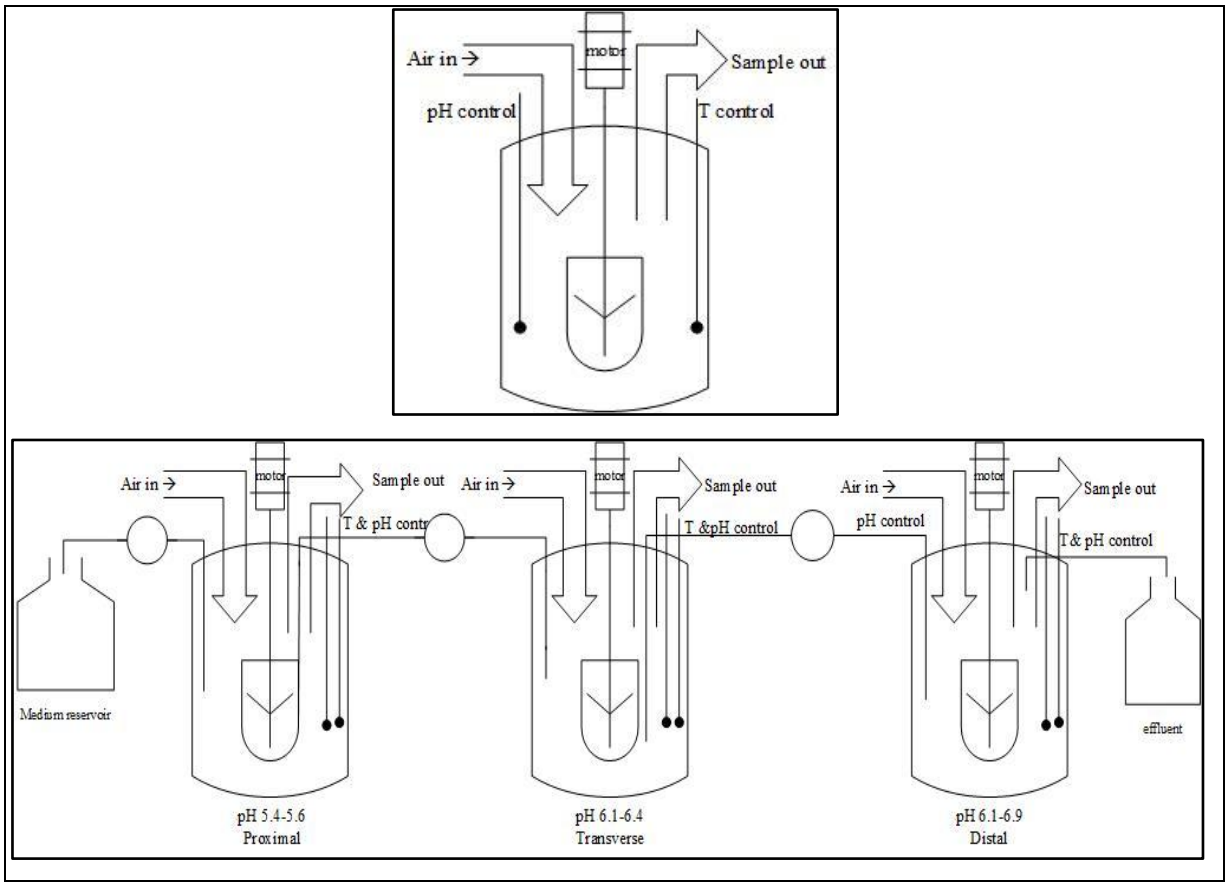


Figure 2.8. Schematic representation of types e.g batch culture closed system (above) and continuous culture systems (below) and process characteristics of *in vitro* colonic fermentation models of different regions of human gut operated at physiological section specific pH, temperature and anaerobic conditions.

2.4.2. *In vivo* Studies

In vivo studies for the investigation of the intestinal fermentation are performed with human intervention studies using healthy volunteers, hospital patients, ileostomists, sudden death victims and human associated animal models. Choice of the target population is the basic aspect of these kinds of studies. More commonly, the study and control groups are chosen from same population. At the beginning of the study, it is crucial to balance both groups at baseline for comparability. This balance can be achieved by either randomization or by reducing the placebo effect. This effect can be measured by giving a placebo treatment (inactive treatment with the same taste, texture, etc. as the active treatment) to participants who are blinded to the fact that they have not received active treatment. When only the participant is unaware, it is single blinded, if both participants and researchers are unaware about the active treatment, it is called double-blinded.

Human intervention studies are done on volunteers being subjected to a relatively long-term study (4 to 10 weeks) and while controlling their diet. Since participation is for a long period, the limitation of these kinds of studies is inadequate number of participants. The analysis of the interaction in these studies is done by collecting samples from human blood, serum, urine, fecal or gut contents, organs and tissues, which are difficult to access and are limited to the practical and ethical reasons [130]. These studies need to have special facilities and are costly and time-consuming. However, the human intervention studies are the best models for investigation of microbe-diet interaction.

Since the accession to intestinal locations in humans is difficult, the use of animal models become necessity. The animals can be the best model to control the diet and allow easy and direct access to their gut contents, tissues and organs as well. Generally, rats and mice have been used for animal models systems [96], [111], [112], [113]. However, the animal intestinal system and its physiology and metabolic activity are different than humans, thus unreliable results are likely to occur. Therefore, human microbiota associated (HMA) animals such as gnobiotic mice were developed by inoculation of human fecal microbiota into animals

intestine to mimic human gut microbial metabolism and provide a reliable model study for human microflora [134], [135], [136], [137]. However, still these do not represent the usual situation in humans. Although animal models have advantages over human model studies, they are expensive and require special handling facilities when compared to *in vitro* model systems.

2.5. ANALYSIS OF PHENOLIC METABOLITE PROFILE

Analytical profiling approaches (metabolomics and metabonomics) have been used for the assessment of the microbial metabolites of phenolic compounds in urine, plasma and *in vitro* models [106], [115], [138]–[140]. Potential methods include nuclear magnetic resonance (NMR)-based metabolite profiling [104] and liquid chromatography with electrochemical detector (HPLC-ECD), liquid chromatography with mass spectroscopy (LC-MS) [91], [122], [141] and gas chromatography with mass spectroscopy (GC-MS) [104], [105], [142]. Most recently, the metabonomic and metabolomic profiling were also developed by coupling of LC-MS, GC-MS, NMR and multivariate statistical analysis [106], [139], [143], [140],. The targeted for quantification and non-targeted for identification of undefined metabolites approaches could be applied for profiling.

For non-targeted metabolite profiling, so called metabonomics, NMR could meet requirements of non-selective detection and quantification [104], [139], [144], [145]. Previously, NMR was applied to detect the major microbial degradation products, including phenolic metabolites from black tea fecal fermentation and short chain fatty acids (SCFAs) [104] and also the metabolites in plasma and urine from black tea and green tea ingestion [139], [146], [147] and the metabolites from feces after the consumption of grape juice extract or a mix of grape juice and wine extract [145]. NMR-based metabonomic approaches contain high-resolution ^{1}H NMR metabolic profiling combined with multivariate statistics and require less pretreatment of sample and is rapid and broad for absolute structural elucidation; however, the identification of these phenolics by NMR was limited due to their low detection

levels in samples and it is an insensitive profiling technique compared to LC–MS or GC–MS [138], [139], [145] [104], [140], [142].

Improved sensitivity may be obtained using targeted detection which involves, focusing on specific compounds. MS systems may provide semi-quantification using commonly available standards and allow detection of low abundant compounds, as well [104]. These have successfully been applied for analyzing microbial bioconversion products in *in vitro* models, feces, urine and plasma [91], [141], [142] [148], [149], [150].

However, GC and LC based metabolomic approaches has some limitations as they rely on deconjugation of metabolites present in urine and plasma, extraction in terms of solid phase extraction (SPE) and liquid–liquid extraction (LLE) of the complex matrix, derivatization and/or fractionation. However, these treatments allow detection of specific compounds as desired. SPE has an advantage over LLE because it is suited for automation. However, the development and selectivity of the method is rather time consuming. LC-based methods are also becoming more widespread due to its high-resolution and highly sensitive mass spectrometric detection for non-targeted analysis [143]. It has been useful to analyze and separate thermally labile and highly polar compounds without any prior derivatization [91], [141]. LC-based metabolite profiling is widely used for analysis of phenolic metabolites in urine and plasma samples [151], and *in vitro* models systems [93]. A large range of pre-identified metabolites of phenolic compounds from *in vitro* fermentation studies could be detected simultaneously using UPLC system coupled to an electrospray interface photodiode array detector and an tandem quadrupole mass spectrometer (UPLC-DAD-ESI-TQ-MS) [91],[92]. Moreover, after consumption of black tea, for deconjugation of metabolites in urine coupling of SPE and HPLC-FTMS^n followed by HPLC-TOFMS-guided SPE-NMR of selected compounds for their structural elucidation were applied for the analysis of metabolites [140].

Due to its high separation, sensitivity and advanced mass spectral libraries, GC–MS is preferred for profiling of (unknown) metabolites. GC systems analyze volatile and semi-

volatile compounds, which undergo a phase transition into intact gas-phase carriers. The compound must be able to resist high temperatures and rapidly transformed into gas phase without degradation or reaction with other compounds. Since GC analysis is dependent on volatility, compounds with multiple polar groups that show poor volatility due to intermolecular interactions must be derivatized. The derivatization reactions commonly used are silylation, acylation, and alkylation-esterification. Phenolic acids should be derivatized before GC–MS analysis. Trimethylsilylation (BSTFA) is the most commonly used method for derivatization due to its low reactivity [104], [142], [150]. GC-MS based profiling was applied to detect the microbial degradation products from black tea in an *in vitro* fecal fermentation [104], [105], [106] and the urine, plasma and fecal samples collected from humans after consumption of green tea and/or black tea were analyzed for metabolite profiling in both a targeted and non-targeted manner by GC-MS [102], [142], [149], [150].

NMR, GC-MS and LC-MS based profiling were capable to identify and quantify the microbial products at micromolecular levels. However, while low abundant phenolic acids were only detected by GC-MS, highly abundant phenolic acids that were in the detection limit of GC-MS were determined by NMR spectroscopy [104]. *In vitro* batch culture fermentation of black tea phenolics, 3-phenylpropionic acid, 3-(3-hydroxyphenyl)propionic acid, 3-(4-hydroxyphenyl)propionic acid were observed as typical end metabolites and also, the conversion from 3-(4-hydroxyphenyl)propionic acid and 3-(3-hydroxyphenyl)propionic acid to 3-(phenyl)propionic acid occurred in some subjects [104]. In another GC-MS metabolite profiling study of black tea phenolic fermentation, it was observed that the main fermentation product was 3-methyl-4-hydroxyphenylacetic acid with smaller amounts of 4-hydroxyphenylacetic acid, 3,3-hydroxyphenylpropionic acid, 2,4-dihydroxyphenylacetic acid and 2,4,6-trihydroxybenzoic acid [105]. Unlike to this study, Roowi et al [102] found 3-methyl-4-hydroxyphenylacetic acid in urine samples not fecal samples in the GC-MS profiling of metabolites in humans after ingestion of green tea. The profile of metabolites in urine differs from fecal fermentation metabolites observed by NMR spectroscopy after the ingestion of green tea and mostly consists of valerolactone and valeric acid conjugates and hippuric acid [140]. Valerolactones and valeric acids were mostly observed in *in vitro*

fermentation of grape seed and wine extracts consisting of monomeric and dimeric flavanols by LC-MS based profiling [86, 88]. In the study of van Dorsten [106], while LC-MS based profiling were performed for the kinetic degradation of phenolic precursors and selectec phenylvalerolactones ((-)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone), GC-MS profile analysis was used for the determination of targeted metabolite profile of black tea phenolics after the fecal fermentation by SHIME model system. Gallic acid, pyrogallol, 3,4-dihydroxyphenylpropionic acid, 4-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid and 4-hydroxyphenylacetic acid were detected during fermentation by GC-MS [106].

2.6. ANALYSIS OF HUMAN GUT MICROBIOTA

Human gut microflora includes diverse and complex microbial community, thus has intense metabolic activity related to human health. The analysis of intestinal microbiota and its ecosystem is required to manage and maintain human health. The composition of the human intestinal microbiota has been extensively studied, by means of culture-dependent techniques and, more recently, culture-independent technologies such as metagenomic analysis. The amplification and direct sequencing of small subunit ribosomal DNA (16S rDNA) sequences has been increasingly developed [9], [17], [152], [153], [154], and discussed by highlighting the uncultured species in recent reviews [7, [155], [156]. The majority of identified species by rRNA sequencing belong to the uncultured and novel microorganims [152]. The omic technologies includes DNA-microarrays (such as Human Intestinal Tract Chip (HITchip)) [157], [158], [159], fluorescent in situ hybridization (FISH) [121], [160], [161], and methods such as PCR coupled to denaturing gradient and temperature gradient gel electrophoresis (PCR-DGGE and PCR-TGGE) [162] [163] and quantitative PCR (qPCR) [9, [164], [165] and pyrosequencing, based on whole genome sequencing [16], [17], [120], [154], [166].

These methods were developed to define the microbial community in human gut and its relation with diet and health. In recent years, the projects performing gut metagenomic studies such as European Metagenomics of the Human Intestinal Tract (MetaHIT) and the US Human Microbiome Project (HMP) have been developed to understand the interaction of human

microbiota, diet and health and intense functional diversity [12], [14], [167]. Rather than identification species levels, the core gene catalogue was established to understand common functionality of microbiota [12], [10], [134].

Although culture dependent techniques may give information about the functionality of cultured species, they have some limitations resulting in the underestimation of bacterial population sizes and microbiota diversity [168], [169]. Moreover, human gut microbiota comprises of many anaerobic bacteria, which are sensitive to long storage and transportation, therefore significantly reduced microbial viability [130]. On the other hand, gene sequencing approaches characterize complex microbial communities more comprehensively and have an advantage over culture-dependent methods of providing more information about commensal and mutualistic microorganisms, and human pathogens, as well [15]. Moreover, the recent developments in high-throughput technologies such as next generation sequencing and phylogenetic microarrays allow more inclusive analysis of phylogenetic diversity of human gut microbiota.

HITchip is a phylogenetic microoarray based on the small subunit ribosomal RNA gene and it was used for the identification of over 1000 phylotypes in a semi-quantitative manner [157]. More recently, it was used to evaluate the microbial diversity in TIM-2 model [158], the stability of microbiota in a short and long term monitoring [159] and to assses the inter-individual variation in the metabolism of black tea and wine/grape phenolics in an *in vitro* fermentation model, attributing the microbial diversity [104].

16S-rDNA sequencing and qPCR and PCR coupled to DGGE were established to assess both the stability of microbiota in a simulator of microbial ecosystem [162] and the influence of dietary changes on microbiota composition in both in *in vivo* [9] and *in vitro* studies [170]. To investigate the interactions between complex dietary phenolic compounds and the gut microbiota, a combination of culture independent techniques including PCR-DGGE, qPCR and pyrosequencing of the 16sRNA gene were applied to identify the gut microbial community [120]. These studies essentially targeted not only direct microbial identification

but also either well-known groups or comparative profile [137]. Characterization of the genes involved in specific metabolic pathways can give an information about functional capabilities of a complex community, however, since RNA, protein and metabolite profiling information were not involved, functionality still remains unknown.

16S-rRNA targeted FISH probes can provide insights into ecologic roles of individual species in the complex microbial ecosystem [160]. rRNA-targeted oligonucleotide probes can also disclose the localization and morphology of cells *in situ* in which the bacterial cells can be detected in their natural habitat. Quantification of the signal obtained by FISH probes may result in the estimation of *in situ* growth rates of individual species [161], [171]. However, this method requires visual counting of microorganisms in mixed community and it is rather time-consuming and dependent on the skills of operator, thus accuracy levels could hardly be reached [172]. Due to those limitations, the automated counting of cells was developed [173]. The fluorescent microscopes has some limitations when the cells are immobilized in or on fluorescent probe and produced biofilms on the slides; therefore, to overcome this problem, laser confocal scanning microscopy could be the solution with 2-D or 3-D images [161], [174] and more recently when the sample is consisted of suspended microorganisms, the flow cytometry could be the solution for the rapid quantification of florescent cells [175]. Moreover, for quantification of cells, digital image analyses were developed [174].

FISH probes have been used to quantify a set of predominant anaerobic bacteria in human fecal slurries [175], [176], [177]. They have been commonly used to study fecal fermentation of carbohydrates in bioreactor systems [178]. Moreover, more recently, they have been used to understand the interaction of human gut microbiota and phenolic compounds such as anthocyanins, flavanols, grape seed flavanols and red wine extract *in vitro* fermentation systems [88], [91], [92], [122] and *in vivo* systems [116], [163].

In the presence of wine extract containing anthocyanins, monomeric and dimeric flavanols in the fermentation of fecal slurry, FISH results showed that *Bifidobacterium* spp., *Lactobacillus/Enterococcus* spp., *Bacteroides* spp., and total bacteria remained constant

during fermentation period and slight but not significant inhibition were observed for *C. histolyticum* [91]. Unlikely, in the presence of monomeric flavanols cocoa flavanols and monomeric and dimeric flavanols in grape seed extract using the same batch fermentation, a positive effect were observed for *Lactobacillus/Enterococcus* spp., [88], [92], [116]. Moreover, malvidin-3-glucoside had significant positive effects on total bacteria *Bifidobacterium* spp., and *Lactobacillus/Enterococcus* spp. and negative but not significant *C. histolyticum* and gallic acid decrease the growth of *C. histolyticum* and *Bacteroides* spp. [122]. No FISH analysis results were establihed for black tea extract. However, selective counting, qPCR and pyrosequencing were generated for black tea extract in SHIME system [120], and the results showed that although selective counting did no reveal conclusive effect, qPCR analysis showed that while numbers of Bacteroides and total bacteria did not change during fermentation, *B. coccoides* group and the bifidobacteria decreased and on the pyla level, a shift between Firmicutes:Bacteroidetes ratio and black tea had a stimulatory role *Klebsiella,* enterococci and *Akkermansia* and reduced bifidobacteria, *B. coccoides, Anaeroglobus* and *Victivallis*. However, the analysis on DNA sequencing is a different approach than FISH and bacterial counting to understand the funtionality.

2.7. AIM OF THE STUDY

The primary aim of this study was to investigate the metabolism of black tea phenolics by intestinal microbiota in a pH-controlled, stirred, batch-culture fermentation system in a simulated human large bowel environment. The changes in the structure of tea phenolics in this competitive bacterial environment and their ability to change the balance of bacterial groups were also studied.

3. METHOD DEVELOPMENT FOR *IN VITRO* FECAL FERMENTATION OF BLACK TEA PHENOLICS

3.1. INTRODUCTION

Human gut microbiota plays an important role in the metabolic, nutritional, and physiological processes in the human body due to multiple enzymatic activities [23], [71]. Dietary substances that escape digestion by host enzymes are the substrates for intestinal microbiota. Phenolic compounds which are abundantly present in the diet, can be absorbed and exposed to alterations in their chemical structure by gut microflora. Interaction of gut microbiota and phenolics was observed in many *in vivo* animal and human studies [116], [139] and with *in vitro* fecal fermentation cultures, as well [78], [88], [122].

Tea leaves (*Camellia sinensis*) contain a variety of phenolic compounds with the main phenolics being the monomeric flavanols (catechins). However, during black tea processing, catechins are oxidized enzymatically, resulting in the formation of dimeric (theaflavins) and polymeric (thearubigins) compounds [40], [48]. For example, water extracts of commercially available green teas in US contained 0.44-10 % wt/wt total catechins and no theaflavins, total catechin and theaflavin content of black tea extracts were in the range of 0.54-6.95 % wt/wt, 0.07-0.9 % wt/wt [42]. In the study of Serpen et al [44], total thearubigin, theaflavin and catechin content of seven grades of Turkish black tea leaves were in the range of 5.92-6.83 %, 0.14-0.42 % and 1.83-2.25 %, respectively.

Although the consumption of black tea is very high, tea brewing habits vary among different countries. Tea solids intake consequently amount of phenolic compounds from tea leaves per cup can thus differ considerably as a result of brew time and initial phenolic concentration of black tea leaves [179], [180], [181].

In vitro fermentations allow the investigation of both existence of microbial species and their functions in the human colon. They are varied from simple batch systems including test tube fermentation to multiple bioreactors with fecal inocula operated under human gut physiological conditions (temperature, pH, and anaerobic conditions). Selection of the appropriate model system depends on the disadvantages and advantages of each type [6].

In this chapter, method development (for sample preparation methods, and analytical methodology) was carried out for the study of *in vitro* metabolism of black tea phenolics by human gut microbiota using fecal suspensions using a static culture system.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals

The reagents for extraction and spectrophotometric assays, ethyl acetate, methanol (HPLC grade, VWR, Belgium), oxalic acid (Merck, Germany), $NaHCO_3$ (Bioshop, Canada) acetic acid (analytical grade), Na_2CO_3 (ACS grade), Folin-Ciocalteu phenol reagent (2N) and gallic acid (ACS grade, ≥ 98 %), were purchased from Sigma Aldrich Co. (USA), unless otherwise indicated. EDTA (Ethylenediaminetetraacetic acid disodium salt, dihydrate) (ACS grade, ≥ 99%), L-ascorbic acid (98%) and the standards used for HPLC analysis, (+)-catechin (C) (≥ 99.0%), gallic acid monohydrate (GA) (≥ 98.0%), caffeine (≥ 99.0%), (-)-epicatechin (EC) (≥98%), (-)-epicatechin gallate (ECG) (≥98%), (-)-epigallocatechin (EGC) (≥ 95%), (-)-epigallocatechin gallate (EGCG) (≥ 95%), (-)-catechin gallate (CG) (≥ 98%), (-)-gallocatechin gallate (GCG) (≥ 98%), tea extract from black tea (≥ 80% theaflavin-TF-basis), were purchased from Sigma Aldrich Co. (USA). HPLC grade acetonitrile and acetic acid were purchased from Fisher Scientific (UK) and JT Bakers (USA), respectively. Peptone water (Merck, Germany), yeast extract (Sigma, France), NaCl (Merck, Germany), dipotassium hydrogen phosphate (K_2HPO_4) (VWR, Belgium), monopotassium phosphate KH_2PO_4 (Bioshop, Canada), $MgSO_4.7H_2O$ (Merck, Germany), $CaCl_2.6H_2O$ (Sigma, Crotia), Tween 80 (Bioshop, Canada), hemin (≥ 98%, Sigma, Netherlands), vitamin K1 (≥ 99%, Sigma,

Switzerland), L-cysteine (Merck, Germany), bile salts mixture 3 (Acumedia, USA), resazurin sodium salt (Sigma, USA) were used for fermentation medium. Sterilization of media and instruments was achieved by autoclaving at 121°C for 15 min. Milli-Q-water from a Millipore Water Purification System (Milli-Q A10, France) was used for all the experiments.

3.2.2. Preparation of Black Tea Samples

Packed bulk black tea leaves were bought from a local market in Istanbul, Turkey. In order to determine the concentration of tea phenolics to use as substrate in fermentation experiments different preparation methods (method 1: extraction, method 2: extraction, drying and reconstitution) were tested. Firstly, to get the maximum extractable phenolic compounds from black tea leaves, the extraction conditions were optimized by brewing black tea with 100 mL double distilled boiling water in a water bath using different sample to solvent ratio and infusion (brewing) time as seen in Table 3.1. The total phenolic content, total theaflavin and thearubigin contents and phenolic profile of the extracts were determined using Folin-Cioccalteu assay, Roberts and Smith method, and HPLC method, respectively as described in Sections 3.2.4.1, 3.2.4.2 and 3.2.4.3. As no phenolics could be detected using HPLC analysis of these extracts when the extracts were diluted 1:10 in basal medium (for the fermentation study), a second method which included extraction, drying and reconstitution was investigated to increase the concentration of tea phenolics in the samples.

Table 3.1. Black tea extraction parameters tested (Method 1)

Sample name	**Amount of tea leaves (g)**	**Brewing time (min)**
A1	2	10
A2	2	20
B1	4	10
B2	4	20
C1	6	10
C2	6	20

In method 2, selected extracts were dried (using spray-drying and freeze drying) and used as described in Gross et al [104]. Briefly, 3125.0 mg powder tea extract was dissolved in 10 mL of distilled water and then stored at 4°C until further use. Samples were diluted to 1/100 (v/v), in basal medium to a final concentration of 3125 mg/L.

3.2.3. Fermentation Experiments

3.2.3.1. Fecal Sample Collection

The fecal samples were collected freshly just before the experiment from one healthy male volunteer with no history of any gastrointestinal disease who had not used antibiotics for at least 6 months prior to the study. Samples were put into bags containing anaerobic phosphate buffer (0.1 M, pH 7.4, containing 0.5 g/L cysteine) and diluted 1:10 (w/v) with same buffer before being homogenized in a stomacher for 2 min. Then, the fecal slurry was immediately inoculated into the fermentation vessels.

3.2.3.2. In Vitro Static Culture Fermentation

The fecal fermentation of Turkish black tea phenolics was performed in falcon tubes filled with 26.7 mL basal medium [88] containing peptone water (2 g/L), yeast extract (2 g/L), NaCl (0·1 g/L), K_2HPO_4 (0·04 g/L), KH_2PO_4 (0·04 g/L), $NaHCO_3$ (2 g/L), $MgSO_4.7H_2O$ (0·01 g/L), $CaCl_2.6H_2O$ (0·01 g/L), Tween 80 (2 ml/L), hemin (50 mg/L), vitamin K_1 (10 mL/L), L-cysteine (0·5 g/L), bile salts (0·5 g/L), resazurin (1 mg/L) and distilled water. The pH of the basal medium was adjusted to 7·0 and it was autoclaved before dispensing it to the tubes. Medium was kept at 37°C in anaerobic jars overnight in order to mimic conditions located in the distal region of the human large intestine (anaerobic; 37°C; pH about 7.0). Three ml fecal slurry samples (1:10, w/v) and 0.3 ml tea extract as substrate (S1) were added and batch cultures were run under anaerobic conditions in anaerobic jars for 48 h at 37°C. During fermentation, samples were collected (one tube for each time interval) at four time points (0, 10, 24 and 48 h) and centrifuged at 2000 g for 10 min. The pellets were discarded and the supernatants were stored at -20°C until analysis. The control experiment was performed under the same coonditions by incubating fecal slurry without tea extract (S2) and without addition

of fecal slurry (S3). The fermentation experiment was performed in duplicate with the fecal samplecollected from two different days from the same person.

3.2.3.3. pH Measurement

The pH of cultures was measured with a Meterlab, pHM210 model pH meter (Radiometer Analytical, France) at analyzed time points (0, 10, 24, 48 h)

3.2.4. Analysis of Phenolics

In order to determine which methods to use for the determination of phenolics in fermentation experiments, and to optimize method steps for best analytical performance the fermentation samples were analyzed for their total phenolic content using Folin-Ciocalteu assay, total theaflavin and thearubigin content using Roberts and Smith method and phenolic profile using HPLC.

3.2.4.1. Analysis of Total Phenolic Content

Total phenolic content of the extracts were determined using Folin-Ciocalteu assay modified by Ainsworth and Gillespie [182]. Gallic acid (0.2 to 0.02 mg/mL) was used as standard. The black tea samples were diluted with water and 100µL of sample was mixed with 200 µL of 10 % Folin-Ciocalteu reagent into microcentrifuge tubes and vortexed thoroughly. Eight hundred µL of 700 mM Na_2CO_3 was then added to each tube and the tubes were incubated for 2 hours in dark. After incubation, 200 µL were transferred to 96 well-plate and the absorbance of each well was read at 765nm using a microplate reader (Thermo Scientific, MultiskanGo, Finland) in duplicate. Water was used as blank. The results were expressed as miligrams of gallic acid equivalents (GAE) per mL extract.

3.2.4.2. Analysis of Thearubigin and Theaflavin Content

The method of Roberts and Smith [48] was used to determine thearubigin (TR) and theaflavin (TF) contents of the samples. The original method was modified (by reducing the volume of sample and dilutions by 10, accordingly) using black tea extracts (obtained using different

brewing time and initial tea leaves amount as described in Table.3.1) to enable analysis of lower sample volumes. The modified method was then applied to fermentation samples to test its performance.

In the original method, 9 grams of black tea leaves were extracted in 375 mL boiling water by boiling for 10 min. Five mL of tea extract was then mixed with 5 mL of ethyl acetate and vortexed for 5 min in test tubes. To break down the emulsion, the tubes were centrifuged at 3000 g for 5 min and then the layers were separated by removing the ethyl acetate layer using pasteur pipettes, leaving the aqueous layer. 0.4 ml and 2.5 ml of ethyl acetate layer was taken into two different tubes. 0.4 ml of ethyl acetate layer were diluted to 2.5 mL with methanol (Solution A, E_A).

2.5 ml of ethyl acetate layer was mixed with 2.5 mL of 2.5 % $NaHCO_3$ solution and shaken for 30 sec, centrifuged at 3000 g for 5 min. The aqueous layer was discarded immediately after separation and 0.4 mL of ethyl acetate layer was taken and diluted to 2.5 mL with methanol (Solution C, E_C).

From aqueous layer which was first separated from ethyl acetate, 0.2 mL was taken and mixed with 0.2 mL of saturated aqueous solution of oxalic acid and 0.6 ml distilled water. The mixture was then diluted to 2.5 mL with methanol (Solution D, E_D).

The absorbance of each sample was measured at 380nm using methanol as blank in UV-VIS spectrophotometer (Thermo Scientific, USA) in duplicate.

According to earlier procedures, the mean absorbance of TR and TF fractions at 380 nm was 0.733 and 2.25, respectively. The following equations (Equations 3.1 and 3.2) were used tocalculate TR % and TF % as in the study of Obanda et al [49] and modified according to extraction and sample amount and volume used in this study.

$$TR\ (\%)=\frac{100\ x\ 0.02\ x\ 6.25\ (2E_D+\ E_A-\ E_C)}{0.733\ x\ m} \quad (3.1)$$

$$TF\ (\%)=2.25\times E_C \quad (3.2)$$

E_A-E_C represents the absorbance due to the ethyl acetate-soluble free acid TR of SI type, where EC represents absorbance of aqueous sodium hydrogen carbonate (NaHCO3)-insoluble TF. Similarly, the value ED represents absorbance of the ethyl acetate-insoluble TR of SII type after acidification with oxalic acid; m is the amount of initial black tea leaves in grams using in black tea extractions.

3.2.4.3. HPLC Analysis

The phenolic compositions of batch culture mixtures were determined using HPLC-PDA (Thermo Scientific, USA) with a Phenomenex Luna phenyl-hexyl column (4.6 mm x 250 mm, 5µm) according to ISO 14502-2:2005 [180]. Solvent A (9 % acetonitrile, 4 % acetic acid and 20 µg/mL EDTA) and solvent B (80 % acetonitrile, 4 % acetic acid and 20 µg/mL EDTA) were used as mobile phase. The parameters of the method were solvent gradient (A): 100% at 0 min, 68% at 15 min, 68% at 25 min, 100% at 35 min. Column temperature was set to 35°C. Flow rate was 1 mL/min and 10 µL of the sample was injected. Chromatograms were recorded between 200−700 nm and the eluent was monitored at 278 nm. Identification of the phenolics was done by comparing the retention times of the sample peaks with those of standards. Quantification of catechins and gallic acid was carried out using standard calibration curves. While catechin and gallic acid amounts were expressed as micrograms per mL culture mixture, theaflavin content was expressed as area %.

Ethyl acetate extraction prior to HPLC analysis was tested to clean up the samples and improve HPLC performance. Two hundred µL fecal supernatant sample was dissolved in 800 µL 0.1M sodium acetate buffer. Samples were acidified by 200 µL acetic acid. One g NaCl was added for separation. Subsequently, 3 mL of ethyl acetate was added and the tubes were vortexed aggressively for 1 min, followed by centrifugation at 1500 g for 10min. The

supernatants were transferred, and the extraction was repeated. Extracts were combined and dried under a stream of nitrogen. The dried samples were dissolved in 1 mL stabilizing solution, vortex-mixed and filtered through a 0.45 μm regenerated cellulose filter and transferred into HPLC vials.

3.2.5. Statistical Analysis

Data were subjected to one way analysis of variance (ANOVA) and Tukey test to determine significant differences ($p < 0.05$) among samples in total phenolic, TR and TF content.For the changes in phenolic precursors with time, data were analyzed using a two-factor repeated-measures analysis of variance with time and treatment as the two factors and Tukey test for multiple comparison with time within same treatment Two-sample t-test were applied for comparison of R1 and R3 (control) at the same time. All statistical analyses were performed with Minitab version 16.

3.3. RESULTS AND DISCUSSION

3.3.1. Black Tea Sample Preparation

In order to determine phenolic content of tea extract to use as a substrate in static cultures, different extraction conditions (method 1) were tested. The total phenolic (TP) contents of six black tea infusions using different sample to solvent ratios and infusion times are given in Table 3.2. When a high amount of tea leaves was used in the extractions (6 g, samples C1 and C2), reliable results could not be obtained due to cream formation and condensation, therefore these results were not included in the analysis. The total phenolic contents of black tea infusions were in the range of 44.36-58.37 mg GAE/g tea leaves however the differences were not significant ($p>0.05$). The concentration of water extracts obtained using 4 g tea leaves (1.77-1.88 mg GAE/ mL extract) were significantly higher ($p<0.05$) than those obtained using 2 g tea leaves (1.03-1.17 mg GAE/ mL extract)

Table 3.2. Total phenolic contents of six different black tea extracts

Samples	Total Phenolics[a] (mg GAE/ g tea leaves)	Total Phenolics[a] (mg GAE/ mL extract)
A1	51.47±6.19a	1.03±0.12a
A2	58.37±3.88a	1.17±0.08a
B1	44.36±1.39a	1.77±0.06b
B2	46.91±1.63a	1.88±0.07b
C1	37.00[x]	2.220[x]
C2	33.89[x]	2.033[x]

[a]Data were expressed as mean ± SD (n = 2) on gallic acid equivalent (GAE), followed by the same letter, within a column, are not significantly different ($p > 0.05$).

[x]Data represented without replicate.

The total thearubigin and theaflavin contents of six black tea extract combinations are given in Table 3.3. The highest TR% was obtained for sample A2 obtained using extraction of 2 g tea leaves for 20 min, and A1 also had higher values, however the differences between TR% of samples were not significant ($p > 0.05$). TF % of B1 and B2 samples was the highest but not significantly different than A1 ($p > 0.05$). The leaf to water ratio used in Roberts and Smiths [48] (3:125 (w/v)) which is closer to 2:100 (w/v) ratio, was estimated to get the maximum amount of extracted TR and TF present in black tea. Although this support the TR % results but not TF % content.

Based on these results, the highest concentration of total phenolics and TF were obtained for samples B1 and B2. However as the phenolic profile of these extracts could not be determined using HPLC analysis when they were diluted in basal medium (1:10) as in the fermentation experiments, a second method including extraction, drying and reconstitution steps was investigated to increase the concentration of tea phenolics in the samples.

Table 3.3. The total thearubigin (TR %) and theaflavin (TF %) contents of six black tea extract combinations with modified method

Samples	TR %	TF %
A1	8.78±1.22a	0.17±0.05ab
A2	10.06±0.13a	0.15±0.03b
B1	8.67±0.39a	0.28±0.00a
B2	8.60±0.54a	0.28±0.00ab
C1	7.92[x]	0.35[x]
C2	7.96[x]	0.40[x]

[x]Data represented without replicate.

[a,b]Data were expressed as mean ± SD (n = 2), followed by the same letter, within a column, are not significantly different (P > 0.05)

For method 2, several batches of black tea infusions were prepared using 2:100 (w/v) leaf to solvent ratio with 10 min brewing time (as this is the most commonly used extraction condition in the literature [180], [181] and freeze dried. The concentration of inoculation was determined to be 3125 mg/L as described in Gross et al [104]. The 3125 mg freeze-dried extracts were initially reconstituted in 10 ml water as a stock solution and diluted in basal medium, but because of the fiberous structure of the dried extracts, solubility problem occured at stock concentration. To overcome solubility problem, spray drying was used to get smaller size of particles than freeze dried extracts [104]. The visual observation indicated that spray dried extract were dissolved and it created better solution than freeze dried one. The fermentation experiments in Section 3.2.2 were done immediately after preparation of stock solution. However, for further experiments it was observed that cold storage of stock solution created precipication and insolubility in the extract

3.3.2. Analysis of Phenolics

As a preliminary work, TP analysis was initially applied to the static cultures at analyzed time points to assess the changes in the total phenolic content of static cultures (A1 sample diluted as 1:10) during fermentation. While TP content of black tea extract (A1) at 0 h time point was as 0.14±0.01 mg GAE / mL batch culture sample, TP content of basal medium without A1

addition were found as 0.10±0.00 mg GAE / mL batch culture sample. Moreover, TP content of undiluted black tea extract was lower than TP content of the extracted diluted with basal medium. (1.03±0.12 mg GAE / mL extract), indicating reactions involving the ingredients present in basal medium. Oxidation substrates in the sample other than phenolics can interfere with the Folin-Ciocalteu assay resulting in inhibitory, additive or enhancing effects [182] Enhancing effects could be from phenols, aromatic amines, high sugar levels, ascorbic acid, sulfites and sulfur dioxide which are also react with the F–C reagent [183]. Basal medium used in this study contains high sugar levels and sulfites (Section 3.2.3.2). Therefore, the Folin-Ciocalteu assay was not carried out for the batch cultures; however, it was used for the optimum extraction conditions.

Black tea contains important dimeric and polymeric phenolic compounds, TF and TR, in addition to catechins. While the HPLC method provides an estimate of the TF content, it does not provide any information on the TR content. Application of a spectrophotometric analysis method (Roberts and Smith method) to the analysis of fermentation samples was tested to be able to investigate the metabolism of TF and TR by gut microbiota in *in vitro* fermentation.

Since static culture fermentations were carried out in small volumes (30 mL), the original method needed to be modified to be able to analyze smaller sample volumes. Initially, the method of Roberts and Smith [48] was tested in this study and optimized for smaller volumes using black tea extracts (Table 3.4). TR and TF contents of the black tea extracts were found as 11.16±1.23 % and 0.24 ±0.08 %, respectively when the standard method was applied. While intraday CVs in thearubigin content were around 10 %, higher CVs were observed in theaflavin content (Table 3.3) due to the small amount of theaflavins analyzed. In the modified method, the TR and TF content were found as 9.98±0.98 % and 0.17±0.10 %, respectively. There was no significant difference in TR content of samples analyzed by both methods ($p>0.05$). These findings indicate that the modified method can be used to determine the TF/TR content of black tea extracts.

When TF/TR method was applied to static cultures to monitor the changes during fecal fermentation of blakc tea extracts, TF/TR contents of fermentation sample at 0 h were higher than that of black tea extract. Moreover, the control sample without black tea addition at 0 h showed absorbance at 380 nm (data not shown) bacause sugars and some proteins such as L-cysteine which are present in basal medium are also compatible with ethyl acetate, resulting in misleading readings. Therefore, the method could not be applied efficiently for batch cultures due to the non-specific nature of ethyl acetate extraction and the insensitivity of this method and no representative results were observed for the static cultures (data not shown).

Table 3.4. Variations in TR and TF (%) content of black tea extracts

	Roberts and Smith[a]			Modified method[b]		
	Mean	**SD**	**CV(%)[c]**	**Mean**	**SD**	**CV (%)[c]**
TR (%)	11.16	1.23	11.06	9.98	0.98	9.83
TF (%)	0.24	0.08	35.44	0.17	0.10	56.73

[a]Method was performed as described in Roberts and Smith [48](1961).

[b]Method was performed with modifications to decrease sample volume.

[c]Intraday CVs represent data from triplicate analyses of each infusion performed on the same day.

3.3.3. HPLC Analysis

Before HPLC analysis, samples S1 and S3 were pretreated with liquid-liquid extraction (LLE) using ethyl acetate to clean up the sample. The changes in the phenolic profile of static cultures (S1) in the presence and absence of of gut microflora (S3) over a 48 hour period and the stability changes in basal medium in 48 h at 37°C under anaerobic conditions are given in Figure 3.1. GA, EGCG, ECG and TF were detected in S1 and S3 samples. While phenolic compounds underwent degradation with colonic microflora, control samples (S3) under the same condition were relatively stable (Figure 3.1).

The composition of spray dried black tea extract were analyzed by HPLC and GA, EC, EGCG, ECG and TF were detected (Appendix A.1). Spray dried black tea extract stock solution (3.125 g/10 mL) were diluted at used concentration in batch cultures (3125mg/L),

treated with LLE and analyzed by HPLC. However, unlike dried sample analysis, only GA, EGCG, ECG and TF were detected in pretreated stock solution. The LLE was used to get better chromatographic resolution in fermentation samples and to substract the basal medium which resulted in integration difficulties in preliminary experiments.

Total catechins and individual catechins underwent rapid degradation in the first 10 h and none of them was observed after 24 h of fermentation (Figure 3.1.a, b, c). The degradation of total catechins and individual catechins (EGCG and ECG) was significant with time ($p \leq 0.05$) and the degradation pattern was significant, as well when compared to those in control sample (S3) ($p \leq 0.05$). However, they were not completely degraded until the end of incubation in S3 samples. For the first 10 h of incubation in S3 samples, total catechins degraded from 7.57 µg/mL to 3.84 µg/mL, but 1.68 µg/mL of catechins remained until the end of incubation (Figure 3.1.a). EGCG were also degraded from 6.32 µg/mL to 2.72 µg/mL (43 % remaining) in 10^{th} h of incubation and only 1.04 µg/mL (16 % of its initial concentration) remained after 48 h (Figure 3.1.b). On the other hand, ECG underwent only 50 % degradation (change from 1.25 µg/mL to 0.65 µg/mL) at the end of 48 h period (Figure 3.1.c). There are some factors such as temperature, pH, oxygen availability and the presence of metal ions and other ingredients that influence the degradation of catechins in basal medium [55], [58]–[60] , [60]–[62]. pH values used in this study might contribute the degradation rate of catechins. (pH>5.0) [63]. EGCG and EGC have lower stability than EC and ECG due to their susceptible hydroxyl group position [67]. In this study, EGCG was less stable than ECG, similar to the results of Su et al [65]. EGCG was completely destroyed at the end of 6 h incubation at pH 7.4 whereas concentration of ECG and EC decreased by 20 and 5% after 3h, respectively. The presence of oxygen and metal ions will lead to oxidation however, since this study was carried out under anaerobic conditions, oxidation of catechins was eliminated. Below 44°C, degradation of catechins was observed and EGCG was degraded by 10 % of its initial concentration at room temperature after 2 days of incubation at pH 5 while in our study the degradation of EGCG was faster after 2 days [58]. The degradation profile of catechins in S1 and S3 for the same time did not reveal significant effects ($p>0.05$) This also implies that beside time effect on

degradation of catehcins, high pH values and changes in the pH values during incubation might lead the degradation of catechins by gut microbiota as well. (Table 3.5)

Gallic acid was significantly degraded in first 10 h of incubation from 28.87 µg/mL to 0.63 µg/mL(remaining 2 % in concentration) in S1 samples and no gallic acid was observed after 48 h ($p \leq 0.05$) (Figure 3.1.d), resulting in a significant difference than the S3 samples at 24^{th} h ($p \leq 0.05$). Gallic acid was also degraded in S3 samples from 24.58 µg/mL to 19.33 µg/mL (46 % remaining) after 24 h, however, unlike S1 samples, there was an accumulation with a large variation due to the degradation of gallated compounds (EGCG and ECG) and theaflavins after 48 h fermentation with 58 % remaining of its initial concentration (Figure 3.1.d).

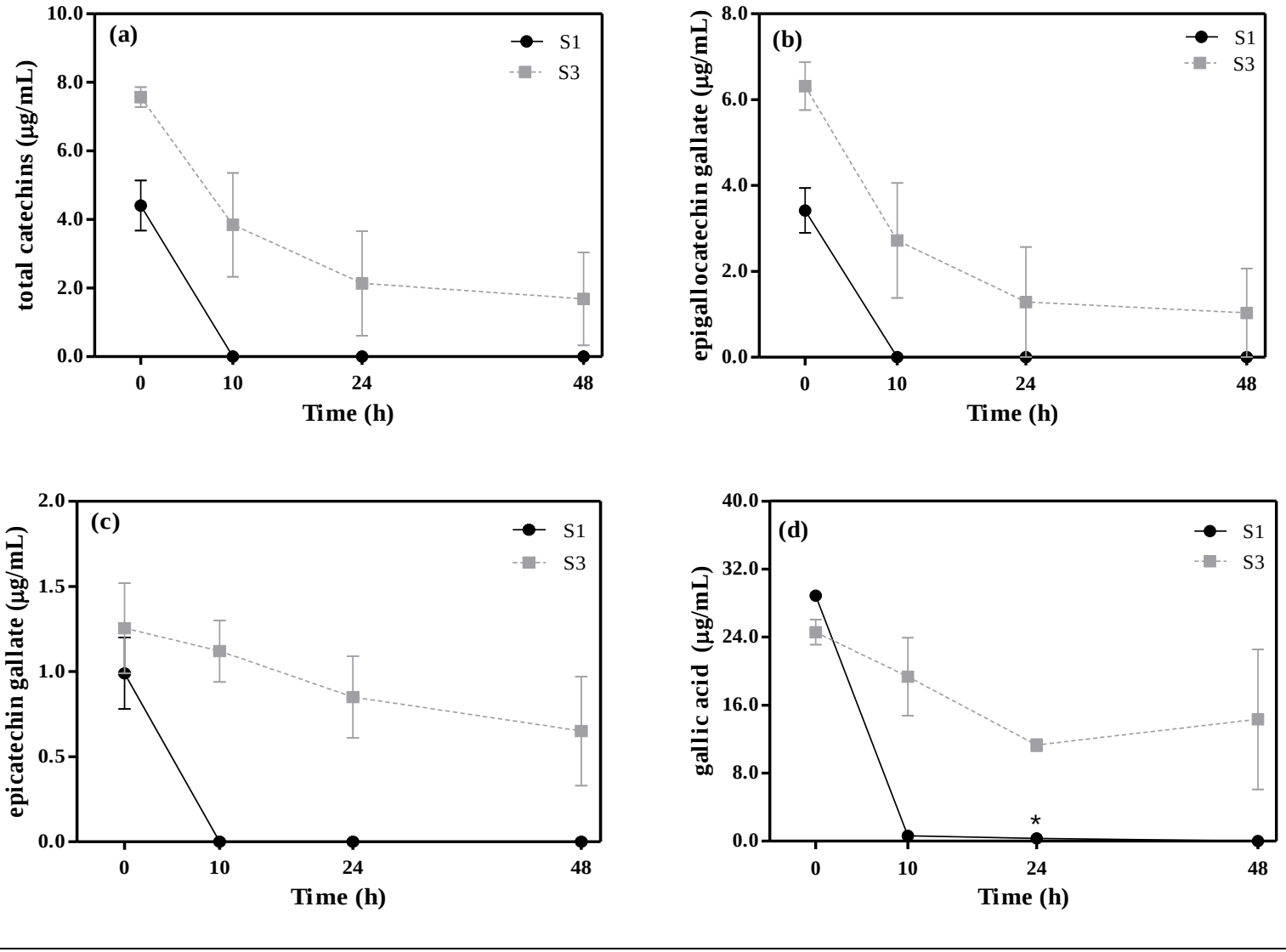
Total theaflavins were significantly degraded over time (p0.05) and underwent 75 % degradation (from 1.23 % area to 0.32% area) during 10 h incubation and there was no theaflavins after 24 h in S1 samples (Figure 3.1.e), resulting in a significant difference from S3 samples ($p \leq 0.05$). Theaflavins seem to be relatively stable than catechins during 48 h period in S3 samples. Even 50 % (from 1.13 % area to 0.94 % area) of the theaflavins were lost after 24 h, 42 % (0.47% area) still remained until the end of fermentation (Figure 3.1.e). Theaflavins also underwent degradation under high pH conditions and 95 % degradation of theaflavins occurred in buffer solution at pH 7.4 at room temperature after 6 h [65]. However, the stability studies suggested the catechins were stable than theaflavins [60], [65], which is contradictory to our results.

3.3.4. pH Measurement of Static Culture Mixtures

The static batch cultures were performed to get preliminary information on the metabolism of black tea phenolics by human gut microbiota only at constant temperature without controlling pH. The pH of the cultures was checked at every sampling time to observe the pH change during fecal fermentation (Table 3.5). While there was a slight increase in the pH of the batch cultures with fecal addition (S1) from pH 7.71 to pH 7.96 during 48 h, pH was relatively stable in S3 samples (Table 3.5).

Table 3.5. The pH change of the batch cultures during fermentation of black tea with fecal (S1) and without fecal addition (S3)

Time (hour)	**pH**	
	S1	**S3**
0	7.71±0.13	7.53±0.02
10	7.81±0.53	7.49±0.01
24	7.86±0.68	7.55±0.23
48	7.96±0.33	7.48±0.33


(a)
total catechins (μg/mL)
10.0
8.0
6.0
4.0
2.0
0.0
0
10
24
48
Time (h)
S1
S3
(b)
epigallocatechin gallate (μg/mL)
8.0
6.0
4.0
2.0
0.0
0
10
24
48
Time (h)
S1
S3
(c)
epicatechin gallate (μg/mL)
2.0
1.5
1.0
0.5
0.0
0
10
24
48
Time (h)
S1
S3
(d)
gallic acid (μg/mL)
40.0
32.0
24.0
16.0
8.0
0.0
*
0
10
24
48
Time (h)
S1
S3

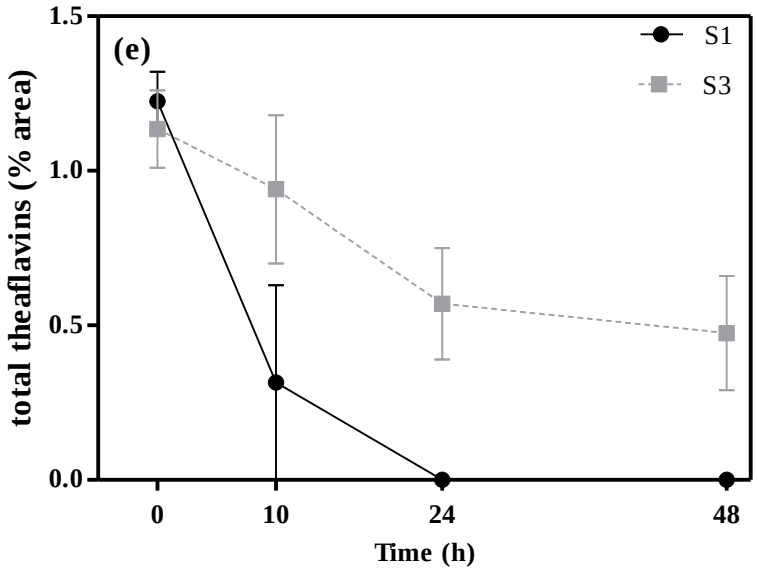


Figure 3.1. The changes in the phenolic profile of static cultures (S1) in the presence and absence of gut microflora (S3) in basal medium during 48 h at 37°C under anaerobic conditions. (a) total catechins; (b) epigallocatechin gallate (EGCG); (c), epicatechin gallate (ECG); (d), gallic acid (GA); (e), total theaflavins (TF). Results are expressed as concentration (µg/mL) in static culture medium. Values are means ±SD of static cultures (n = 2). *Significant differences ($p < 0.05$) between S1 and S3 at the same time.

3.4. CONCLUSION

In conclusion, in this chapter, some issues such as solubility, concentration of extract and fermentation optimization and analysis for degradation of precursor phenolics have been resolved and baseline knowledge was provided for the study of fermentation of black tea phenolics by gut microbiota. Preparation of black tea samples used in the fermentation was optimized considering factors such as phenolic content, solubility and analytical performance.Since black tea extracts used in basal medium remained diluted (1:10 v/v), drying and reconstitution of extract were performed to increase the concentration of tea phenolics in the samples

The spray-dried form of black tea infusion was chosen to increase the solubility of black tea extracts although it was not cost-efficient choice. For the first reconstitution, the solubility increased However, the cold-storage of the extract resulted in an insolubility and a precipitation of particles. As a clear advantage could not be attained using spray drying. Freeze dried samples showed higher concentrations in composition (Apendix A.1) and considering the higher cost of spray drying. Freeze-dried black tea extracts was chosen for further studies. However, to address the solubility problems of freeze dried samples, instead of starting with a stock solution for the inoculum of black tea extract, powder samples were inoculated directly at the desired final concentration for further fermentation studies.

In HPLC analysis, the basal medium sometimes created some unresolved chromatographic images in the culture samples therefore, ethyl acetate extraction was used as sample pretreatment for the batch culture samples before HPLC analysis to get rid of basal medium background. However, it was not found as efficient and resulted in variation between replicates and it was rather time-consuming. Therefore, ethyl acetate extraction of the samples was not included in the final methodology, however, in order to overcome this issue, a guard column was used for HPLC analysis in further fermentation studies.

4. METABOLISM OF BLACK TEA PHENOLICS BY HUMAN GUT MICROBIOTA IN AN *IN VITRO* CONTROLLED FERMENTOR

4.1. INTRODUCTION

The human gut is a highly colonized part of the human digestive system consisting of 10^{12}-10^{14} cells per g of colonic content, of which almost 99 % are anaerobic with diverse activity changes due to environmental and diet differences [2], [17]. Due its diverse metabolic activity which is resulted from dietary compounds, including phenolics, the microbiota residing in the colon can pose both beneficial and harmful effects on the human body. Phenolic compounds are known for their abundance in many fruis, vegetables and beverages and their bioactivity in the human body [34]. Flavanols are the most abundant bioactive molecules among phenolic compounds with monomeric (e.g catechins), dimeric (e.g theaflavins), oligomeric (e.g proanthocyanins) and polymeric structures (e.g thearubigins). Their chemical and structural diversity affects bioavailability of flavanols. For example, monomeric ones are more susceptible to absorption in the small intestine, whereas oligomeric and polymeric forms reach the colon where they can be transformed by the gut microbiota into metabolites such as aglycones and aromatic acids that could result in a difference in their bioavailability and bioactivity [74]. In general, a small amount of these compounds can be absorbed from the small intestine as aglycones by enzymatic activity of human genes, therefore, a big portion reaches the colon and is further degraded by gut microbiota into simpler phenolic acids and microbial metabolites which can be absorbed easily through epithelium and release their health effects into human body [23], [31], [32], [35]–[37], [71], [78], [97], [184]. The interaction of gut microbiota and phenolics has another pathway. Exposure to phenolics may also have an influence in the bacterial community in human gut, simulating the benefical microbes and having anti-microbial effects on susceptible ones [88], [91], [92], [114], [185], [186], [187].

Black tea is one of the most consumed beverages in Turkey with significant phenolic content containing representative phenolic compounds such as catechins, gallic acid, theaflavins (TF) and thearubigins (TR). In the last few years, microbe-derived phenolic metabolites of black tea phenolics have been reported to exert beneficial effects on the intestinal microbiota [88], [105], [110].

There have been many *in vivo* and *in vitro* studies that investigate the interaction of phenolics and gut microbiota [88], [91], [116]. *In vitro* batch and continuous culture fermentation systems with fecal inoculums have mostly been used as a gut model system due to the limitation in *in vivo* studies. The batch culture systems are very simple and provide a convenient way to gain basic information about the metabolite profile occur in human gut.

To date, many *in vitro* gut fermentation studies varying from batch culture fermentations such as tubes and serum bottles [104], [110] to more complex continuous systems such as TIM-2 [105] and SHIME [106], [120] were performed to examine the interaction of black tea phenolics with human gut microbiota. In addition, microbial metabolism of black tea phenolics were analyzed by GC-MS, LC-MS and NMR-based profiling [104], [105], [106]. Gallic acid, pyrogallol, 3,4-dihydroxyphenylpropionic acid, 4-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid and 4-hydroxyphenylacetic acid were detected as metabolites from fecal fermentation of black tea. In addition to gut fermentation of black tea, gut fermentation of other beverages and fruits such as wine and grapes which also include monomeric and dimeric catechins were performed and similar metabolites were observed [88], [91], [92], [95], [188]. Furthermore, there have been many *in vivo* studies to understand the metabolism of tea phenolics and the circulation of metabolites in human body by collecting urine, plasma and fecal samples from humans after ingestion of black tea. In addition to metabolites found in colonic fermentation, sulfated, methylated and glucuronated forms of catechins and typically huppiric acid were excreted in urine and plasma samples [102], [139], [143] [147], [151]. Moreover, similar pathways were suggested for the appearance of metabolites after administration of either individual catechins or black/green tea extracts observed in human intervention, animal and *in vitro* gut fermnentation studies

[95], [102]. The colonic fermentation of theaflavins in mice resulted in the production of gallic acid and pyrogallol [189], [190]. However, the specific microorganims which involved in the metabolism of catechins remains unclear to date, although some isolates such as *Eggerthella lenta* and *Flavonifractor plautii from* human gut can degrade both epicatechin and catechin by cleaving their C-ring, resulting in the formation of 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)-propan-2-ol and further conversion to 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol to 5-(3,4-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3,4-dihydroxyphenyl)-γ-valeric acid [103], which were also observed *in vitro* colonic fermentation of tea [102].

In addition to metabolism of phenolic compounds by gut microbiota, phenolics showed anti-microbial and/or growth stimulation effects on bacteria, resulting in the changed in the bacterial community in the colon. Bacterial changes after administration of black tea to *in vitro* fermentation systems were observed by plate counting, qPCR, PCR-DGGE and pyrosequncing [110], [120]. Although FISH analysis was commonly performed for the bacterial community changes by individual catechins [88], the effects of black tea administration on microbiota were not established using FISH method. It was shown that catechins resulted in an increase in the growth of the *Clostridium coccoides–Eubacterium rectale group, Bifidobacterium* spp. and decrease in the growth of the *C. histolyticum* group [88], [110]. Moreover, although plate counting did not reveal conclusive effects on gut microbial community after administration of black tea extract, in pyrosequencing analysis, black tea extract caused a shift in the Firmicutes: Bacteroidetes ratio by stimulating *Klebsiella*, enterococci and *Akkermansia* and reducing bifidobacteria, *B. coccoides*, *Anaeroglobus* and *Victivallis* in *in vitro* gut fermentation [120].

In this study, the interaction of Turkish black tea phenolics and gut microbiota collected from a male volunteer living in Turkey, whose diet is specific to this region was investigated in an *in vitro* pH-controlled, batch-culture fermentation system and the analysis of degradation of precursor phenolics and formation of their microbial metabolites were performed using HPLC and GC-MS, respectively. Moreover, the effects of black tea relative to control on gut

microbiota were assessed by selective plate counting and fluorescent in situ hybridization (FISH).

4.2. MATERIALS AND METHODS

4.2.1. Chemicals

EDTA (Ethylenediaminetetraacetic acid disodium salt, dihydrate) (ACS grade, ≥ 99%), L-ascorbic acid (98%) and the standards used for HPLC and GC-MS analysis (+)-catechin (C) (≥ 99.0%), gallic acid monohydrate (GA) (≥ 98.0%), caffeine (≥ 99.0%), (-)-epicatechin (EC) (≥ 98%), (-)-epicatechin gallate (ECG) (≥ 98%), (-)-epigallocatechin (EGC) (≥ 95%), (-)-epigallocatechin gallate (EGCG) (≥ 95%), (-)-catechin gallate (CG) (≥ 98%), (-)-gallocatechin gallate (GCG) (≥ 98%), tea extract from black tea (≥ 80% theaflavin-TF-basis), 3,4-dihydroxybenzoic acid (≥ 98%), 3,4-dihydroxyphenylacetic acid (≥ 98%), 4-hydroxyphenylacetic acid (≥ 98%), 3,4-dihydroxycinnamic acid (caffeic acid) (≥ 98%), 2,4,6-trihydroxybenzoic acid (≥ 90%), pholoroglucinol (≥ 99%), 2,4,5-trimethoxycinnamic acid (≥ 98%) were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). 3-(3-hydroxyphenyl) propionic acid (≥ 98%), Pyrogallol, pyrocatechol and hippuric acid were from Alfa Aesar, GmbH & Co KG (Germany) HPLC grade acetonitrile and acetic acid were purchased from Fisher Scientific (UK) and JT Bakers (USA), respectively.

Peptone water (Merck, Germany), yeast extract (Sigma, France), NaCl (Merck, Germany), dipotassium hydrogen phosphate (K_2HPO_4) (VWR, Belgium), monopotassium phosphate (KH_2PO_4) (Bioshop, Canada), $MgSO_4.7H_2O$ (Merck, Germany), $CaCl_2.6H_2O$ (Sigma, Crotia), $NaHCO_3$ (Bioshop, Canada), Tween 80 (Bioshop, Canada), hemin (≥ 98%, Sigma, Netherlands), vitamin K1 (≥ 99%, Sigma, Switzerland), L-cysteine (Merck, Germany), bile salts mixture 3 (Acumedia, USA), and resazurin sodium salt (Sigma, USA) were used for fermentation medium. Brain Heart Infusion, Coliform, Violet red bile glucose, ROGOSA agar were purchased from Merck (Germany) Agar for microbiological cultivation and Reinforced Clostridium Medium were purchased from Sigma (USA) and Mannitol Salt Agar were from

Lab M (UK). Sterilization of media and instruments was achieved by autoclaving at 121°C for 15 min. Milli-Q-water from a Millipore Water Purification System (Milli-Q A10, France) was used for all the experiments.

Oligonucleotide probes used for fluorescent in situ hybridization (FISH) were commercially synthesized and labeled with the fluorescent dye Cy3 at the 5' end (AlphaDNA, Canada)

4.2.2. Black Tea Sample Preparation

Commercially available bulk Turkish black tea leaves were purchased from a local market in Istanbul, Turkey. Ten g tea in 500 ml boiling water were brewed for 10 min, filtered through glass-wool and then cooled to room temperature and freeze dried and stored at -20°C until further analysis.

The inoculation amount of the black tea extract for fermentor cultures was used as described by Gross et al [104]. Black tea extract that was used in fermentor cultures was analyzed for total phenolic content (using Folin Ciocalteu assay [182]), thearubigin and theaflavin content (using Roberts and Smith method [48]), and phenolic profile (by HPLC). The freeze dried black tea extract was inoculated into the fermentor vessels at a concentration of 3125 mg/L, yielding a final phenolic concentration of 1000 mg/L, which corresponds to the daily phenolic intake for humans, 1 g [30].

4.2.3. Fecal Sample Collection

The fecal samples were collected freshly just before the experiment from one healthy male volunteer with no history of any gastrointestinal disease who had not used antibiotics for at least 6 months prior to the study. Samples were put into bags containing anaerobic phosphate buffer (0.1 M, pH 7.4, containing 0.5 g/L cysteine) and diluted 1:10 (w/v) with same buffer before homogenized in a stomacher for 2 min. Then, the fecal slurry was immediately inoculated into the fermentation vessels.

4.2.4. *In vitro* Fermentor Cultures

The schematic representation of fermentation systems are illustrated in Figure 4.1. The fecal fermentations were performed in 135 mL of sterile basal medium in 300 mL glass vessels, which was run under controlled pH conditions (Figure 4.2). Control of pH was performed using an automatic pH controller (Biosis, Turkey) (Figure 4.2). The entire system was placed inside an incubator (New Brunswick, USA) to keep temperature constant during fermentation and agitated slowly with N_2 sparging. The basal medium [88] contained peptone water (2 g/L), yeast extract (2 g/L), NaCl (0·1 g/L), K_2HPO_4 (0·04 g/L), KH_2PO_4 (0·04 g/L), $NaHCO_3$ (2 g/l), $MgSO_4.7H_2O$ (0·01 g/L), $CaCl_2.6H_2O$ (0·01 g/L), Tween 80 (2 mL/L), hemin (50 mg/L dissolved in 1 ml 1M NaOH), vitamin K_1 (10 mL/L), L-cysteine (0·5 g/L), bile salts (0·5 g/L), resazurin (1 mg/L) and distilled water and the pH of the basal medium was adjusted to 7·0. After sterilization, medium was flushed with N_2 overnight and its temperature was set 37°C and its pH was maintained at 6.8 by addition of either sterile 0.1M HCl or 0.1 M NaOH with a digital pH controller. Fifteen mL of fecal slurry samples (1:10, w/v) and freeze dried tea extract (3125 mg/L) were added to the vessels as substrate and the systems were run for 48 hours. During this period, 5 ml samples (R1, R2, R3) were collected at 7 time points (0, 4, 8, 10, 24, 30, 48 h) for analysis of microorganisms (FISH, plate counting) and phenolic compounds. The control vessels contained either fecal slurry without tea extract (R2) or tea extract without fecal slurry (R3) in basal medium. The fermentation experiments were performed in triplicate using fecal collected from three different days.

For FISH analysis, samples (500µL) were immediately fixed with 4 % paraformaldehyde and kept at +4°C overnight. For plate counting, 0.1 mL of the samples was plated immediately on appropriate agar after serial dilution in PBS. For phenolic profile analysis, samples were centrifuged at 2000 X g for 10 min, and 500 µL aliquots were dispensed into eppendorf tubes and stored at -20°C until further analysis.

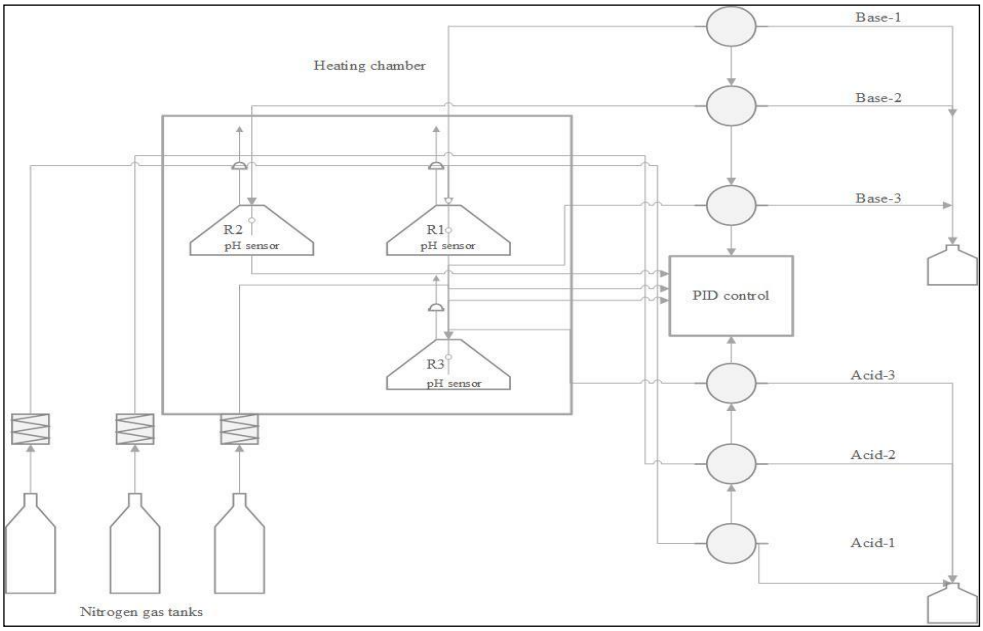


Figure 4.1. Schematic representation of fermentor system used in this study.

Figure 4.2. The bioreactor system used in the study. 100 % N_2 gas tanks each for each reactor vessels (left) and pH controller with pumps next to heating chamber including vessels (right).

4.2.5. Determination of Phenolic Composition of Fermentor Cultures Using High Performance Liquid Chromatography (HPLC)

Phenolic profile (gallic acid, catechins and theaflavins) of fermentor cultures was analyzed using an HPLC-PDA (Thermo Scientific, USA), a Phenomenex Luna phenyl-hexyl column (4.6 mm x 250 mm, 5µm) coupled with a Phenomenex SecurityGuard and 4 mmX 3.0 mm Phenyl-Hexyl cartridge according to ISO 14502-2:2005 [180]. Culture supernatants were diluted 1:5 in stabilizing solution (L-ascorbic acid, EDTA (500 µg/mL) and acetonitrile (10 % v/v), and then filtered through 0.45 µm regenerated cellulose filter (Sartorius, Germany). The mobile phases consisted of: A (9 % acetonitrile, 4 % acetic acid and 20 µg/ml EDTA) and B (80 % acetonitrile, 4 % acetic acid and 20 µg/mL EDTA) and the gradient profile used was as follows: (A): 100% at 0 min, 68% at 15 min, 68% at 25 min, 100% at 35 min. Column temperature was set to 35°C. Flow rate was 1 mL/min and 10 µL of the sample was injected. Chromatograms were recorded between 200−700 nm and the eluent was monitored at 278 nm. Identification of phenolics was done by comparing the retention times of the sample peaks with those of standards. Quantification of catechins and gallic acid was carried out using standard calibration curves. While catechin, gallic acid and caffeine amounts were expressed as micrograms per mL culture mixtures, theaflavin content was expressed as % area.

4.2.6. Determination of Degradation Products in Fermentor Cultures by GC-MS Analysis

The phenolic metabolite profiles of fermentor cultures were determined using GC-MS as described in Gross et al [104] and Grün et al [142]. 500 µL sample, 80 µL 1.5 M sodium acetate were mixed and then acidified with 65 µL 1 M HCl. After addition of 50 µL of internal standard (100 µg/mL in 1:1 methanol/water), samples were stored at 4 °C for 10 min. The samples were extracted with 2 mL of ethyl acetate, three times, vortexed for 30s, and centrifuged at 3000 × g for 10 min. The supernatants were then transferred to new glass tubes and dried under a stream of nitrogen using a heating block at 40 °C. Samples were further

dried by subsequent addition and evaporation of 1 mL of dichloromethane. Dried samples were derivatized with 100 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 10 % trimethylchlorosilane (TMCS) for 30 min at 90 °C. After cooling down to room temperature, 400 µL of hexane was added and the samples were vortexed and then, 200 µL of the derivatized samples was transferred to GC vials.

Calibration standards were prepared from a phenolic stock solution containing 8 phenolics (Table 4.3) at a concentration of 100 µg/mL. The stock solution was diluted in 1:1 methanol/water mixture and 50 µL of mixture standard plus 50 µL of internal standard at a concentration 100 µg/mL were dried under a stream of nitrogen and further dried twice with 1 mL dicholomethane and the following procedure were performed as described above.

Derivatized samples were analyzed on a Thermo ISQ mass spectrometer (Thermo Scientific, UK) equipped with a Thermo Model Trace GC Ultra gas chromatograph and Thermo TRI PLUS RSH auto sampler (Thermo Scientific, Switzerland). The separation were carried out on a fused silica capillary column, VF-5MS (30 m x 0.25 mm i.d, coated with 5% Phenyl Methylpolysiloxane (film thickness: 0.1 µm) (Varian, Agilent Technologies, Netherlands)). Helium was the carrier gas with a constant flow rate of 1 mL/min. The samples (1 µL) were injected into GC injector port operated in split mode at a split ratio of 1:10. The injection port was maintained at a temperature gradient from 80 °C (held for 1 min) to 300 °C (held for 5min) at 10 °C/s. The interface and MS source temperature were 250 °C and 230 °C, respectively. The temperature of column was initially kept at 45°C for 1 min, then increased from 45 to 100 °C at 10 °C/min, then from 100 to 250 at 5 °C/min, and finally from 250 to 300 °C at 20 °C/min, where it was held for 6 min. Mass spectra were recorded in EI mode at 70 eV from 8 to 45 min at a scan time of 0.2 s, and a scan range of *m/z* 50–500.

The identification of the phenolic metabolites was carried out by comparing their ion spectrum with the NIST library and with that of targeted standards. Quantification was done using calibration standards.

4.2.7. Selective Bacterial Enumeration Using Plate Counting

The composition of bacterial groups in fermentor cultures collected at time 0 and 48 h of incubation was determined by plating on selective culture media as described by Possemiers et al [162]. The bacterial groups and the specific media that were used are given in Table 4.1. The samples were serially diluted in PBS (0.1 M, pH 7.4) and plated on appropriate agar and incubated at 37°C under the conditions given in Table 4.1. Anaerobic incubation of plates was performed in an anaerobic cabinet (Don Whitley Scentific, UK) with a gas atmosphere (80% N_2, 10% CO_2 and 10% H_2). To observe the changes in bacterial community, the results were expressed as log CFU/g.

Table 4.1. Analyzed bacterial groups with the selective media and incubation conditions used

Bacterial Groups	**Medium**	**Condition**	**Time (h)**
Total Aerobes	Brain Heart Infusion (BHI) Agar	Aerobic	24
Total Anaerobes	BHI Agar + 0.5 g/L L-cysteine	Anaerobic	72
Lactobacillus spp.	Rogosa Agar	Anaerobic	72
Total Coliforms	Coliform Agar	Aerobic	24
Clostridia	Reinforced Clostridium Medium (RCM)	Anaerobic	48
Enterobacteria	Violet Red Bile Glucose (VRBG) Agar	Aerobic	24
Staphyloccus aureus	Mannitol Salt Agar (MSA)	Aerobic	24

4.2.8. Bacterial Enumeration Using Fluorescence *In Situ* Hybridization (FISH)

To observe the changes in bacterial community in fermentor cultures, fluorescent in situ hybridization (FISH) method was used with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA. The probes were commercially synthesized and labeled with the fluorescent dye Cy3. The bacterial groups studied for enumeration, probe sequences and hybridization conditions are listed in Table 4.2. Batch culture samples (500 μL) were fixed in

1.5 mL of 4% (w/v) filtered paraformaldehyde at 4°C overnight were then centrifuged at 13000 g for 5 min, washed twice with PBS (0.1 M, pH 7.0), resuspended in 500 µL of PBS−99% ethanol mixture (1:1, v/v), and stored at −20 °C until analysis. Hybridization of cell suspensions was performed as described by Hidalgo et al[122]. Briefly, the cell suspension was diluted appropriately for each probe and 20 µL was pipetted onto each well of a Teflon- and poly-L-lysine-coated 10-well slide (EMS, USA). Slides were dried 46-50 °C in a desktop plate incubator (Binder, UK), dehydrated in ethanol (50%, 80%, and 100% (v/v) ethanol, 3 min each), and dried further.

To increase cell permeability for gram positives used with Lab158 and Bif164 probes, 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, then washed in water and before being dehydrated in the ethanol series (50%, 80%, and 96% (v/v) ethanol, 3 min each).

Twenty µL of hybridization mixture containing 0.9 M NaCl, 20 mM Tris-HCl, 0.1 % SDS, pH 7.2) was added onto the surface of each well, and 5µL of 50 ng/mL probes were mixed on the surface. The slides were placed onto the slide tray, which was sealed and left for overnight in the hybridization oven set at the appropriate temperature for each probe (Table 3.2). After hybridization, slides were transferred into 50 mL of wash buffer (containing 0.9 M NaCl, 20 mM Tris-HCl and warmed at the appropriate temperature for each probe) for a further 15 min. Slides were then dipped into ice-cold distilled water for 2−3 s. 15 µL of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 50 ng/mL; Sigma, USA) were added and kept in dark for 10 min and dipped into ice-cold water again and dried. Five µL of antifade solution (DABCO (Sigma, USA)) was then added to each well, and a coverslip was applied. Slides were stored in the dark at 4°C until cells were counted on Confocal Microscope (Zeiss, Germany). For each slide, five fields of view were counted.

Table 4.2. FISH oligonucleotide probes used in this study [122]

Probe name	Target bacterial group	Sequence from 5' to 3' end	T_H (°C)
Bac 303	*Bacteroides* spp	CCAATGTGGGGGACCT T	46
Bif 164	*Bifidobacterium* spp	CAT CCGGCAT TACCACCC	50
Erec482	*Eubacterium rectale−Clostridium coccoides* group	GCT TCTT AGTCARGTACCG	50
Lab 158	*Lactobacillus−Enterococcus* spp	GGTATTAGCAYCTGTTTCCA	50
EUB 338	Total bacteria	GCT GCCT CCCGTAGGAGT	46
EUB338II	Total bacteria	GCAGCCACCCGTAGGTGT	46
EUB338III	Total bacteria	GGT CGGT CT CTCAACCC	46

4.2.9. Statistical Analysis

For the changes in phenolic precursors within time in HPLC, the data were analyzed using a two-factor repeated-measures analysis of variance with time and treatment as the two factors and Tukey test for multiple comparison with time within same treatment. Two-sample t-test were applied for comparison of R1 and R3 (control) at the same time. For metabolite analysis, GC-MS data were subjected to repeated measure analysis of variance to observe the changes with time and Tukey test were performed for multiple comparison of different time points. Univariate correlations were calculated using Pearson's correlation to find the correlation between production of metabolites and their phenolic precursors. For bacterial enumeration, changes in bacterial community were expressed as mean values and standard deviations by applying a paired Student's t test. All statistical analyses were performed with Minitab version 16 and GraphPad Prism 5 was used to interpretation of results.

4.3 RESULTS AND DISCUSSION

In this study, the metabolism of phenolics present in black tea (gallic acid, catechins and theaflavins) by human microflora over 48 hours during *in vitro* fecal fermentation was investigated. While the changes in the profiles of the precursor compounds in black tea (gallic

acid, catechins, theaflavins) were determined using HPLC, GC-MS was used for the investigation of the produced metabolites.

4.3.1. Changes in Phenolic Composition of Fermentor Cultures Determined by HPLC Analysis

The TP content of black tea was found as 51.47 mg GAE/ g tea leaves by Folin assay and TR and TF content was found as 8.78 ±1.22 % wt/wt and 0.17 ± 0.05 % wt/wt of extractable solids, respectively. Moreover, in HPLC analysis, EC, EGCG, GCG, ECG, TC, GA and TF were detected in the concentration of 5.18, 6.17, 1.83, 1.14, 14.32, 11.36 mg/g dry extract, 2.17 % Area (Appendix A.1). In R1 and R3 samples, catechins, gallic acid and theaflavins were detected. While only EC, EGCG and ECG were detected in R1 and R3 during incubation, no epimerized catechins (gallocatechin (GC), catechin (C), gallocatechin gallate (GCG) and catechin gallate (CG)) were observed.

The changes in the concentration of individual and total catechins, gallic acid and theaflavins during 48 h fecal fermentation of black tea (R1) are illustrated in Figure 4.3. Although a restricted diet was not applied to the male volunteer whose fecal inocula was used in the fermentation experiments, no black tea phenolics (catechins, gallic acid, theaflavins) were detected in any of the fecal samples used for fermentations (R2). The stability behavior of black tea phenolics was also investigated by analyzing the changes in the concentration of catechins, theaflavins and gallic acid in basal medium without fecal inoculation under same experimental conditions (37°C, N_2 flushing, pH: 6.8) during 48 h (Figure 4.3).

Degradation of total catechins, individual catechins (EC, EGCG and ECG), gallic acid and theaflavins was significant with time ($p \leq 0.05$). Although degradation of phenolics in basal medium was observed over a 48 h period, catechin, gallic acid and theflavin loss rate caused by microbiota was significantly higher in fecal fermented cultures (R1) than control samples (R3) ($p<0.05$)

Total catechins were degraded significantly from 22.08 µg/mL to 4.49 µg/mL with 20 % remaining in 8 h of fermentation ($p<0.05$) and completely degraded after 24 h. Significant difference in the concentration between R1 and R3 was observed starting at 8 h of incubation ($p<0.05$) (Figure 4.3.a). Total catechin concentration in R3 samples decreased only from 24.23 µg/mL to 18.88 µg/mL (75 % of its initial concentration) after 10 h and 9.35 µg/mL (40 % of its initial concentration) remained at the end of fermentation. In literature, the studies related to stability of catechins showed that catechins underwent degradation at pH higher than 5 [63], [65], [66]. For example, while no changes were observed in the concentration of catechins at pH 5 in 18 h, at pH 7.4, 80 % loss was observed after 3 h and they were completely degraded after 18h [63].

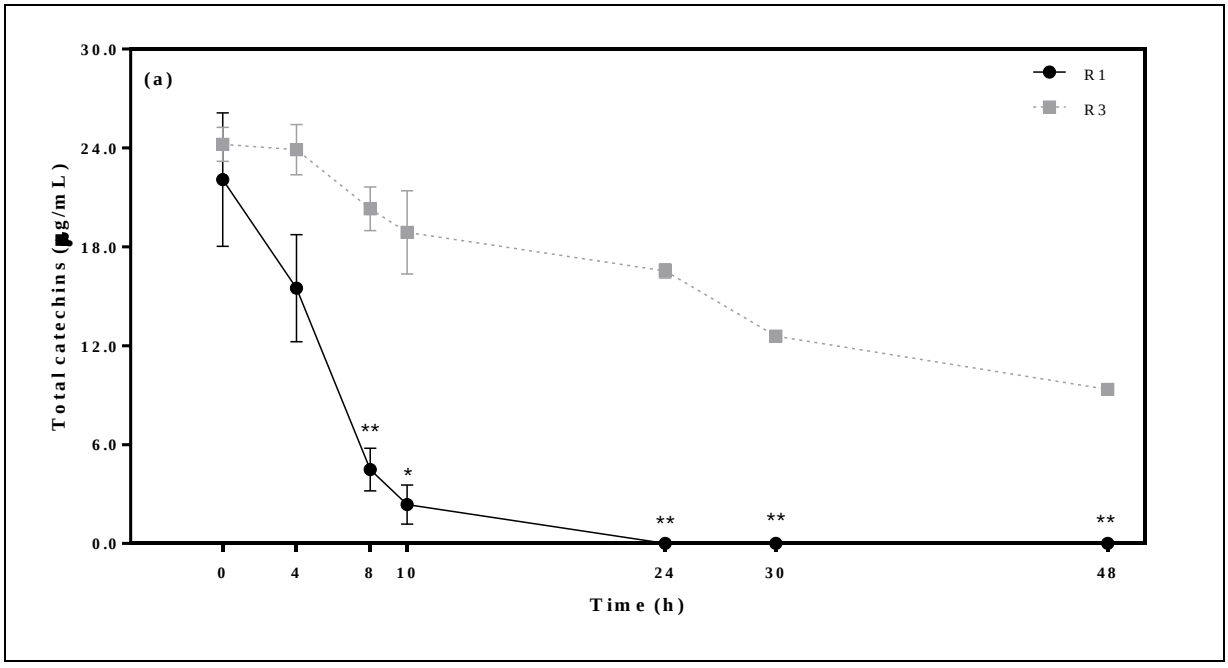
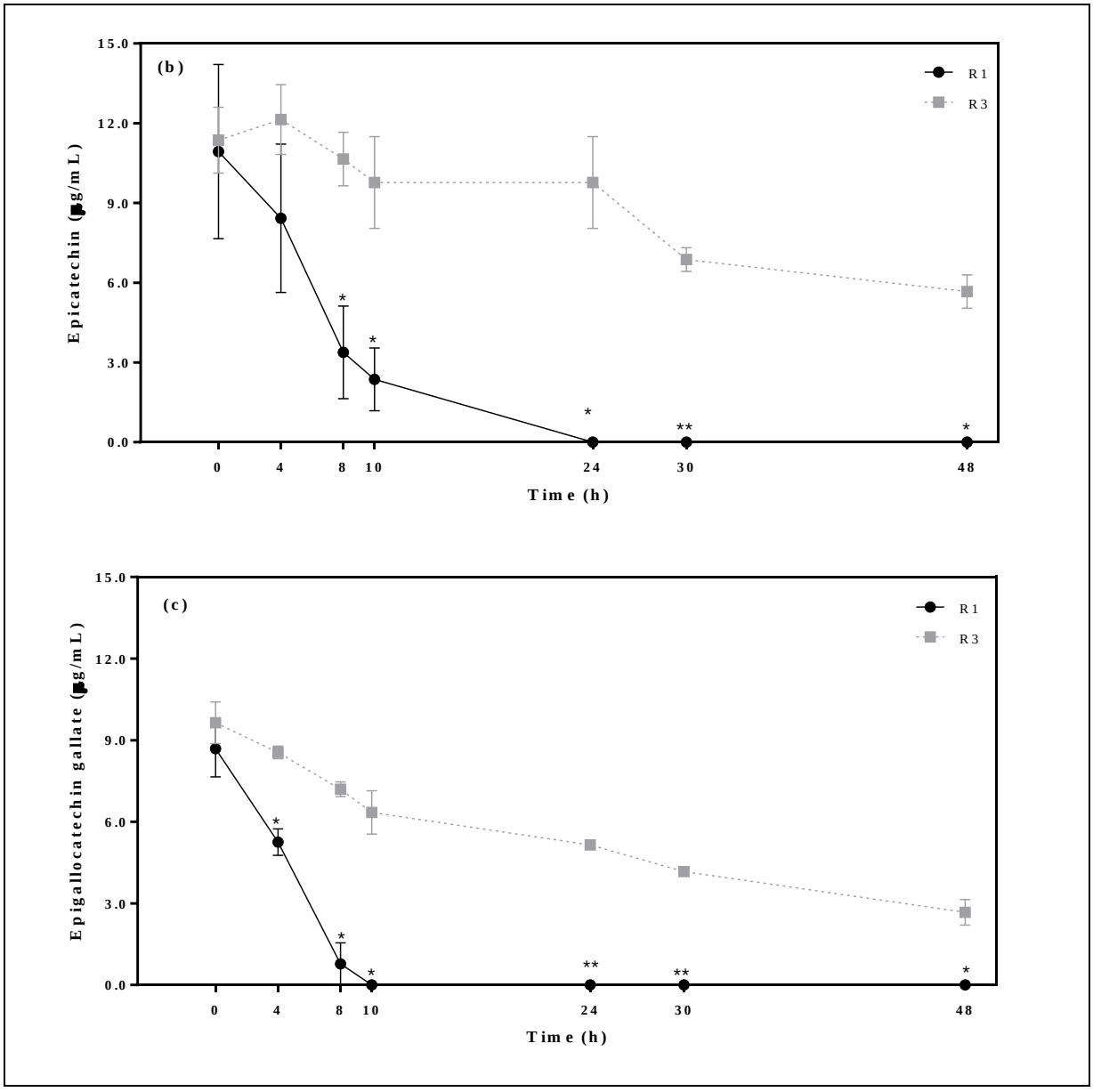


Figure 4.3.a. The changes in the total catechin amounts in fecal fermentor cultures (R1) and in basal medium containing tea extract without fecal slurry (R3) during 48 h at 37°C under anaerobic conditions at pH 6.8. Values are means ±SD of three individual fermentations (n = 3). *Significant differences between R1 and R3 at the same time, $p< 0.05$, ** Significant differences between R1 and R3 at the same time, $p< 0.005$.

The degradation profiles of individual catechins in R1 and R3 are demonstrated in Figure 4.3.b, c, d. In fecal fermentor cultures (R1), EC showed slow degradation during the first 8 h and degradation reached significant levels (22 % remaining, 2.36 µg/mL) at 10 h ($p<0.05$) following complete degradation after 24 h of incubation (Figure 4.3.b). On the contrary, EC concentration in basal medium containing tea extract without fecal slurry (R3) was relatively stable during 24 h and 50 % of its initial concentration remained thereafter suggesting that degradation of EC in R1 samples was caused by activity of gut microorganims showing significant levels at 8 h compared to R3 ($p<0.05$). Compared to EC, degradation of EGCG in R1 samples was faster in the first 8 h (9 % remaining, 0.77 µg/mL) and after 10 h, no EGCG was observed (Figure 4.3.c). In R3 samples, although EGCG showed degradation during incubation, it was much more slower that in R1 samples and 2.67 µg/mL of EGCG was still present at the end of 48 h. Similar to degradation profile of EC the microbial degradation of EGCG in R1 samples was significantly higher when compared to R3 samples starting from 4 h of incubation ($p<0.05$).

The concentration of ECG declined to 1.82 µg/mL (74 % of its initial concentration) within 4 h of fermentation reaching significantly lower levels at 10 h ($p<0.05$) and no ECG was observed after 24 h in R1 samples (Figure 4.3.d). ECG concentration in R1 samples was significantly diffrent than control (R3) at 24 and 30^{th} h. In R3 samples, the degradation profile of ECG was relatively stable and 60 % of ECG remained at the end of incubation. Consequently, EC and ECG were less affected than EGCG by the incubation conditions (37 °C, pH 6.8). While 50 % and 60 % of EC and ECG remained, respectively, only 28 % of EGCG remained at the end of incubation. Therefore, it can be concluded that in fecal fermented cultures (R1) due to the action of microflora, the utilization of individual catechins was observed much faster than R3 samples.


(b)
R1
R3
Epicatechin (μg/mL)
15.0
12.0
9.0
6.0
3.0
0.0
0
4
8
10
24
30
48
Time (h)
(c)
R1
R3
Epigallocatechin gallate (μg/mL)
15.0
12.0
9.0
6.0
3.0
0.0
0
4
8
10
24
30
48
Time (h)

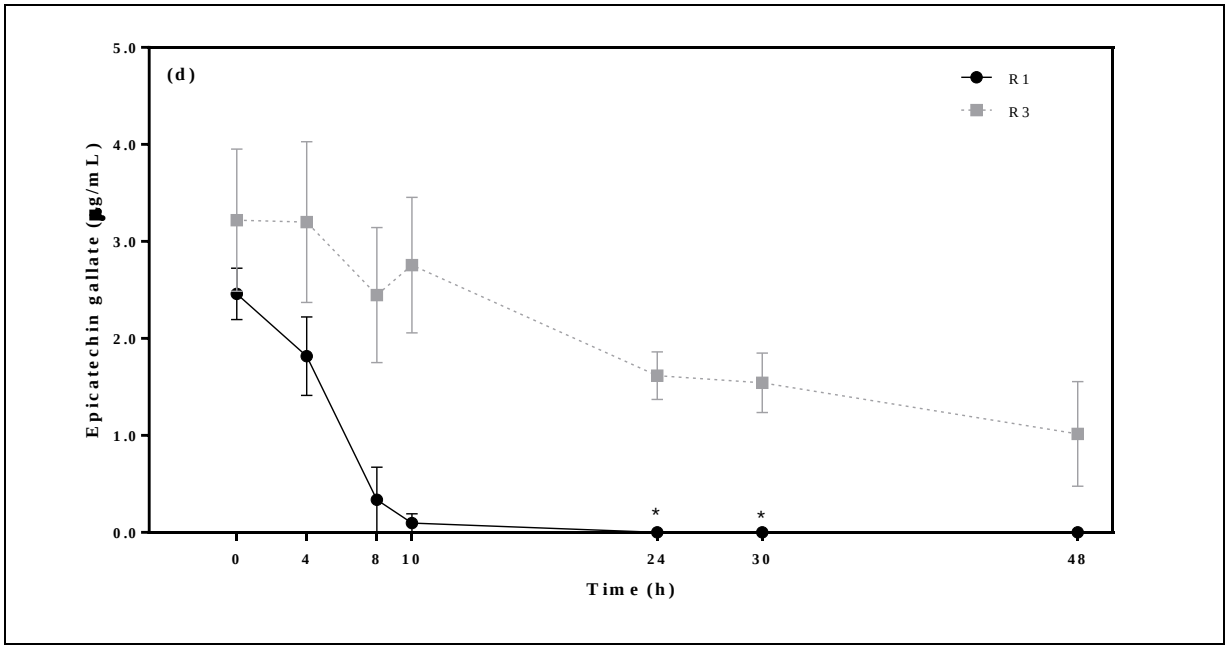


Figure 4.3.b. c. d. The changes in the epicatechin (EC) (b); epigallocatecchin gallate (c) (EGCG); epicatechin gallate (ECG) (d) amounts in fecal fermentor cultures (R1) and in basal medium containing tea extract without fecal slurry (R3) during 48 h at 37°C under anaerobic conditions at pH 6.8. Values are means ±SD of three individual fermentations (n = 3). *Significant differences between R1 and R3 at the same time, $p < 0.05$, ** Significant differences between R1 and R3 at the same time, $p < 0.005$.

Degradation of individual catechins has also been observed in *in vitro* fecal fermentation studies using either standards or black tea as substrate [88], [104], [105]. In the study of Tzounis et al [88], EC inoculated at a concentration of 1000 mg/L degraded over a 24 h period with 15 % remaining during fecal fermentation. Moreover, the metabolism study of C, EC, ECG, EGCG and dimer B2 using *in vitro* colonic fermentation with rat colonic content showed that EC, EGCG and ECG were mostly metabolized in 24-48 h and EC and ECG were not detected at 48 h [95]. In the study of Gao et al [105], black tea colonic fermentation was investigated in TIM-2 model and EGCG, EC and ECG decreased to 2, 60 and 4 % of their initial black tea powder content over a 28 h period. These results are in agreement with our results, suggesting that EGCG and ECG underwent faster microbial degradation than EC by the microbial gut flora. On the contrary, in the study of van't Slot and Humpf [191], in which metabolism of C, EC, EGC, EGCG, GC, GCG, procyanidin B2, and gallic acid in pig cecum model was investigated, after 8 h of incubation, 8 % of EC, 3 % of EGC and 17 % of EGCG remained, which indicated that EC degraded more rapidly in pic cecum fermentation suggesting that the utilization was also affected by the microflora involved in fermentation. In the study of Sanchez-Patan [91], EC, C and ECG in wine extract during *in vitro* fecal fermentation was completely degraded after 5 h of fermentation, however, C, EC and ECG in grape seed extract was completely degraded between 10-24 h [92] These findings indicate that the utilization rate of these precursor compounds was dependent on the composition and concentration of phenolics as determined by their source (ie. wine extract, grape seed extract, black tea extract and individual standards).

In terms of stability of individual catechins in R3 samples under anaerobic conditions without presence of fecal cultures at pH 6.8, as expected EC and ECG seemed to be more stable than EGCG. Lower stability of EGCG and EGC was attributed to the presence of three vicinal hydroxyl groups at positions 3', 4' and 5' in their structure, which make them more susceptible to destruction than the two vicinal hydroxyl groups at positions 3' and 4' in ECG and EC [67]. Moreover, although elimination of oxygen may result in epimerization of epi forms to non-epi forms such as EGCG to GCG in phosphate buffer (pH 7.4) either at room temperature or 37°C [56], not observing epimerization reaction in this study, is in agreement

with the results in the study of Wang and Helliwell [62], in which no epimerization was observed when the temperature was kept at 40°C. A kinetic study on epimerization suggested two turning points at 44°C and 98°C such that below 44°C degradation was more intense than epimerization whereas epimerization dominated above 44°C and epimerization from non epi to epi form was faster than other reactions above 98°C [58].

Gallic acid slowly degraded during the first 10 h compared to other time intervals and reached small levels at 24 h of fermentation (with 2 %, 0.86 µg/mL remaining) ($p<0.05$) and trace amounts were observed at the end of the fermentation (Figure 4.3.e). When compared to R3 samples, gallic acid concentration of fecal fermented samples was significantly lower at 24 and 30th h and the extent of degradation over a 48 hour period was lower ($p>0.05$). R3 samples were more stable for the first 10 hour with 12 % lost (3.2 µg/mL lost), but , 50 % (15.13 µg/mL) of its initial concentration remained after 48 hours (Figure 4.3.e).

Degradation of gallic acid by human microflora has been studied [91], [122]. In the metabolism of gallic acid at a concentration of 1000 mg/L during 24 h fermentations with fecal samples collected from three different humans, it was observed that gallic acid concentration remained constant for the first 5 h of incubation, but, it underwent degradation with higher rates between the 5th and 24th h [122]. However, a slower degradation was observed in one of the cultures with 50 % remaining after 24 h period [122]. In the study of Sanchez-Patan et al [91], gallic acid (1.06 mg/g) present in wine extract containing catechins and anthocyanins, as well, was completely degraded after 30 h of fermentation. These results are in agreement with those in our study. In some studies, gallic acid appeared as a metabolite in fecal fermentation of other sources of galloylated flavan-3-ols (e.g. green tea and grape seed extract) due to the degradation of gallated catechins (such as EGCG and ECG) and theaflavins [102], [189]. In our study, although there was high variation between replicates for the first 10 h incubation, formation of gallic acid due to the degradation of gallated catechins and theaflavins was not clearly observed because gallic acid was also degraded by gut microbiota during this period.

The degradation profiles of total theaflavins are illustrated in Figure 4.3.f. In R1 saples, they were significantly degraded from 2.81 % area to 0.98 % area in 8 h fermentation (with 35 % remaining) and after 10 h, the concentration of theaflavins decreased to 15 % (0.42 % area), no theaflavins were detected after 24 h of incubation. Theaflavin content in fermentation samples was significantly different than control samples (R3) after 4^{th} h ($p<0.05$). In R3 samples, it remained stable until 24 h incubation (changes from 2.94 to 2.60 % area) but, at the end of the incubation, 60 % of its initial concentration was still in basal medium (Figure 4.3.f). According to stability studies of theaflavins, degradation of theaflavins occurred in buffer solution at pH 7.4 at room temperature such that 95% of TFs were degraded after 6 h [65]. However, in our study in which a lower pH value was used (pH 6.8) degradation of theaflavins was not observed in the control samples (R3) due to the culture conditions.

In literature, the microbial degradations of theaflavins was also investigated in different systems. In the study of Gao et al [105], microbial degradation of black tea in TIM-2 colonic model was investigated and it was observed that the amount of total theaflavins did not change over 28 h period. However, the studies conducted with germ free mice by collecting their fecal samples after being fed with black tea theaflavins, and the *in vitro* fermentation of human fecal slurries showed that theaflavin digallate was metabolized to theaflavin, theaflavin-3-gallate and theaflavin-3'-gallate, gallic acid, and pyrogallol by human microbiota [189], [190]. Moreover, both theaflavin-3-gallate and theaflavin-3'-gallate were metabolized to theaflavin, gallic acid, and pyrogallol.

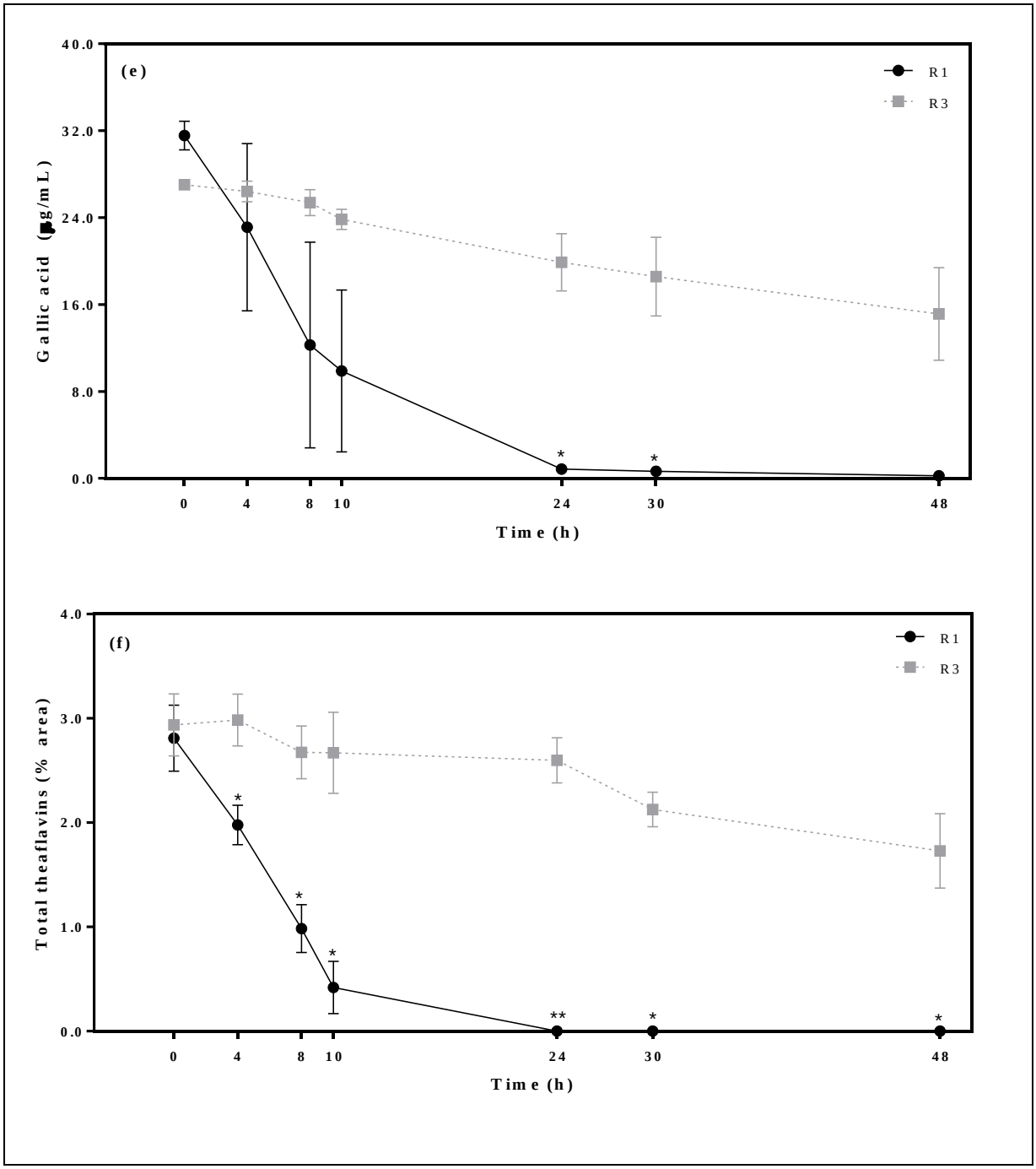


Figure 4.3.e. f. The changes in the gallic acid (e) and total theaflavins (f) amounts in fecal fermentor cultures (R1) and in basal medium containing tea extract without fecal slurry (R3) during 48 h at 37°C under anaerobic conditions at pH 6.8. Values are means ±SD of three individual fermentations (n = 3). *Significant differences between R1 and R3 at the same time, $p < 0.05$, ** Significant differences between R1 and R3 at the same time, $p < 0.005$.

In R1 samples, while catechins and theaflavins were completely degraded at 24 h, trace amounts of gallic acid still remained at the end of fermentation. In R3 samples, the stability of theaflavins and gallic acid were similar but catechins were less stable than others. For the first 10 h, gallic acid and theaflavins were more stable (10 % degradation) than catechins (22 % degradation) and at the end of incubation while approximately 50 % of gallic acid and theaflavins remained in the medium, catechins were degraded to 40 % of its initial concentration. These are contradictory to stability studies in which theaflavin standards in sodium phosphate buffer at pH 7.4 for 6 h led to 95 % degradation whereas catechins underwent to 65 % degradation [65].

4.3.2. Analysis of Phenolic Metabolites in Fermentor Cultures by GC-MS

Identification of fecal fermentation derived phenolic metabolites from black tea was performed by GC-MS using respective standards and their mass spectrometric fragmentation. When the standards were not available, identification was done by reference to literature [102], [150] and Wiley 7 library. Moreover, the background peaks originating from basal medium and solvent were eliminated by subtracting the chromatogram of basal medium. After background subtraction was performed, the peaks for each sample were analyzed. Since retention time drift was observed (0.01 min) between chromatograms, full subtraction could not be performed but the identified peaks originating from background were not taken into account. A summary of phenolic metabolites identified in fecal fermentation of black tea cultures is given in Table 4.3.

Table 4.3. Retention time and characteristic ions of phenolic metabolites in fermentor cultures

Phenolic acids and metabolites	**Retention time (min)**	**Base ion (*m/z*)**	**Qualifier ions (*m/z*)**	**Identification**
Pyrocatechol	10.03	254	239;73	Standard, Wiley 7
3-phenylpropionic acid	12.14	222	104;207	Wiley 7, Jenner et al 2005
2-hydroxybenzoic acid	14.04	267	147;73	Wiley 7, Jenner et al 2005
Pyrogallol	14.63	342	239;73	Standard, Wiley 7
4-hydroxyphenylacetic acid	16.89	296	252;179	Standard, Wiley 7
Phloroglucinol	17.09	342	327;147	Standard, Wiley 7
3-(3-hydroxyphenyl)propionic acid	18.72	310	205;192	Standard, Wiley 7
3,4-hydroxyphenylpropionic acid	19.38	310	192;179	Wiley 7
3,4-dihydroxybenzoic acid	20.6	355	193;311	Standard, Wiley 7
3,4-dihydroxyphenylacetic acid	20.75	384	179;267	Standard, Wiley 7
Hippuric acid	21.01	251	105;206	Standard, Wiley 7
3,4-dihydroxycinnamic acid	22.98	398	179;73	Standard, Wiley 7
Gallic acid	23.36	458	281;73	Standard, Wiley 7
2,4,6-trihydroxybenzoic acid	23.76	354	369;443	Standard, Wiley 7
2,4,5-trimethoxycinnamic acid (I.S)	27.82	220	278;310	Standard, Wiley 7

Identification of catechins was also performed by analyzing the chromatograms of a mixture of catechin standards (GC, EGC, C, EC, EGCG, GCG, ECG). Silylated catechins that are not available in the Wiley 7 mass spectral library (silylated epigallocatechin, epicatechin gallate and epigallocatechin gallate) were identified by comparing their ion mass spectrums with those found in the literature [192]. From the six catechins in the mixture of catechin standards, only 3 peaks appeared with respective retention times of 37.44, 37.69, 38.04 min (Figure 4.4) with similar ion mass spectrum. Since full scanning was performed in the range of 50-500 *m/z* and silylated catechins have higher molecular weight fragmentation (in the range of 600-1100 *m/z*), they could not be identified individually. The phenolic profile of freeze dried black tea extract (reconstituted in distilled water (3125 mg/L)), which had been previously analyzed using HPLC, was also investigated using GC-MS (Figure 4.5). The compounds that eluted at 21.05 and 23.34 min were identified as caffeine and gallic acid, respectively. The compounds that were detected at 37.44 and 38.04 min with small relative abundance (Figure 4.5) had similar ion mass spectrums with those in the catechin mixture (Figure 4.4). In addition to those identified as catechins, a compound with catechin ion mass spectrum in the range of 50-500 *m/z* was also eluted at 39.14 min. However, this compound was not observed in the catechin mixture (Figure 4.4) and could not be identified using the Wiley 7 Library.

In order to investigate the source of the peaks in fecal fermented cultures, initial samples of all fermentations (R1, R2 and R3) (0^{th} h), and black tea sample used in fermentation were compared. The identified peaks in fecal fermented black tea sample are shown in Figure 4.6. The peaks labeled as 2, 7, 8, 10, 11, 17, 24, 25, 31 originated from the fecal slurry (R2). Peaks 2, 24, 25 and 31 could not be identified as they were not present in the library and their standards were not available, peaks 7 and 8 were identified as 3-phenylpropionic acid, 2-hydroxybenzoic acid using their ion mass spectrum matches with library and according to base and qualifier ions listed in Jenner et al [150]. The other peaks belonging to the fecal source (peak numbers 10, 11) were identified using standards as 4-hydroxyphenylacetic acid and 3-(3-hydroxyphenyl) propionic acid. These metabolites initially present in fecal contributed to non resricted diet of a person [95]. Peaks 12, 13, 15, 16, 19, 20, 28, 29, 30 that were present in the 0^{th} hour fecal fermented black tea culture originated from black tea. Peaks

12, 13 and 16 eluted at retention times 20.63, 21.06 and 23.36 were identified as 3,4-dihydroxybenzoic acid, caffeine and gallic acid, respectively. The peaks 28, 29, 30 gave ion spectrum same with catechins identified in black tea sample in distilled water and catechin mixture. The remaining peaks were unidentified although the basal medium was substracted from chromatogram.

After identification of the peaks of the 0^{th} h samples, the changes in the phenolic profile of R1, R2 and R3 during a 48 h period were investigated. According to the HPLC analysis of the control experiment performed only with black tea (R3), gallic acid, catechins and theaflavins were degraded to a certain extent in the absence of microbial degradation. So, it was necessary to identify the degradation products of black tea (R3) to be able to interpret the changes in the fecal fermented black tea samples (R1) correctly. Therefore, the phenolic profile of seven time point fractions of R3 samples were monitored using GC-MS. None of phenolic metabolites of fecal fermentation of black tea reported in the literature [102], [104], [105] were observed in the control black tea fermentor culture (R3) over a 48 h period. The peaks that were present at the 0^{th} hour were still observed over 48 h periods (data not shown). Therefore, we can say that the degradation of black tea in basal medium (R3) over a 48 hour period did not yield the microbial degradation products detected in sample R1 at the same time frame. In the fecal control (R2), the compounds identified in the 0^{th} samples (peaks 10,11) also appeared in seven time fractions but their quantity did not change over time (data not shown).

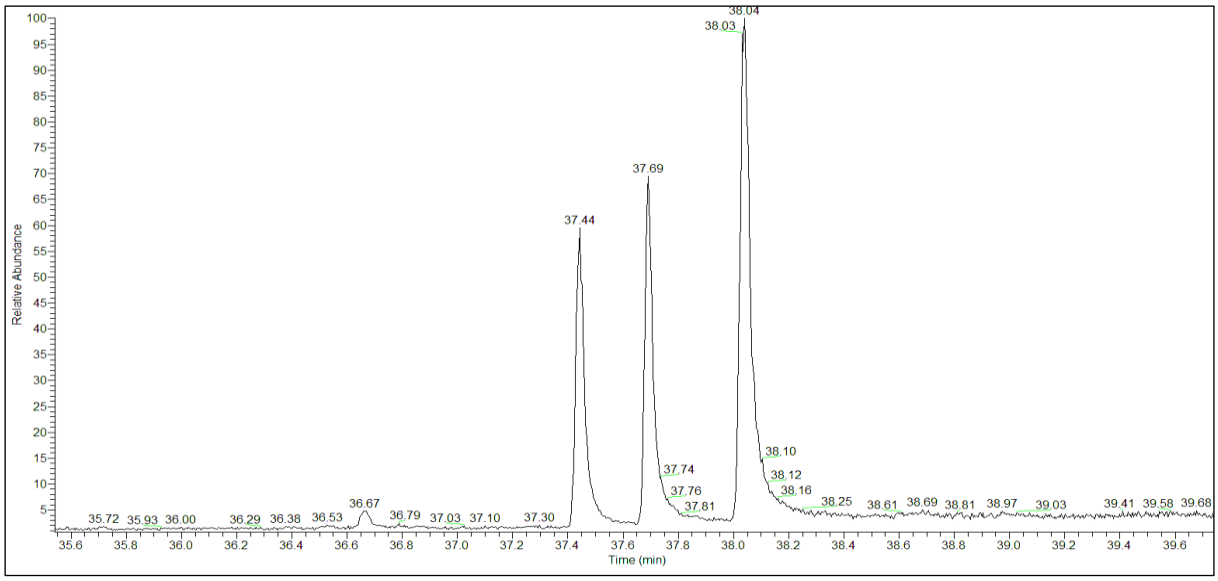


Figure 4.4. The chromatogram from catechin mix in stabilized solution (L-ascorbic acid, EDTA and acetonitrile).

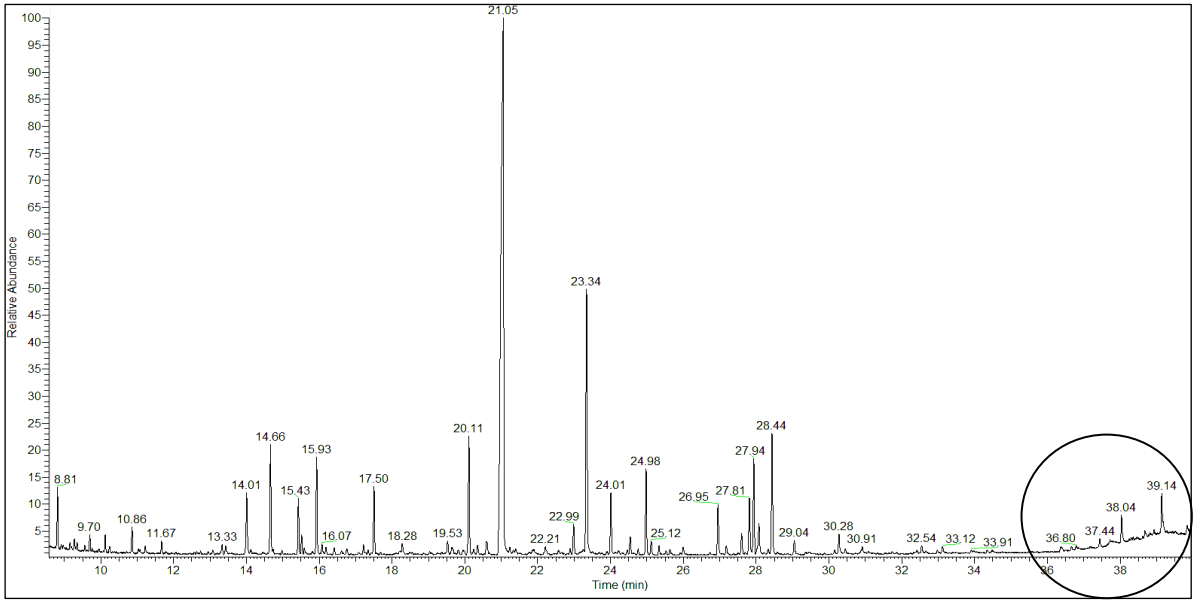


Figure 4.5. Chromatogram of black tea sample (3.125 mg/ml), circle shows the catechin peaks which give same retention time and mass spectrum with those identified in catechin mix.

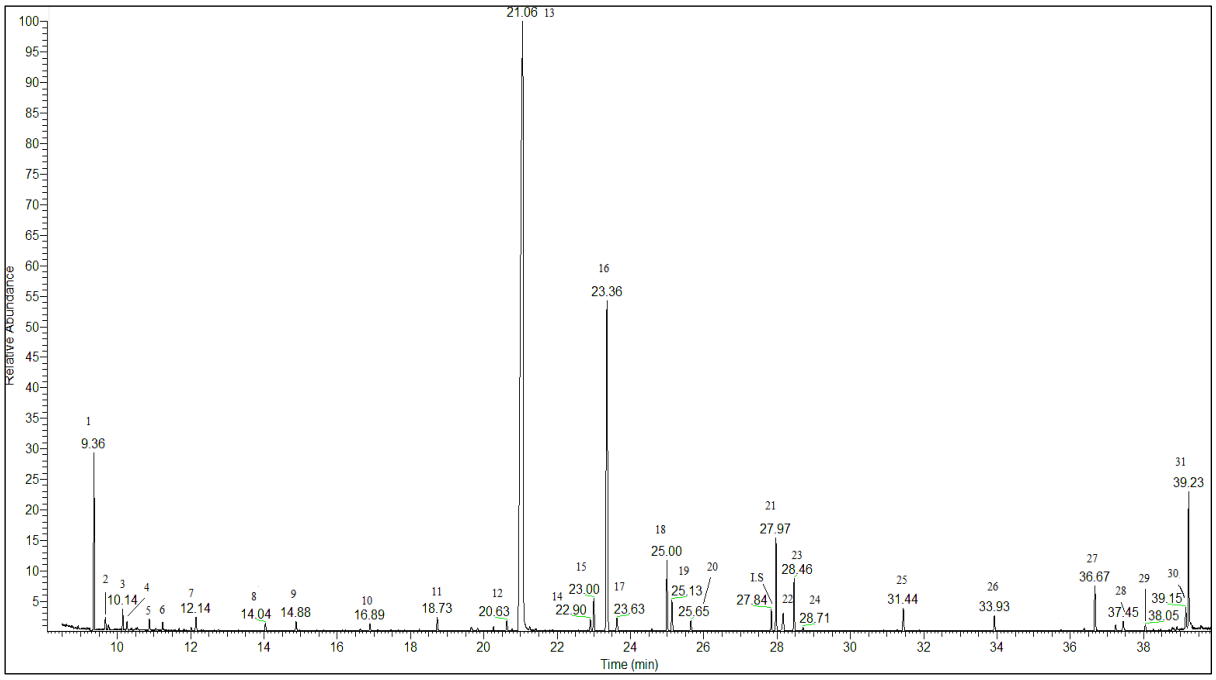


Figure 4.6. A typical chromatogram of fecal fermented black tea sample at 0 h. The peaks were identified according to their sources as follows: 1: (9.36) basal medium, 2: (9.67) unidentified (fecal), 3: (10.14) basal medium, 4: (10.25) basal medium, 5: (10.53) basal medium, 6: 10.86 basal medium, 7: (12.14) 3-phenylpropionic acid (fecal), 8: (14.04) 2-hydroxybenzoic acid (fecal), 9: (14.88) basal medium, 10: (16.89) 4-hydroxyphenylacetic acid (fecal), 11: (18.73) 3-(3-hydroxyphenyl)propionic acid (fecal), 12: (20.63) 3,4-dihydroxybenzoic acid (black tea), 13: (21.06) Caffeine (black tea), 14: (22.90) basal medium, 15: (22.99) unidentified (black tea), 16: (23.36) gallic acid (black tea), 17: (23.63) unidentified (fecal), 18: (25.00) basal medium 19: 25.13 unidentified (black tea), 20: (25.65) unidentified (black tea), 21: (27.97) solvent, 22: (28.15) solvent, 23: (28.46) solvent, 24: (28.71) unidentified (fecal), 25: (31.44) unidentified (fecal), 26: (33.93) solvent, 27: (36.67) basal medium, 28,29,30 (37,45, 38.05, 39.15) catechins, 31: (39.23) unidentified (fecal), I.S: 2,4,5-trimethoxycinnamic acid.

The composition of metabolites of fecal fermentation of black tea during 48 h period using GC-MS is illustrated in Figure 4.7. The quantification of metabolites with available standards listed in Table 4.3 was performed. The fermentation of fecal slurries with black tea (R1) resulted mainly in the appearance of pyrocatechol, pyrogallol, phloroglucinol, 4-hydroxyphenylacetic acid, 3-(3-hydroxyphenyl) propionic acid and 3,4-dihydroxyphenylacetic acid. The disappearance of 3,4-hydroxybenzoic acid which is originally present black tea was quantified using GC-MS. Moreover, the dissappearance of gallic acid was also confirmed by GC-MS. Gallic acid was also quantified using HPLC and the results were in agreement with the findings in HPLC analysis, it decreased gradually during fermentation showing same trend (data not shown). Hippuric acid, 3,4-dihydroxycinnamic acid and 2,4,6-trihydroxybenzoic acid which were previously detected in fecal fermentation of black tea [105], [147] were not observed in R1 samples in this study.

As stated previously, pyrogallol and pyrocatechol are the main fermentation products of gallic acid and gallated catechins (EGCG, ECG) and theaflavins [102], [189]. Pyrocatechol was produced in smaller quantities compared to pyrogallol during the fermentation of black tea phenolics by gut microbiota. It was initially absent in fermentor cultures, but it appeared at the 4^{th} h and reached its highest concentrations (3.02 µg/mL) at 24^{th} h however, after 24 h of incubation, its concentration did not change significantly (from 3.02 to 2.77 µg/mL) until the end of incubation ($p>0.05$) (Figure 4.7.a). Moreover, the increase in the concentration of pyrocatechol in fermentor cultures strongly correlated with the degradation of gallic acid ($r = -0.987$, $p<0.001$), gallated catechins such as EGCG ($r=-0.889$, $p<0.01$), ECG ($r=-0.910$, $p<0.005$) and theaflavins ($r=-0.971$, $p<0.001$). Furthermore, its production was also correlated with EC ($r=-0.971$, $p<0.0001$).

Pyrogallol was the highest in concentration among other metabolites of black tea fermentation. The production of pyrogallol was significant over 48 h period ($p<0.05$). It was not initially present at the 0^{th} h, however, it appeared at the 4^{th} h with a concentration of 65.57 µg/mL and reached its highest concentration (95.45 µg/mL) at the 24^{th} h, then decreased consistently until the end of incubation (17.09 µg/mL) (Figure 4.7.b), suggesting that

pyrogallol might have also been utilized by gut microflora. The increase in the concentration of pyrogallol were strongly correlated with utilization of gallic acid ($r = -0.903$, $p<0.05$), EGCG ($r = -0.946$, $p<0.05$), ECG ($r = -0.906$, $p<0.05$) and theaflavins ($r = -0.921$, $p<0.005$).

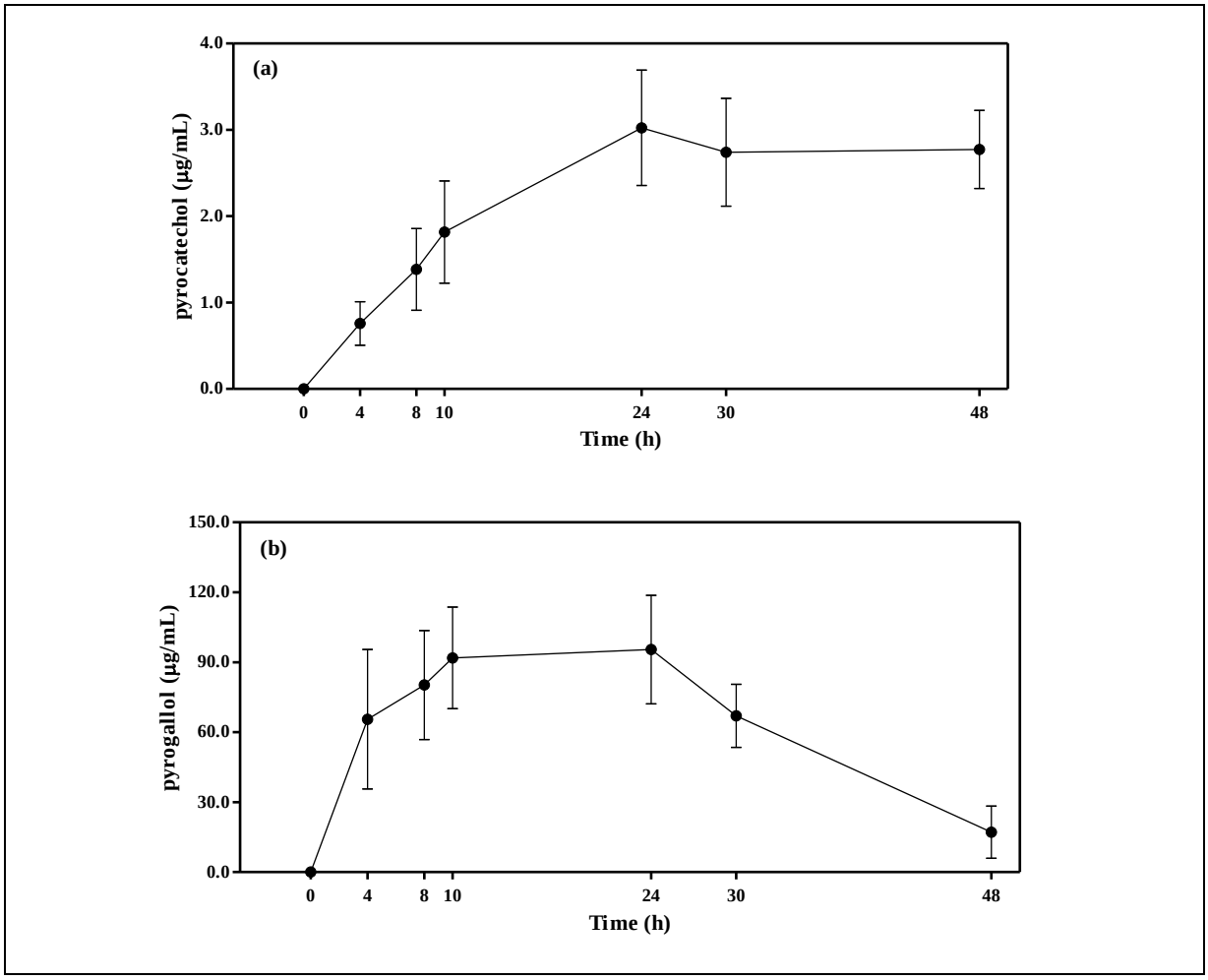


Figure 4.7.a.b. The profile of pyrocatechol (a) and pyrogallol (b) in fecal fermentation of black tea during 48 h period using GC-MS. Data are expressed as mean±SD of three individual fermentations (n=3).

Gallic acid underwent rapid degradation in the first 4 h of incubation resulting in rapid formation of pyrogallol and pyrocatechol for the same time frame. Moreover, no EGCG, ECG and theaflavins were observed after 24 h incubation, that's why pyrogallol and pyrocatechol

reached their higher concentration (95.45 and 3.02 µg/mL, respectively) at 24 h. Since none of precursor compounds were observed after 24 h, there was no pyrogallol production and its concentration started to decrease. Since gallic acid was available at smaller quantities until the end of incubation, the presence of pyrocatechol were attributed to gallic acid degradation after 24 h.

Pyrocatechol and pyrogallol are derived from the gallic acid moiety of 3-O-gallated catechins and theaflavins [102], [189]. The incubation of EGCG with fecal slurries yielded both pyrogallol and pyrocatechol [102]. Pyrogallol production in fecal slurries from EGCG started at 24th hour and decreased after that for one subject, it increased between 4-6 h, and then none were observed until the end of 48 h incubation for another subject. The production of pyrogallol was observed in the study of van Dorsten et al [106] evidenced by an increase in the concentration of pyrogallol in the first part of SHIME colon compartments after dosing of black tea extract, with the highest levels observed at 8th h and a reduction in the other parts of SHIME with further conversion. Gross et al [104] also showed that pyrogallol was not quantitatively produced from gallic acid and was further degraded by the gut microbiota and it was still detectable after 72 h in the batch cultures where black tea fecal fermentation occurred. In the presence of gallic acid *Eubacterium oxidoreducens* G41 which was isolated from the rumen was responsible for the production of pyrogallol [193]. In the study of Sanchez-Patan et al [87], EGCG were incubated with *L. plantarum* IFPL935 and the results showed that pyrogallol were produced after 24 h of fermentation, as the subsequent decarboxylation product of gallic acid, and its concentration increased until the end of incubation. In the study of Chen et al [189], in which the metabolism of theaflavins by human gut microbiota were investigated by incubating with fecal slurries collected from three healthy individuals, theaflavins were metabolized to gallic acid and gallic acid was further metabolized to pyrogallol. While gallic acid was almost completely degraded to pyrogallol after 48 h incubation with fecal slurries collected from one individual, very little gallic acid was degraded to pyrogallol even after 72 h incubation with fecal slurries collected from another individual [189]. This also shows the difference of metabolism of phenolics by different gut microbiota. While pyrocatechol was observed starting from the 4th h, reaching its

highest concentration at the 24th h and decreasing to some extent until the end of incubation for one subject, the appearance of this compound was at the 24th h and it increased until the end of incubation for the other subject [102]. Pyrocatechol resulted from the dehydroxylation of gallic acid during the fecal fermentation of grape seed extracts (flavanol monomers) for 48 h and it increased gradually to 0.2 µg/mL until the 30th h of incubation and reached a plateau between 30 h and 48 h [92].

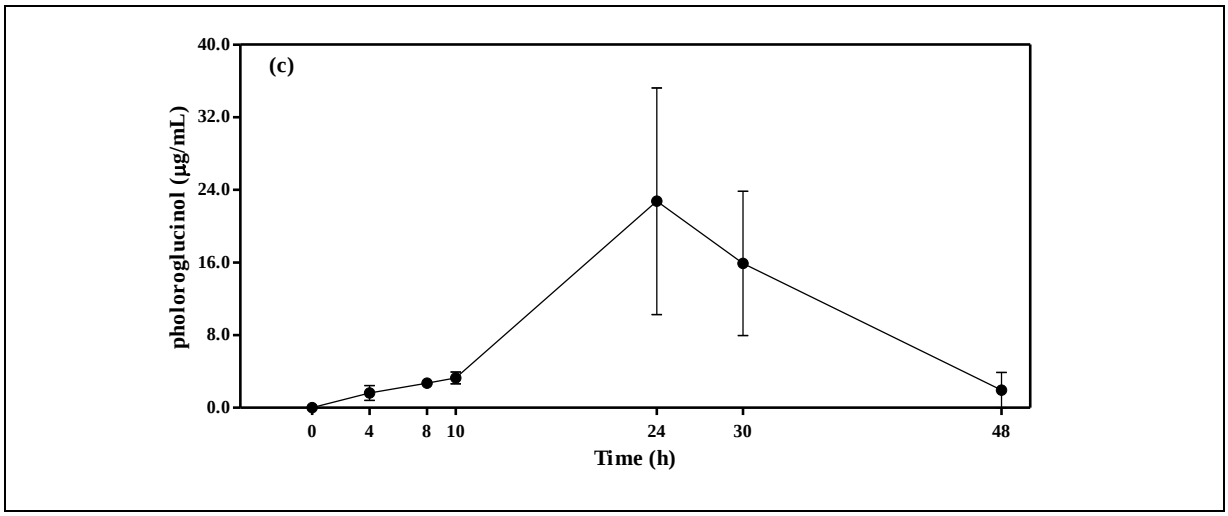


Figure 4.7.c. The profile of phloroglucinol in fecal fermentation of black tea during 48 h period using GC-MS. Data are expressed as mean±SD of three individual fermentations (n=3).

Phloroglucinol was also observed as an intermediate product during 48 h fermentation of R1 samples (Figure 4.7.c) It was not present initially but it was observed after 4 h fermentation reaching to 22.75 µg/mL at 24th h and decreased to 1.95 µg/mL at the end of incubation, but the levels are not significant over time ($p>0.05$). In the study of Braune et al [194], it was shown that phloroglucinol formed from the *in vitro* degradation of neohesperidin dihydrochalcone by human gut microbiota, underwent further rapid degradation by human gut microorganism, *Eubacterium ramulus*. Krumholz et al [193] investigated the metabolism of gallic acid and phloroglucinol by human fecal bacteria, *Eubacterium oxidoreducens* and it was found that gallic acid was metabolized to pyrogallol and pyrogallol were further

metabolized to phloroglucinol. However, in this study, no correlation was established between pyrogallol and pholoroglucinol.

3,4-dihydroxybenzoic acid (protocatechuic acid) and gallic acid were initially present in black tea. 3,4-dihydroxybenzoic acid decreased gradually during fermentation was and not observed at the end of fermentation ($p<0.05$) (Figure 4.7.d). Although in a study in which rat colonic fermentation were performed protocatechuic acid was shown as a metabolite of gallic acid and epicatechin [95], in this study, it was not observed as a metabolite.

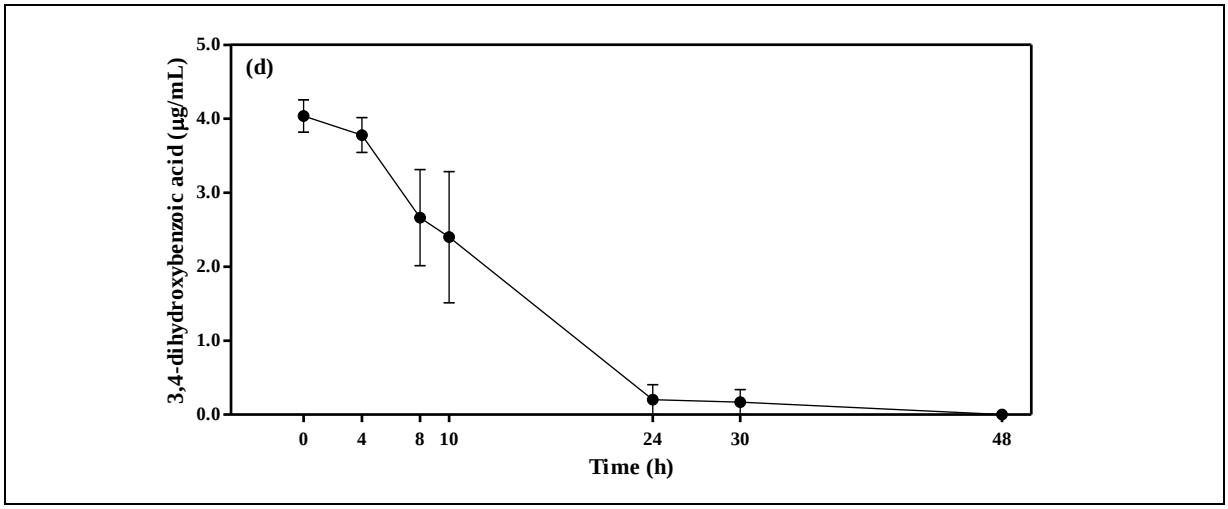


Figure 4.7.d. The profile 3,4-dihydroxybenzoic acid of in fecal fermentation of black tea during 48 h period using GC-MS. Data are expressed as mean±SD of three individual fermentations (n=3).

4-hydroxyphenylacetic acid and 3-(3-hydroxyphenyl)propionic acid, previously reported as the main metabolites of epicatechins [102], were initially present in all fecal slurries (2.50 and 4.78 µg/mL, respectively) and their concentration changed significantly over time ($p<0.05$) and reached to 14.84 and 22.90 µg/mL, respectively after 48 h of fermentation (Figure 4.7.e and Figure 4.7.f). The production 4-hydroxyphenylacetic acid were well correlated with the decrease in the concentration of EC ($r=-0.773$, $p<0.05$) and theaflavins ($r=-0.757$, $p<0.05$) during 48 h fermentation but not correlated to that of EGCG. Moreover, the correlation

between 3-(3-hydroxyphenyl)propionic acid and EC (r=-0.778, $p<0.05$) and TF (r=-0.763, $p<0.05$) indicated that its production resulted from degradation of epicatechin and theaflavin. EC and TF were completely degraded after 24 h incubation, and its potent metabolites 4-hydroxyphenylacetic acid and 3-(3-hydroxyphenyl) propionic acid reached its maximum at 24 h. These results are in aggreement with the findings in the literature [102], [104].

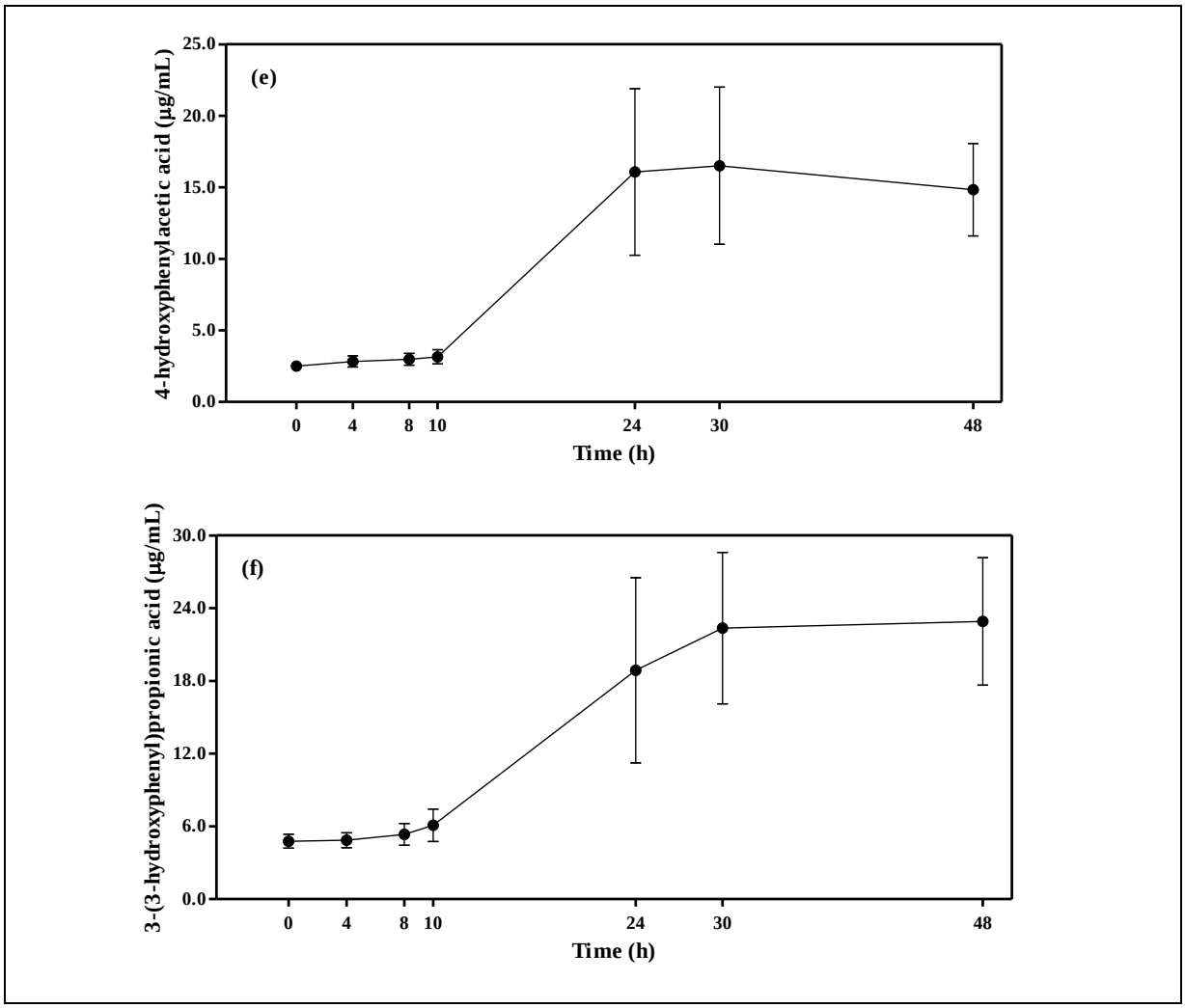


Figure 4.7.e.f. The profile of 4-hydroxyphenylacetic acid (e) and 3-(3-hydroxyphenyl)propionic acid (f) in fecal fermentation of black tea during 48 h period using GC-MS. Data are expressed as mean±SD of three individual fermentations (n=3).

In the study of Roowi et al [102], in which metabolites of EC, EGC and EGCG during 48 h fecal fermentation were investigated, 4-hydroxyphenylacetic acid was formed. During fermentation of EC, it was observed from 24th h of fermentation in one donor, whereas in two other donors it appeared from 4th h of incubation. In EGC fermentation, while the concentration of 4-hydroxyphenylacetic acid did not significantly change after 4 h through 48 h for two donors, it was observed after 24 hour incubation and increased gradually for one donor. EGCG also yielded 4-hydroxyphenylacetic acid after 24 h fermentation and it increased until the end of incubation for all donors. Moreover, the incubation of EC with fecal slurries collected from three different donor yielded 3-(3-hydroxyphenyl)propionic acid [102]. Furthermore, the appearance of 3-(3-hydroxyphenyl)propionic acid was also observed in the incubation of black tea containing 1000 mg/L phenolics with fecal slurries collected from 10 different healthy volunteers and the formation of this metabolite showed differences among the subjects [104]. The formation of 3-(3-hydroxyphenyl)propionic acid favored by dehydroxylation reactions of 3-(3,4,- dihydroxyphenyl)-propionic acid, which were also produced by epicatechins [91]. In the *in vitro* fecal fermentation of red wine extract, the formation of 3-(3-hydroxyphenyl)-propionic acid were observed as a gradual increase till 48 h of fermentation for a volunteer. Similarly, in the same study, 4-hydroxyphenylacetic acid showed progressive increase until the end of 48 h period for two volunteers [91]. Likewise, in the study of Cueva et al [92], in which the degradation of grape seed extracts by human gut microflora was shown, the production of 4-hydroxyphenylacetic acid increased progressively during the fermentation of both grape seed extracts. Concentration of 3-(3-hydroxyphenyl)-propionic acid also increased in small increments during 48 h fermentation for both extracts.

3,4-dihydroxyphenylacetic acid was also initially present at the 0th h (2.57 µg/mL), however,the concentration changed significantly over time ($p<0.05$). It reached its highest concentration at the 24th h (8.45 µg/mL) and then decreased to a certain extent (3.97 µg/mL) until the end of fermentation (Figure 4.7.g). However, the formation of this metabolite was not correlated with any of the identified precursor compounds ($p>0.05$) in this study. However, this results are in agreement with the findings in literature [91], [92], [105]. The intermediate precursor compounds of 3,4-dihydroxyphenylacetic acid was previously

identified as 3-(3,4,-dihydroxyphenyl)-propionic acid, however, 3,4-dihydroxyphenylacetic acid was not produced from EGCG, EC and ECG during rat colonic fermentation [95]. However, black tea contains other phenolic compounds such as quercetin to a certain extent [131], not identified in this study, which also strongly produces 3,4-dihydroxyphenylacetic acid as a fecal fermentation metabolite [97], [149], [195]. The presence of 3,4-dihydroxyphenylacetic acid was observed in small quantities during fecal fermentation of black tea in a dynamic *in vitro* model of the colon (TIM-2) during 30 h [105]. Moreover, in the study performed with healthy humans with an intact colon, after black tea solid administration, 3,4-dihydroxyphenylacetic acid was also found as metabolite [149]. In the *in vitro* fecal fermentation of grape seed extracts, 3,4-dihydroxyphenylacetic acid reached its highest concentration in the first 10 h of fermentation and then decreased [92]. Similarly, in the in vitro fecal fermentation of red wine extract, although it was initially absent, 3,4-dihydroxyphenylacetic acid started to increase until 24 h of incubation, then rapidly decreased [91].

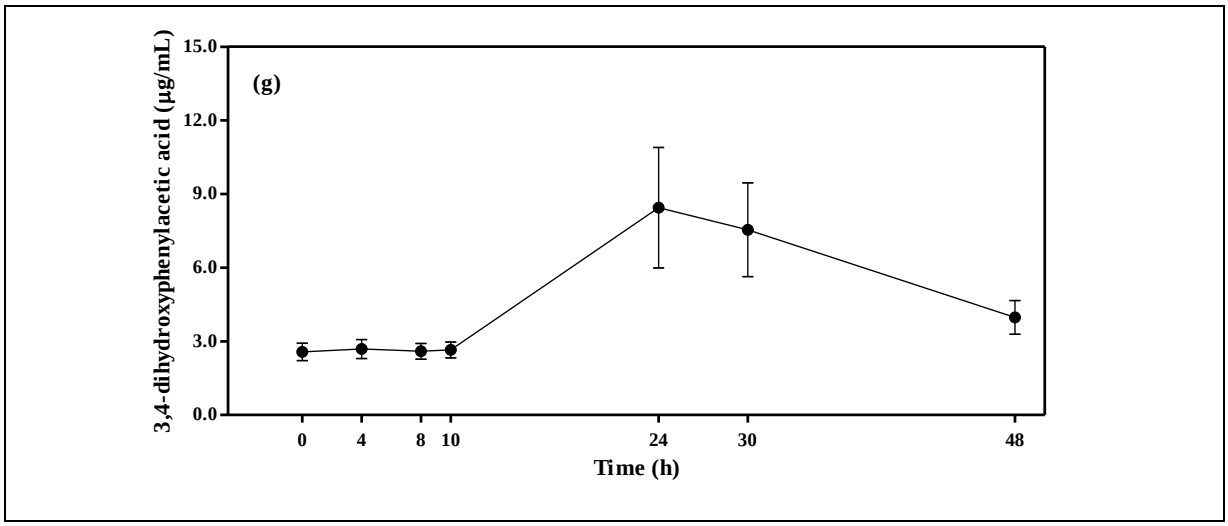


Figure 4.7.g. The profile of 3,4-dihydroxyphenylacetic acid in fecal fermentation of black tea during 48 h period using GC-MS. Data are expressed as mean±SD of three individual fermentations (n=3).

Moreover, in this study, although they were not quantified, the appearance and increase in the concentration of resorcinol and 3,4-hydroxyphenylpropionic acid was observed by GC-MS during 48 h fermentation. Resorcinol is formed from pyrogallol and phloroglucinol resulting in a decrease in the concentration of both pyrogallol and phloroglucinol [196]. 3,4-hydroxyphenylpropionic acid is formed from both the fecal fermentation of catechin and epicatechin from red wine and grape seed extracts which contains high level of catechins [91], [92], [104].

However, the predominant fecal degradation products of EC and EGCG have previously been identified as (-)-5-(3',4'-dihydroxyphenyl)-γ-valerolactone and 5-(3,4-dihydroxyphenyl)-γ-valeric acid and 5-(3',4',5'-trihydroxyphenyl)-γ-valerolactone as intermediate metabolites after that yielding 3-(3-hydroxyphenyl)propionic acid, 4-hydroxypehenylacetic acid [91], [92], [102], they were not identified by library in this study due to the lack of standards.

4.3.3. Analysis of Bacterial Community in Fermentor Cultures by Plate Counting and FISH

The effects of black tea phenolics on human microflora were investigated by performing selective enumeration (plate counting) at 0^{th} and 48^{th} hour and fluorescent *in situ* hybridization (FISH) analysis at 0, 8, 24 and 48 h of incubation.

The changes in the bacterial population by selective plate counting performed for fecal fermentation of black tea samples (R1) and fecal cultures without black tea addition (R2) at 48^{th} h are illustrated in Figure 4.8. The changes in bacterial community by black tea extract addition did not reveal conclusive effects. When compared to control (R2), after 48 h fermentation, the growth of total aerobes, *Lactobacillus* spp. and *Clostridia* group showed a slight increase upon black tea extract addition. On the other hand, a slight supression were observed on the growth of total anaerobes, *Enterobacteria* and coliform. However, *Staphylococcus aureus* was not detected in either culture (R1 and R2). The changes in the

bacterial groups were not significant when compared to control group (R2) ($p>0.05$). Contradictory results on the community changes in the presence of either individual phenolics or black tea extracts were reported in literature [110], [120]. Epicatechin, catechin and their metabolite 4-hydroxyphenylacetic acid positively affected the growth of commensal *Clostridium* spp., *Bifidobacterium* spp. and *Lactobacillus* spp. but not pathogenic *Clostridium* spp. [110]. However, culture dependent techniques (plate counting) were difficult to draw conclusive effects of the phenolic metabolism on the bacterial growth whereas clear trends could be observed using culture independent methods, Nevertheless, plate counting was performed to investigate the stability of microorganism during long-term fecal fermentation in an *in vitro* models in literature [120], [162]. On the other hand, the *in vitro* gut fermentation study after addition black tea phenolics results showed that the growth of *Bacteroides* and *Bifidobacterium* spp. were affected by black tea phenolics in the observation on qPCR while they were not reduced by plate counting [120]. In phyla level, the black tea addition to the fermentation resulted in a shift in the Firmicutes: Bacteroidetes ratio by stimulating *Klebsiella*, enterococci and *Akkermansia* and reducing bifidobacteria, *B. coccoides*, *Anaeroglobus* and *Victivallis* [120].

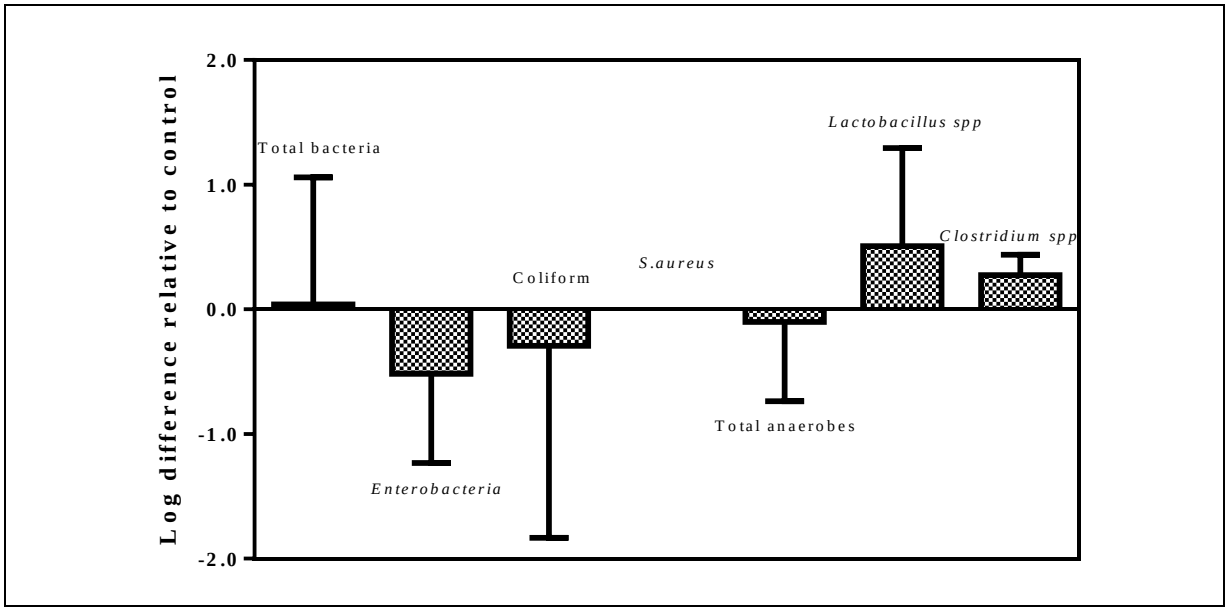


Figure 4.8. Selective enumeration of microorganism by plate counting. The changes in growth of bacterial group were expressed by comparing the number of a specific bacterial group in a fecal fermentor sample with the number found in the control group, at 48^{th} h.

Although culture dependent techniques such as plate counting were not well-established for the effect of catechins and its metabolites on gut microbiota, culture independent techniques such as FISH, PCR-DGGE and qPCR techniques were launched for analysis of the interaction of catechins and gut microbiota. According to FISH analysis, whereas no significant changes were not observed for bacterial groups (*Bifidobacterium* spp., *Lactobacillus/Enterococcus* spp., *Bacteroides* spp., and total bacteria) in the presence of wine extracts including catechins and gallic acid [91], significant increase in *Lactobacillus/Enterococcus* spp. and a slight decrease in *C. histolyticum* in the presence of momeric or dimeric flavanols were observed for the same *in vitro* fermentation system [88], [92]. Differences between results were attributed to some factors such as the concentration of extracts used in the system and time to exposure of phenolic compounds [91]. The concentration of catechins (22.0±7.0 mg/L) which was found in this study was lower compared to that of catechin /epicatechin standards (150− 1000 mg/L) used by Tzounis et al. [88] and close to 5.94, 4.12, and 20.94 mg/L of catechin, epicatechin and flavan-3-ols in wine extract used by Sanchez-Patan et al [91].

Since selective counting were only applicable for culturable microorganisms and since bacterial enumeration were performed for the beginning and end of fermentations, the changes in the bacterial communities during incubation period at specific time points could not be observed in this study, there should be a need to observe the microbial profile changes by culture-independent techniques such as FISH.

In FISH analysis, the microbial profile were observed at 8, 24, 48 h. The time points were selected according to GC-MS results, in which the metabolite profilings were changed siginificantly. the analysis of total bacteria (EUB mix; three EUB probe used in equimolar concentration), *Lactobacillus−Enterococcus* spp. (Lab 158), *Clostridium coccoides−Eubacterium rectale* group (Erec 482), *Bifidobacterium* spp. (Bif 164), *Bacteroides* spp. (Bac 303) were performed. The representative FISH images in this study for each probe are given in the Figure 4.9-4.14)

The FISH procedure is usually performed in 6 steps (fixation, sample preparation, hybridization, washing, mounting ,visulization). When the target molecule is 16S rRNA, the fixation and hybridization steps are important. Fixation step, which is crucial for succesive results, is performed for the permeability of bacterial cells walls to allow the penetratation of the probe protecting RNA from degradation. When the paraformaldehyde solution allows the sufficient fixation for gram negatives, ethanol fixation can be used for gram positives. paraformaldehyde fixation reqiured additional enyzmatic and/or heat treatment step for gram positives to open the pepdidoglycan layer, allowing permeability [161], [197], [198], [199]. In the begining ofthis study, the bacterial cells were fixed with either ethanol or paraformaldehyde. Ethanol fixation was also performed for gram negatives, however, ethanol fixation caused to short term storage of the samples and insufficient results (no images for Cy-3 dye) were obtained. Therefore, paraformaldehyde fixation was done for all bacterial groups. No clear images were obtained by using fluorescence microscope, therefore, confocal microscope was chosen for better images. For Lab 158 and Bif 164 probes, enzymatic reactions were performed by trial for different concentration of lysozyme and different incubation temperatures. Lysozyme can break down β(1→4) linkages between *N*-acetylmuramic acid and *N*-acetyl-d-glucosamine residues in peptidoglycan and between *N*-acetyl-D-glucosamine residues in chitodextrin. The cells for Lab 158 and Bif 164 probes were incubated in 1 mg lysozyme/mL 100mM Tris-HCl for 15 minutes at room temperature (Figure 4.9) after sample preparation step. In sample preparation, the concentrations of cells in the wells were optimized. The most significant problem of FISH analysis is to be hampered by unspecific binding [197]. The basal medium, mounting and the FISH procedure result in the false signal intenstities [200]. Although FISH analysis were used by many research groups for the investigation of fecal bacteria [88], [92], [122], the quantification of hybridized bacteria in fecal preparation is strongly dependent on the skills of worker. In the study of Jansen et al [173], the error assay was introduced by accounting the one homogenized sample by 20 times and visual counting were found to be less accurate than automated methods.

In this study, although all the steps were performed in FISH analysis, due to the nonspecific binding of the probes in the background, the counting were not successful for each time point,

therefore, the analysis should be confirmed for conclusive counting and the prepresentative images and the problems encountered during the analysis in this study built up a baseline information for further studies.

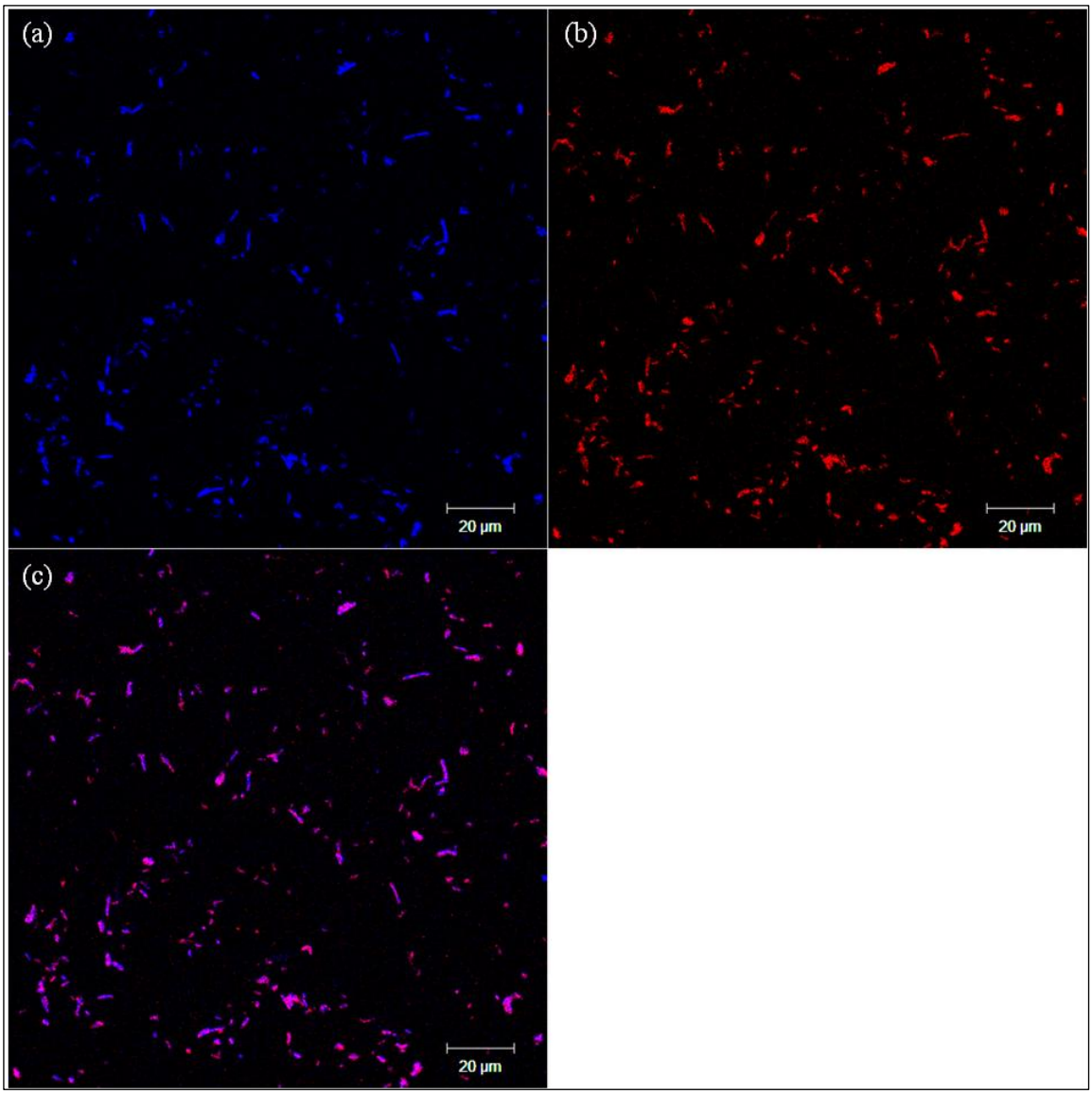


Figure 4.9. Confocal microscope image of Lactobacillus-Enteroccus spp. (Lab 158) of fecal fermentation (R1, 0^{th} h) after lysozyme treatment (1 mg/mL). a: DAPI-counting, b: Cy3-dye c: Merge image of DAPI and Cy-3

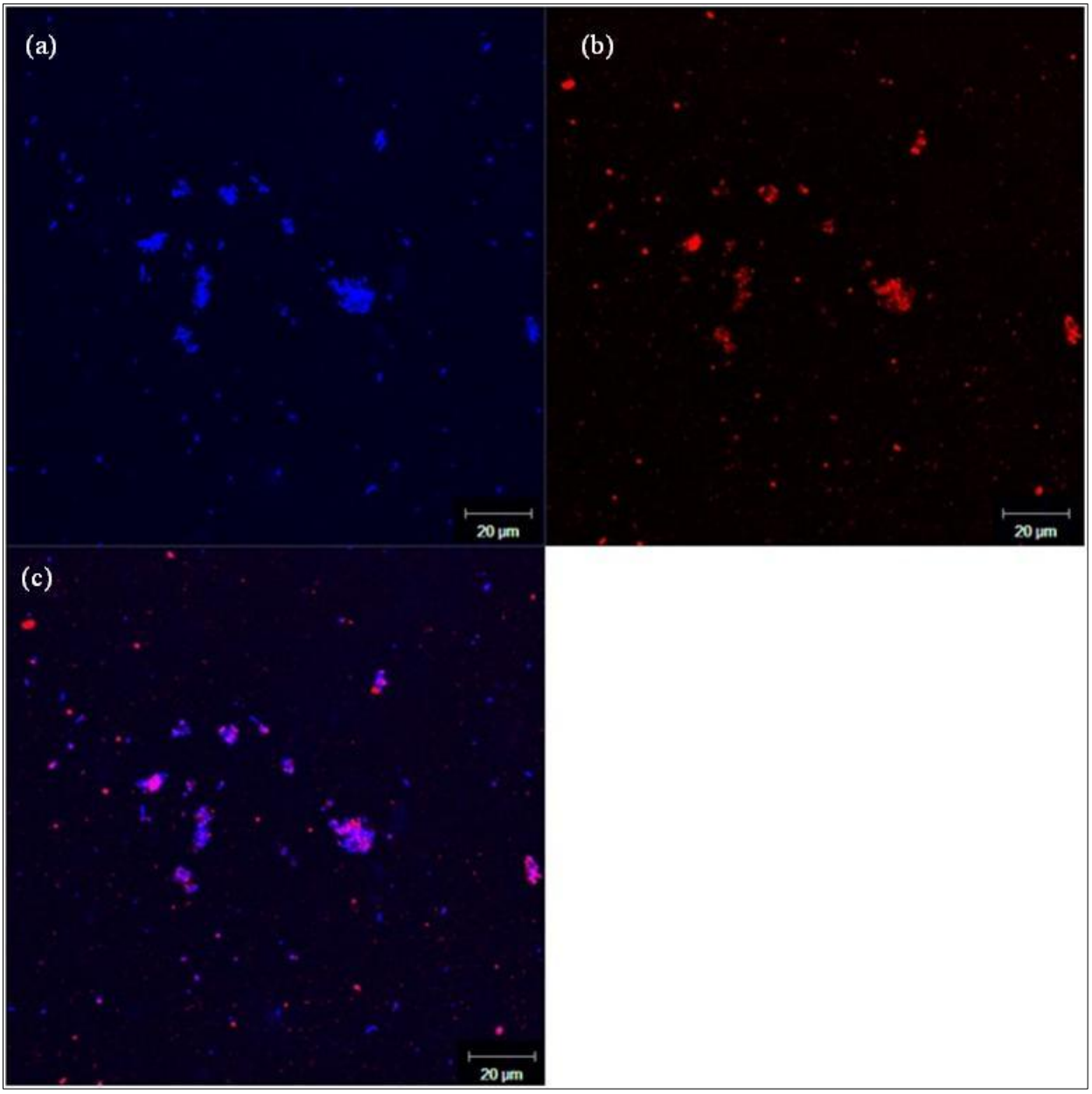


Figure 4.10. Confocal microscope image of *Bacteroides* spp. (Bac 303) of fecal fermentation (R1, 8th h). a: DAPI-counting, b: Cy3-dye c: Merge image of DAPI and Cy-3

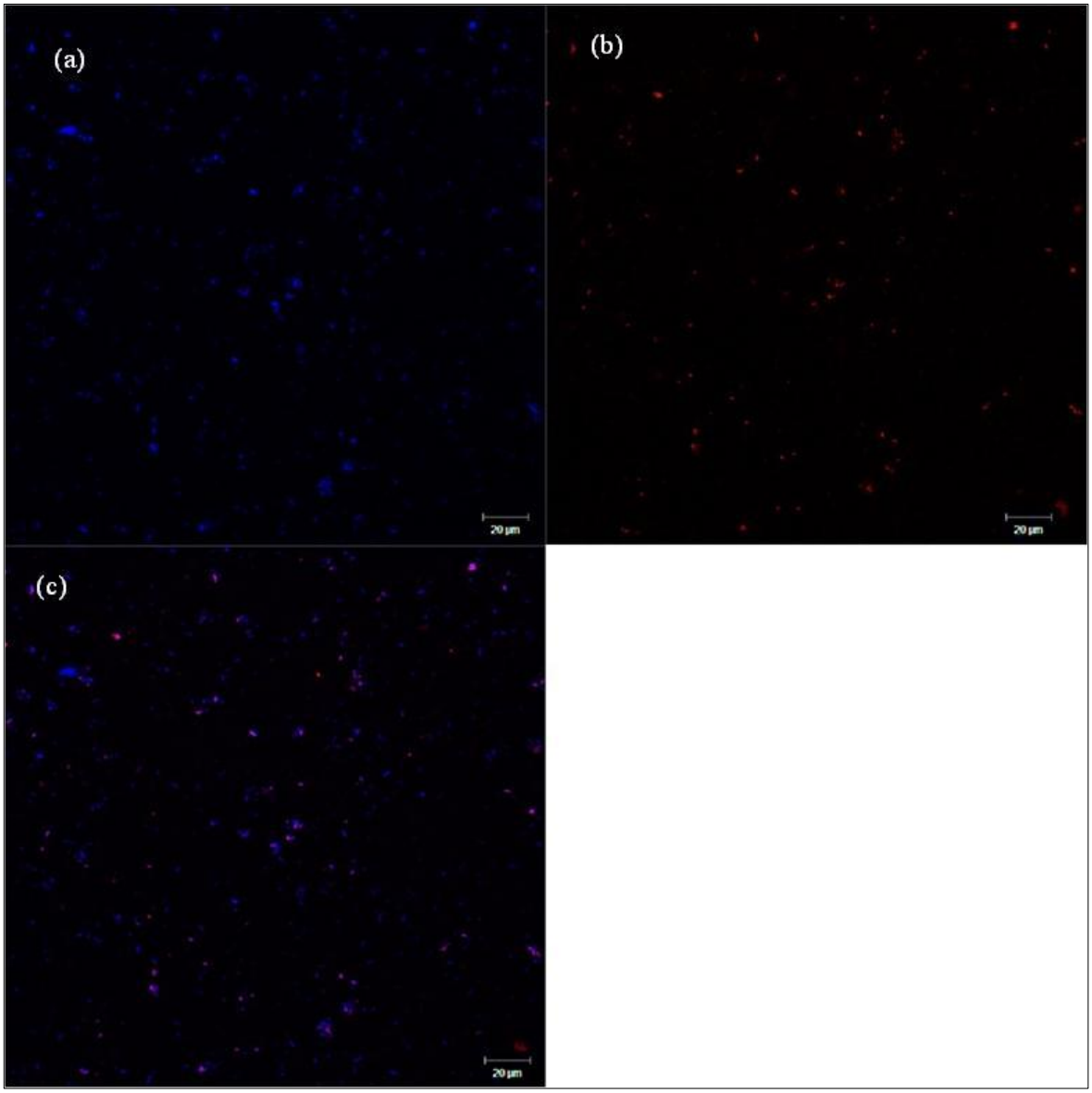


Figure 4.11. Confocal microscope image of *Bifidobacterium* spp. (Bif 164) of fecal fermentation (R2, 24th h) after lysozyme treatment (1 mg/mL). a: DAPI-counting, b: Cy3-dye c: Merge image of DAPI and Cy-3

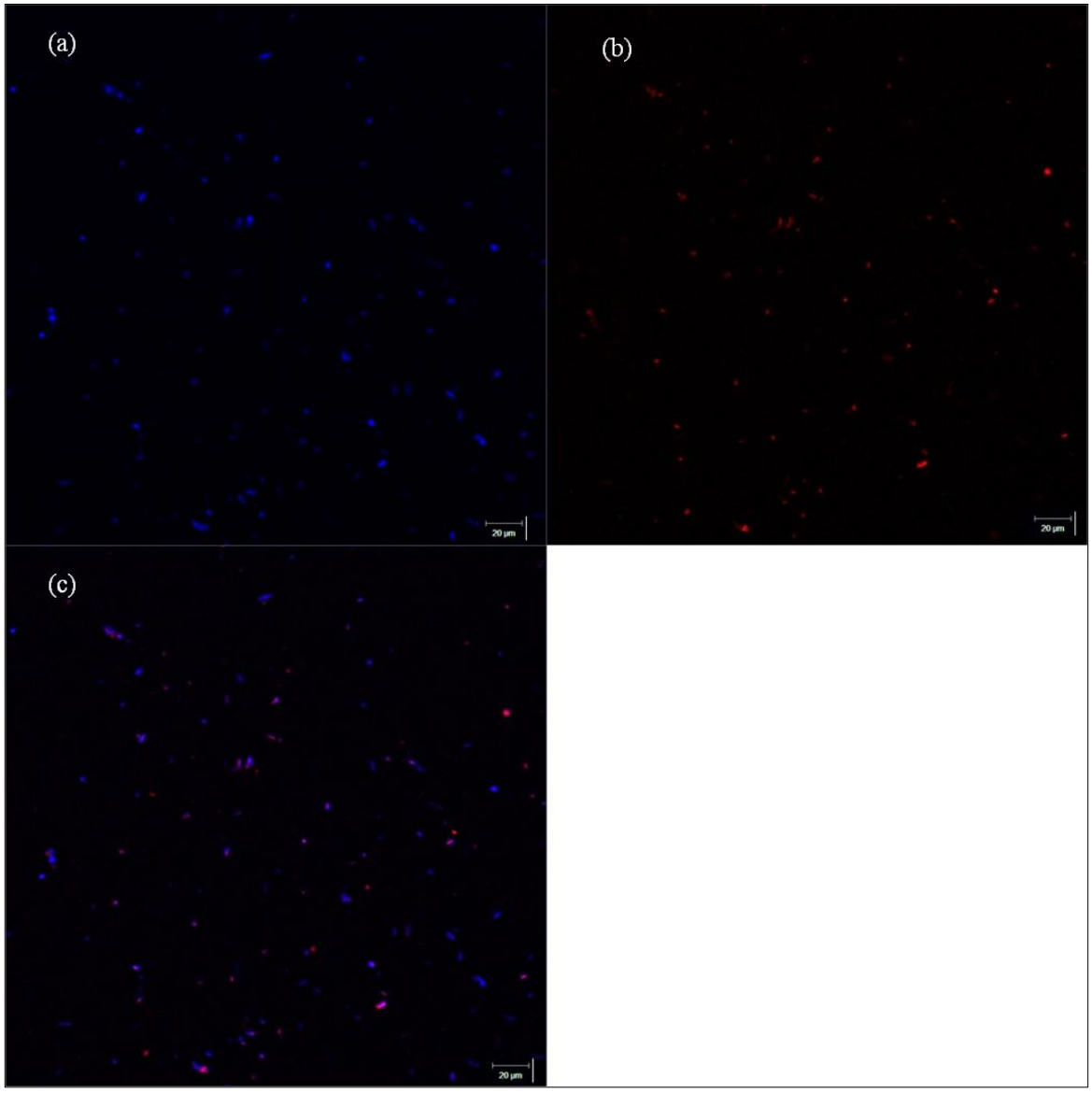


Figure 4.12. Confocal microscope image of *Eubacterium rectale−Clostridium coccoides* group (Erec 482) of fecal fermentation (R2, 0^{th} h). a: DAPI-counting, b: Cy3-dye c: Merge image of DAPI and Cy-3

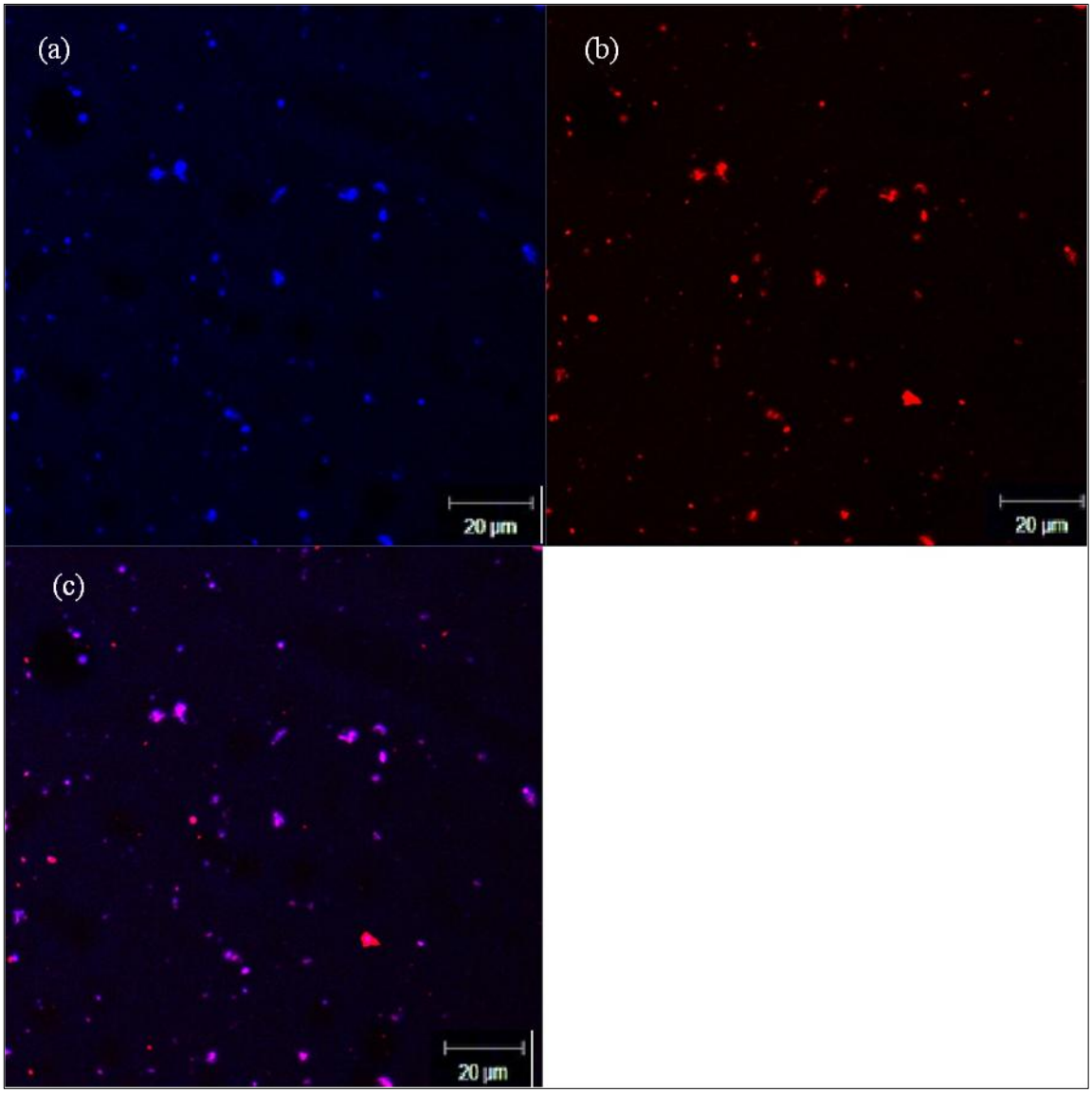


Figure 4.13. Confocal microscope image of Total bacteria (Eubmix: EUB 338, EUB 338II, EUB 338III) of fecal fermentation (R1, 48th h). a: DAPI-counting, b: Cy3-dye c: Merge image of DAPI and Cy-3

5. CONCLUSION

In this study, the interaction between human colonic microflora and phenolic compounds in Turkish black tea were investigated by analysis of microbial bioconversion of tea phenolics in *in vitro* pH-controlled fermentor cultures using HPLC, GC-MS-based profiling of tea phenolic metabolites and the changes in the bacterial population by selective plate counting and fluorescent in situ hybridization to reveal the effects of phenolic profile changes on the gut microflora.

The results of this study showed that human gut microflora have a potent capacity to metabolize the Turkish black tea phenolics and increase digestion of phenolics by breaking down into more easily absorbed phenolic compounds such as phenolic acids, phenylacetic acids, phenylproppionic acids. The production of pyrogallol and pyrocatechol were correlated with the degradation of gallic acid, gallated catechins (EGCG and ECG) and theaflavins. 4-hydroxyphenylacetic acid and 3-(3-hydroxyphenyl)propionic acid were found correlated with EC and theaflavins.

Moreover, in return, the Turkish black tea phenolics have an influence on the colonic microflora profile. At the end of fermentation, there is an increase in the growth of *Lactobacillus* spp., and *Clostridium* spp. and decrease in the *Enterobacteria* and coliform organisms which have harmful effects on the human gut health, but not significantly ($p>0.05$) compared to control. Also, FISH analysis needed to be confirmed for the microflora profile at certain time points.

As a conlusion, although this study provided detailed and valuable information about the interaction of black tea phenolics and human colonic microflora in vitro, the specific microorganisms involved in the metabolism of black tea phenolics and the potent health benefits of the phenolic metabolites is still not clear and needs to be studied in future studies, both *in vitro* and *in vivo*.

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APPENDIX A: PHENOLIC COMPOSITION OF BLACK TEA EXTRACTS

Table A.1. Phenolic composition of freeze-dried and spray dried black tea extracts.

	Black Tea Extract Samples	
Catechins (mg/g dry extract)	Freeze dried	Spray dried
GC	ND[a]	ND
EGC	ND	ND
C	ND	ND
EC	5.18	3.69
EGCG	6.17	5.74
GCG	1.83	1.52
ECG	1.14	1.02
Total catechins	14.32	11.97
GA	11.36	12.77
TF (% Area)	2.17	1.27

[a]ND: Not determined.