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Fate of dietary phytosteryl/-stanyl esters upon digestion by humans: Analysis of individual intact esters in feces

Tim Lubinus

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ABBREVATIONS

5αC	5α-cholestane
ABCG5/G8	ATP-binding cassette transporter (ABC) heterodimer G5/G8
ACAT2	acyl-CoA:cholesterol-acyltransferase 2
AD	androst-4-ene-3,17-dione
ADD	androst-1,4-diene-3,17-dione
C16:0	palmitic acid (in intact esters)
C18:1	oleic acid
C18:2	linoleic acid
C18:3	linolenic acid
C20:0	eicosanoic acid
CA	caffeic acid
CC	cholesteryl cinnamate
CD	cholesteryl dodecanoate
CF	cycloartenyl ferulate
CHD	coronary heart diseases
CM	chylomicrons
CP	cholesteryl palmitate
CS	cholesterol
CVD	cardivascular diseases
DHCA	hydrocinnamic acid (dihydrocinnamic acid)
Eq	equation
EF	ethyl ferulate
EtOAc	ethyl acetate
EtOH	ethanol
ExS	extraction standard
FA	ferulic acid
FID	flame ionization detection
FPP	farnesyl pyrophosphate
GC	gas chromatography
GI	gastrointestinal
HAD	9α-hydroxyandrost-4-ene-3,17-dione
HCA	hydrocaffeic acid (dihydrocaffeic acid)
HDL	high density lipoprotein
HDL-C	high density lipoprotein cholesterol
HPA	3-(3-hydroxyphenyl)propionic acid
IPP	isopentenyl pyrophosphate

IS	internal standard
LDL	low density lipoprotein
LDL-C	low density lipoprotein cholesterol
LXR	liver X receptor
MCF	24-methylenecycloartanyl ferulate
MPA	3-(3-methoxyphenyl)propionic acid
MS	mass spectrometry
MTP	microsomal triglyceride transfer protein
NB	nutritive buffer
NPC1L1	Niemann-Pick C1-like 1 protein
PA	palmitic acid
PCE	pancreatic cholesterol esterase
PS	phytosterols/-stanols
PSA	phytostanyl fatty acid esters
PSE	phytosteryl fatty acid esters
PSF	phytosteryl/-stanyl ferulic acid esters
ROS	reactive oxygen species
RRF	relative response factor
RRT	relative retention time
SA	stearic acid
SGA	syringic acid
SIM	selected ion monitoring
TC	plasma total cholesterol
TG	plasma triacylglycerols
THB	thioglycollate broth
TMS	trimethylsilyl
TS	testosterone
VLDL	very low density lipoprotein

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1 INTRODUCTION AND OBJECTIVES

According to the World Health Organization (WHO), cardiovascular diseases (CVD) including any disorders of the heart and blood vessels are the leading causes of death worldwide [1]. The majority of CVD deaths are attritbuted to coronary heart diseases (CHD) and strokes, both predominantly caused by atherosclerotic plaque formation within the arteries. Hypercholesterolemia characterized by increased plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels is one of the major risk factors for the development of CHD. Hypercholesterolemia treatment conventionally involves medication with either statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) or Ezetimibe (intestinal cholesterol absorption inhibitor) as well as a combination therapy of both. In addition to the medical treatment, dietary supplementation of phytosterols/-stanols (PS) and their esters via the consumption of fortified food products is increasingly offered as an auxiliary therapeutic treatment. The cholesterol-lowering properties of phytosterols/-stanols and their fatty acid esters commonly used in functional food products have been described in several human studies [2-7]. Similar effects on plasma cholesterol in humans have also been reported for phytosteryl/-stanyl ferulic acid esters (PSF) using γ-oryzanol, a ferulate mixture obtained from rice bran [8-11].

The mechanisms by which these bioactive compounds impart their effect are not completely elucidated. According to the present knowledge the mechanisms discussed in the literature all require phytosterols/-stanols in their free form. Thus, the rate of intestinal hydrolysis of the respective phytosteryl/-stanyl esters by digestive enzymes, e.g. pancreatic cholesterols esterase (PCE), appears to be a limiting factor for the cholesterol-lowering efficiency. *In vitro* data [12-15] and animal studies [16, 17] indicate that the hydrolysis of these esters is affected by both the phytosterol structure and the acid moiety. So far, research in humans was either confined to oral administrations with ileostomy patients [18-20] or to intubation studies in subjects with normal intestinal tract [21-23]. The data arising from these human trials do not provide insights regarding the metabolic fate of individual phytosteryl/-stanyl esters *in vivo* because they exhibit three major limitations: (I) Phytosteryl/-stanyl ferulic acid esters have not been employed as

substrates in any of the human studies; (II) a potential impact of the colon microflora on the metabolization was disregarded by the exclusive investigation of small intestinal excreta although microbial conversion products have been reported at least for phytosterols in their free form [24-29]; (III) the approaches commonly used for the calculation of hydrolysis rates of total fatty acid esters were based on the separate quantitation of liberated PS and total phytosterols/-stanols subsequent to alkaline saponification of the remaining esterified fraction.

Therefore, the aim of this thesis was to investigate the metabolic fate of individual phytosteryl and -stanyl esters varying in their molecular structures upon consumption and digestion by healthy human subjects. To approach this objective a randomized human trial (n = 15) employing functional food products enriched with complex mixtures of phytosteryl/-stanyl esters as food carriers of the target substances should be performed; hydrolysis rates of individual phytosteryl/-stanyl fatty acid and ferulic acid esters should be determined by comparison of the respective amounts of intact esters recovered in feces with the amounts consumed. Prior to the human trial, *in vitro* fermentation experiments using human feces as inoculum and esters varying in their molecular structures as subtrates should be conducted to investigate whether steryl and stanyl esters are subject to bacterial transformation processes when entering the human colon in intact form.

2 BACKGROUND

2.1 PHYTOSTEROLS/-STANOLS

2.1.1 STRUCTURE AND NATURAL OCCURRENCE

Phytosterols are natural constituents of plants and as steroid alcohols they are part of the broad group of isoprenoids, more precicesly triterpenoids. In analogy to cholesterol in animals, the amphiphilic phytosterols/-stanols have many essential functions in plant cells. Predominantly, they regulate fluidity and permeability of cell membranes and are involved in activities of mebrane-bound enzymes and signal transduction [30-32]. In addition, they are substrates for the synthesis of a wide variety of secondary plant metabolites such as glycoalkaloids, cardenolides and saponins [30]. Futhermore, they play important roles in cellular and developmental processes in plants as precursors to the brassinosteroids [32].

Overall, more than 250 distinct types of phytosterols and related derivatives containing more than 100 different phytosterols/-stanols have been reported in plant and marine species [31-33]. In general, phytosterols exhibit molecular structures made up of a tetracyclic cyclopenta[a]phenanthrene ring, two methyl groups at C10 and C13, a long flexible carbon side chain with 9 – 10 carbons at C17 and a hydroxyl group an C3 with β-stereochemistry (Fig. 1) [34].

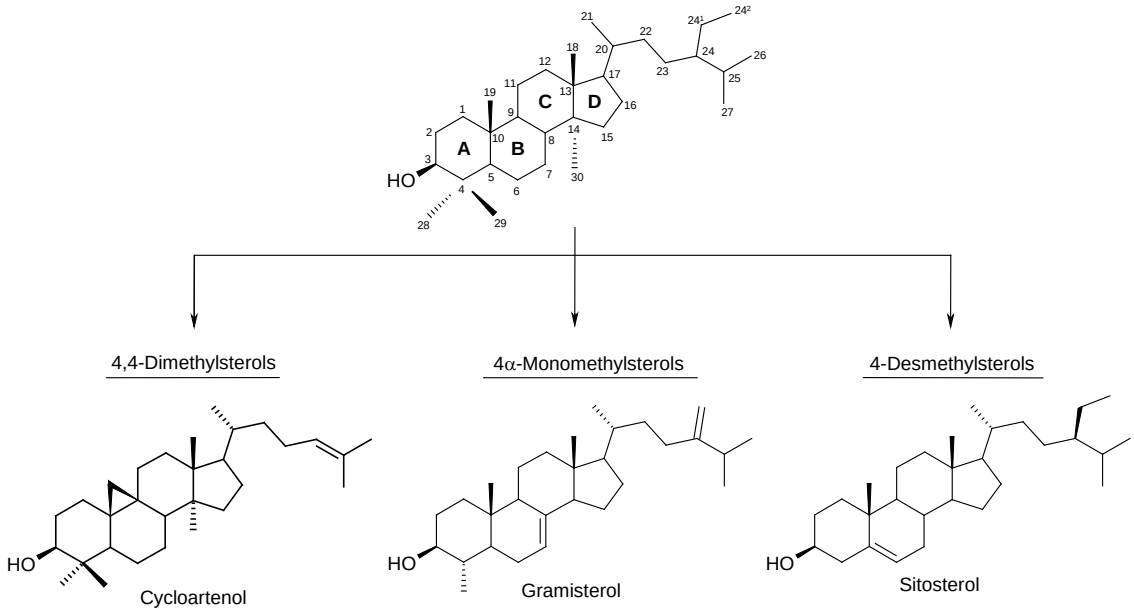

Fig. 1 General molecular structure of phytosterols (according to [34]) and representatives for the three different groups: 4,4-Dimethylsterols, 4α-Monomethylsterols, and 4-Desmethylsterols

Based on structural and biosynthetic grounds, phytosterols can be divided into 4,4-dimethylsterols, 4α-monomethylsterols, and 4-desmethylsterols (Fig. 1). The 4,4-dimethylsterols and 4α-monomethylsterols are biosynthetic intermediates existing at lower levels than the end-products 4-desmethylsterols. The most abundant 4-desmethylsterols comprising compounds without methyl substitution at C4 are synonymous with the term phytosterols commonly used.

Phytosterols can be further categorized according to the position and number of the contained double bonds into Δ^5-sterols (double bond between C5 and C6), Δ^7-sterols (double bond between C7 and C8), and $\Delta^{5,22}$-sterols (double bonds between C5 and C6 as well as C22 and C23). Saturated phytosterols without double bonds are called phytostanols (Fig. 2).

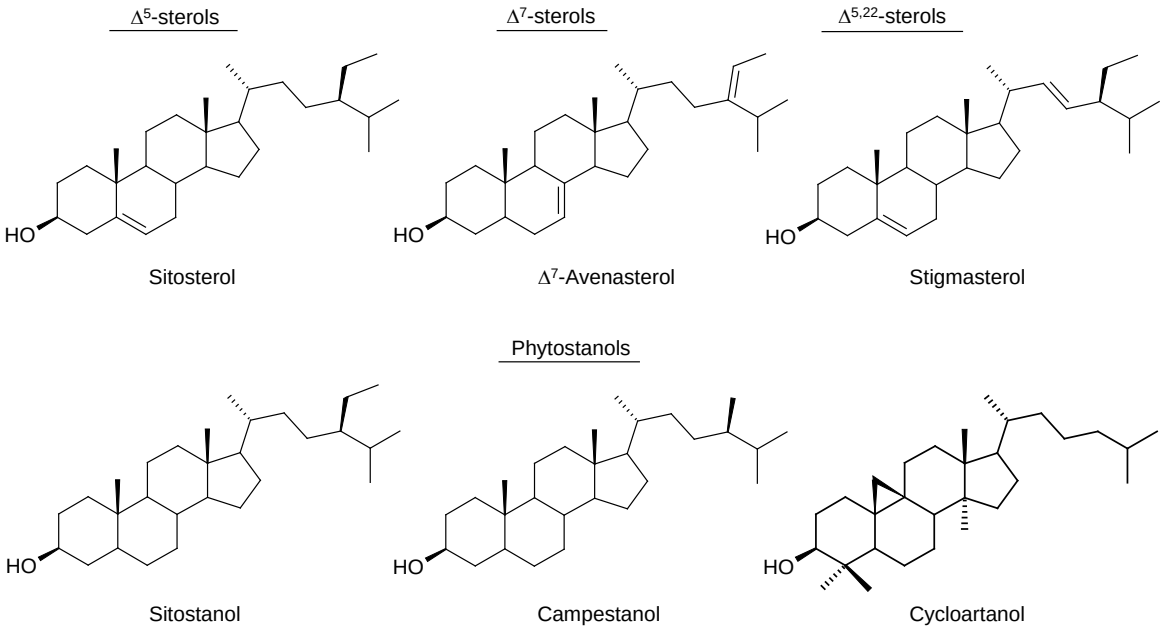

Fig. 2 Representatives of the phytosterol categories: Δ^5-sterols, Δ^7-sterols and $\Delta^{5,22}$-sterol and molecular structures of selected phytostanols

The structural similarity between phytosterols and mammalian cholesterol is astonishing. They only differ by the side chain structure. In contrast to cholesterol, 4-desmethylsterols exhibit an additional methyl or ethyl group at C24 or an additional double bond in the side chain. The main sources of phytosterols in the human diet are vegetable oils, cereal grains, cereal-based products, nuts and

seeds [32, 35-40]. Depending on biological conditions and technological processes, total phytosterol contents in vegetable oils range between 50 and 1560 mg/100 g of oil, with corn and rapeseed oils containing the highest, and palm oil containing the lowest amounts [32, 37, 39]. Crude rice bran oil is exceptional in that it can contain phytosterols in concentrations up to 3225 mg/100 mg of oil [32]. Among cereal-based products, the richest sources of phytosterols are wheat germs (344 mg/100 g raw material) and rice bran (450 mg/g raw material) [35, 36, 40]. In nuts and seeds, total phytosterol concentrations ranged between 95 mg/100 g in pumpkin seeds and brazil nut up to 404 mg/100 g in sesame seeds [37, 38].

In general, the most abundant phytosterol in the human diet is β-sitosterol (up to 62 % of the total amount), followed by campesterol (24 %) and stigmasterol (7 %) [36, 37]. Estimations of average daily intakes of phytosterols from natural sources range between 150 and 440 mg [41, 42] depending on the type of diet. Intakes of phytosterols by vegetarians are much higher and can be twice as much in exceptional cases [32, 42]. Thereby, the dietary intake of phytostanols appears to be maximum 10 % of total daily intake [32, 42].

Phytosterols in plants not only occur in their free form but also as conjugates in which the hydroxyl group at carbon C3 is either esterified with fatty acids or hydroxycinnamic acids (section 2.2) or derivatized to glycosides and acylated glycosides (Fig. 3) which occur together with free sterols/stanols as structural components in various membranes [31, 32, 43-46].

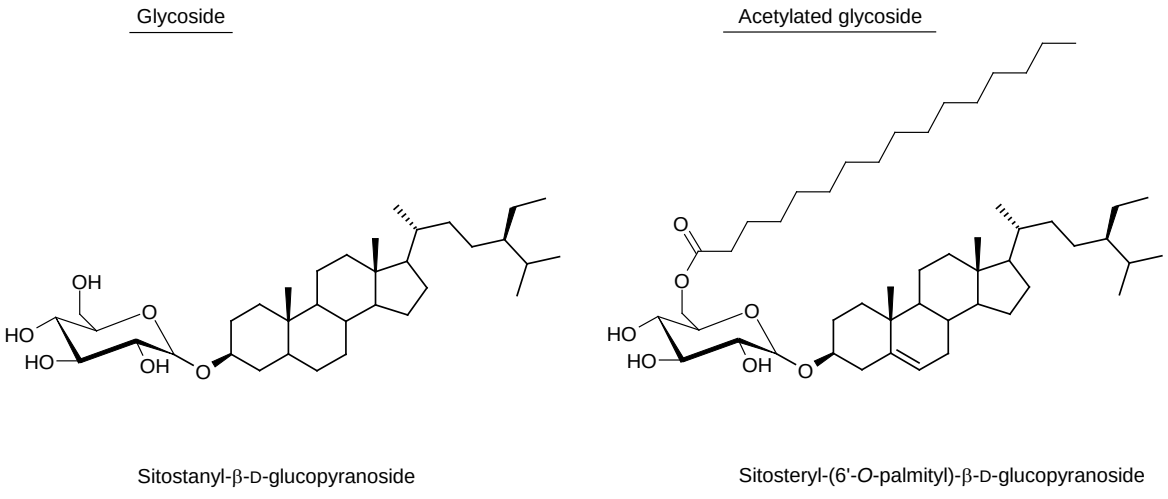

Fig. 3 Examples for naturally occurring phytosteryl/-stanyl glycosides and acetylated glycosides

2.1.2 BIOSYNTHESIS OF PHYTOSTEROLS/-STANOLS

Phytosterols are synthesized in a sequence of more than 30 enzyme-catalyzed reactions during the isoprenoid biosynthetic pathway in plant cytoplasm. Basic building block is isopentenyl pyrophosphate (IPP) which is made up of acetyl-CoA in the mevalonate pathway [32]. A simplified biosynthetic pathway in higher plants is illustrated in Fig. 4. At first, conversion of three molecules of IPP

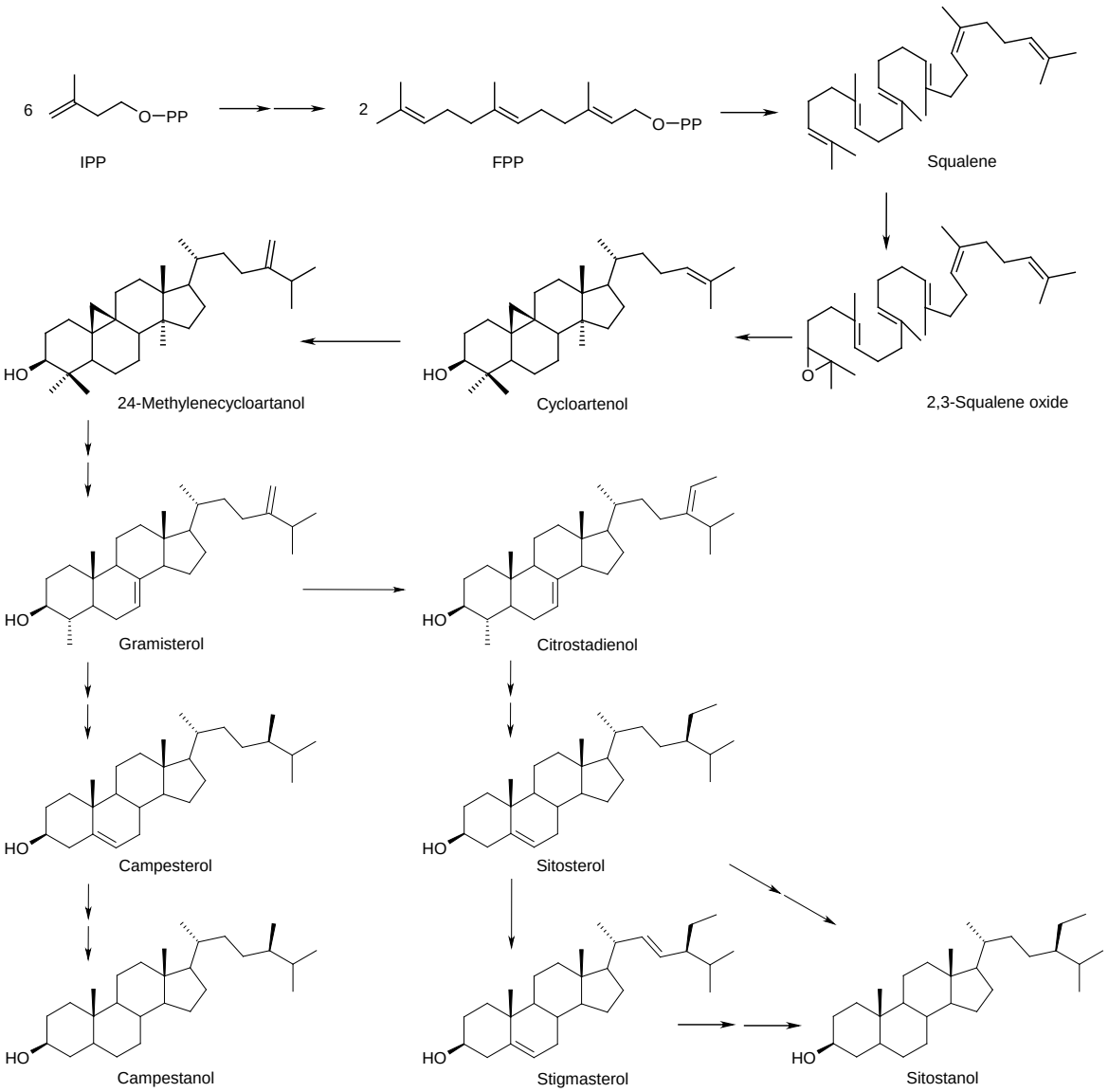

Fig. 4 Simplified biosynthetic pathway of phytosterols/-stanols in photosynthetic plants [32, 47, 48]; IPP = isopentenyl pyrophosphate; FPP = farnesyl pyrophosphate

leads to the squalene precursor farnesyl pyrophosphate (FPP). Then, squalene is synthesized via condensation of two molecules FPP. By oxidation to 2,3-squalene oxide and add-on cyclization [47, 49] cycloartenol, a 4,4-dimethylsterol, is

synthesized first. Therefore, cyclartenol is the precursor of phytosterols in photosynthetic plants. Starting from cycloartenol, various interacting pathways including side chain modifications, demethylation at carbon C4 or C14, opening of the C9/C10-cyclopropane ring, and implementation of double bonds in the phytosterol ring structure are involved in the synthesis of the 24-methyl- and 24-ethylphytosterol end-products [32, 47].

2.1.3 METABOLISM

Despite their structural similarity, the general metabolism of cholesterol (CS) and phytosterols/-stanols (PS) differs in several aspects. In contrast to cholesterol, PS cannot be synthesized by humans endogenously [50] and the intestinal absorption of phytosterols/-stanols is much lower. Whereas absorption of cholesterol in humans amounts to 20 – 80 % [51-53], only 0.04 % (sitostanol) up to 16 % (campesterol) of PS are absorbed [50, 54-57]. Reduced intestinal absoprtion of PS is attributed to an increased hydrophobicity depending on both an additional methyl/ethyl group at the C24 position of the side chain and the degree of saturation of the ring structure [54]. The precise molecular mechanisms are not well defined [58, 59]. Fig. 5 summarizes the commonly described major metabolic pathways.

Intestinal absorption requires solubilization of free CS and free PS into mixed micelles, interaction of the sterol-laden micelles with the apical brush border membrane thereby facilitating uptake by enterocytes, and finally incorporation of re-esterified phytosterols/-stanols and cholesterol into chylomicrones followed by basolateral secretion into bloodstream via lymphatic system and transport to liver. Thus, esterified CS and PS need to be hydrolyzed by digestive enzymes like pancreatic cholesterol esterase. Once incorporated into micelles, PS and CS are mainly absorbed by the influx transporter protein Niemann-Pick C1-like 1 (NPC1L1) which is highly expressed in small intestine, mostly in the apical brush border membrane of enterocytes [60-63]. NPC1L1 is also localized in the canalicular membrane of hepatocytes. Furthermore, data has been published suggesting that besides NPC1L1 other proteins, e.g. the scavenger receptors class B type I (SR-BI) and cluster determinant 36 (CD36) are involved in mediating

at least cholesterol uptake [64, 65]. After absorption, phytosterols and cholesterol are esterified in the endoplasmic reticulum by acyl-CoA:cholesterol acyltransferase 2 (ACAT2); PS are poor substrates compared to CS and only a very small part is esterified [66-68].

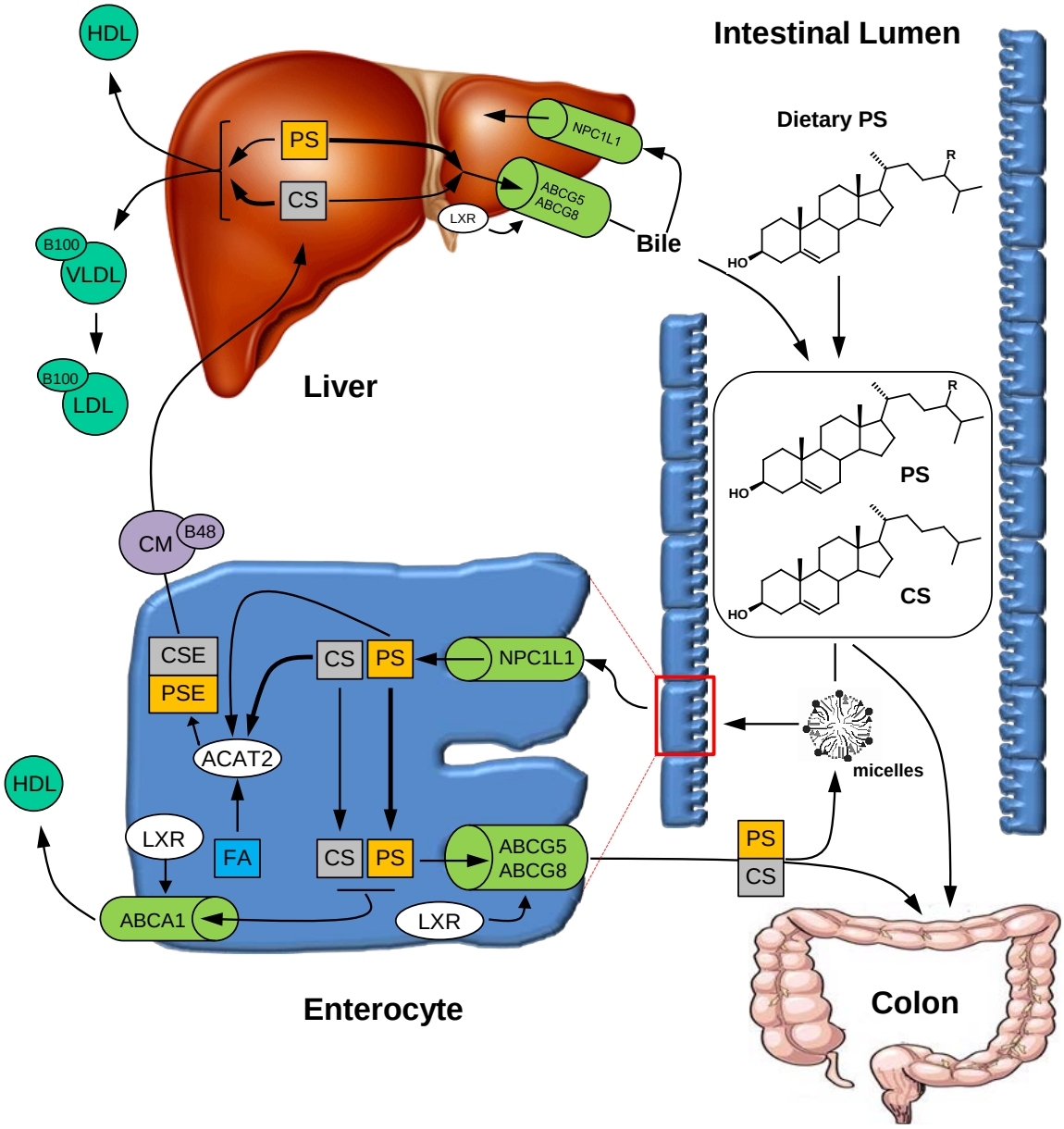

Fig. 5 **Major metabolic routes of cholesterol and phytosterols/-stanols in the human body (adapted & modified from [69]); PS = phytosterols/-stanols; CS = cholesterol; PSE = phytosteryl/-stanyl ester; CSE = cholesteryl ester; NPC1L1 = Niemann-Pick C1-like 1 protein; ABCG5/G8 = ATP-binding cassette transporter (ABC) heterodimer G5/G8; LXR = liver X receptor; ABCA1 = ATP binding cassette transporter A1; FA = fatty acids; ACAT2 = acyl-CoA:cholesterol acyl-transferase 2; CM = chylomicrons; B48/100 = apolipoprotein B48/100; VLDL = very low density lipoprotein; LDL = low density lipoprotein; HDL = high density lipoprotein**

Phyterosteryl/-stanyl and cholesteryl esters are then assembled along triglacylglycerols into apolipoprotein B48 containing chylomicrons (CM) in a microsomal triglyceride transfer protein (MTP)-dependent manner for secretion into the circulation via the lymphatic system and portal vein. Most of unesterified phytosterols and cholesterol are transported back into the intestinal lumen by the efflux transporter protein called ATP-binding cassette transporter (ABC) heterodimer G5/G8 (ABCG5/8) [70-73] which is regulated by liver X receptor (LXR). Data also indicate that phytosterols and cholesterol may be transported to the basolateral membrane of the enterocyte for the ABCA1-mediated biogenesis of high density lipoprotein (HDL) [74, 75]. During circulation chylomicrons are hydrolyzed by lipoprotein lipase and the PS and CS are delivered to the liver in chylomicron remnants.

After uptake into the liver a large amount of cholesterol is converted into bile acids for hepatobiliary secretion. To date, there is no evidence for phytosterol-derived bile acids [76]. Hepatic pathway shared by PS and CS is the esterification by ACAT2 followed by the incorporation into very low density lipoprotein (VLDL) particles in an MTP-dependent manner and finally secretion into the circulation. Unesterified phytosterols and cholesterol are either transported to ABCA1 for the biogenesis of HDL or in a majority to the canalicular membrane of hepatocytes for direct secretion into bile via ABCG5/8 [59]. The efficiency of hepatic clearance via these efflux transporters has been reported by SUDHOP *et al.* [57]. They could show that the relative secretion rates of phytosterols, namely sitosterol and campesterol, is significantly higher compared to cholesterol. In addition, sitosterol was secreted twice as much than campesterol. The low secretion rate of cholesterol could be caused by the hepatic NPC1L1 assuming that the function of NPC1L1 may be the prevention of an excessive loss of endogenous cholesterol by transporting secreted biliary cholesterol back into hepatocytes [59]. They also concluded that the interplay of intestinal absorption and hepatic clearance of phytosterols is responsible for the higher plasma concentrations of campesterol compared to sitosterol, although the dietary intake of campesterol is on average one third compared to sitosterol (section 2.1.1).

Typical plasma campesterol and sitosterol concentrations reported in adults over the last decade range from 0.3 – 0.5 mg/dL and 0.2 – 0.3 mg/dL, respectively. Plasma concentrations of the saturated counterparts are 10 – 30 times lower [56, 57, 77-80]. Resutling plasma total cholesterol levels in normocholesterolemic humans are 500- to 1000-fold higher than plasma phytosterol concentrations [81]. Drastically increased plasma phytosterol concentations are characteristic for phytosterolemia (sitosterolemia), a rare autosomal recessive inherited disease which is induced by increased intestinal absorption and decreased intestinal and biliary excretion. Mutations and polymorphisms in the ABCG5 or ABCG8 gene in the enterocytes and hepatocytes are responsible for the hyperabsorption and hyposecretion of PS [70, 82-84].

Phytosterols and cholesterol which are secreted back into the lumen by the ABC transporters can either become part of the instestinal and enterohepatic circulation or enter the colon together with non-absorbed PS and CS. In the colon phytosterols can be subject to microbial transformations analogous to cholesterol (section 2.1.5). Furthermore, NISSINEN *et al.* [23] described that free phytosterols/-stanols administered as triacylglycerol or diacylglycerol infusates experienced partial esterification and sedimentation during their intestinal passage after infusion to the duodenum of healthy human subjects.

2.1.4 CHOLESTEROL-LOWERING PROPERTIES

Since the 1950s, when POLLAK [85] demonstrated a lowering in serum cholesterol upon ingestion of sitosterol due to the prevention of intestinal cholesterol absorption in humans, phytosterols have been used for treatment of hypercholesterolemia. Cholesterol-lowering action of PS is closely associated to the intestinal and hepatic metabolic events shared by cholesterol (Fig. 5) [33, 69, 86]. In general, the cholesterol-lowering effect of phytosterols and –stanols is mainly based on their ability to reduce intestinal cholesterol absorption [87] and seems to be limited to 4-desmethylsterols/-stanols [88-91]. Thereby, total luminal cholesterol (1000 – 2000 mg/d) is composed of dietary (300 – 400 mg/d) as well as biliary cholesterol (800 – 1400 mg/d) [92]. The exact mechanisms by which unesterified PS inhibit cholesterol absorption are not completely understood

[69, 93]. The proposed mechanisms can be differentiated according to three aspects: (I) competition with cholesterol for solubilization into mixed-micelles within the intestinal lumen, (II) co-crystallization with cholesterol to form insoluble non-absorbable crystals, and (III) regulation of intestinal cholesterol transporters [86, 87]. Consequently, this results in an increase in fecal excretion of cholesterol and its metabolites.

The inhibition of intestinal cholesterol absorption by competitive solubilization into dietary mixed micelles appears to be the main mechanism [93-95]. The inhibition is based on the competition with dietary cholesterol for micellarization as well as the displacement of micellarized biliary cholesterol due to the higher hydrophobicity of phytosterols compared to cholesterol, respectively. Data on co-crystallization of phytosterols and cholesterol in the intestinal lumen, the initially pronounced mechanism [85], are confliciting. Whereas the *in vitro* study of CHRISTIANSEN *et al.* [96] suggested that co-crystallization may occur, MEL'NIKOV *et al. [97]* concluded in a more realistic *in vitro* setup that both CS and PS are highly soluble in hydrolysis products of dietary lipids, making co-crystallization unlikely. To date, effects on direct regulation of intestinal genes and accompanying regulation of sterol transporters by phyotsterols remain unclear. Recently, JESCH *et al*. [98] reported that sitosterol reduced mRNA levels of NPC1L1 in FHs 74 Int cells, a human small intestine epithelial cell line, to the same extent as cholesterol suggesting that sitosterol exhibits comparable regulatory properties as cholesterol. In Caco-2 cells, a heterogeneous human epithelial colorectal adenocarcinoma cell line, it had been shown that phytosterols may activate LXR leading in an increased expression of the ABC transporters [99-101]. In contrast, animal studies indicate that inhibition of cholesterol absorption by phytosterols/-stanols is unrelated to transcriptional changes in ABCA1, ABCG5, ABCG8 or NPC1L1 [102-105]. So far, the described mechanisms require phytosterols/-stanols in their free form. Therefore, the interaction of dietary phytosteryl/-stanyl esters with digestive enzymes, e.g. pancreatic cholesterol esterase, in terms of ester hydrolysis is important (section 2.2.2).

A consequence of the reduced intestinal cholesterol absorption is a state of relative cholesterol deficiency within the circulation, followed by a compensatory upregulation in endogenous cholesterol biosynthesis and LDL receptor activity

(Fig. 6). Despite this increased cholesterol *de novo* synthesis, the net effect of PS dietary supplementation is a reduction in plasma LDL concentrations based on higher LDL receptor acitvity, decreased apolipoprotein B levels and by association decreased LDL formation along the apolipoprotein B cascade [33, 69, 106, 107].

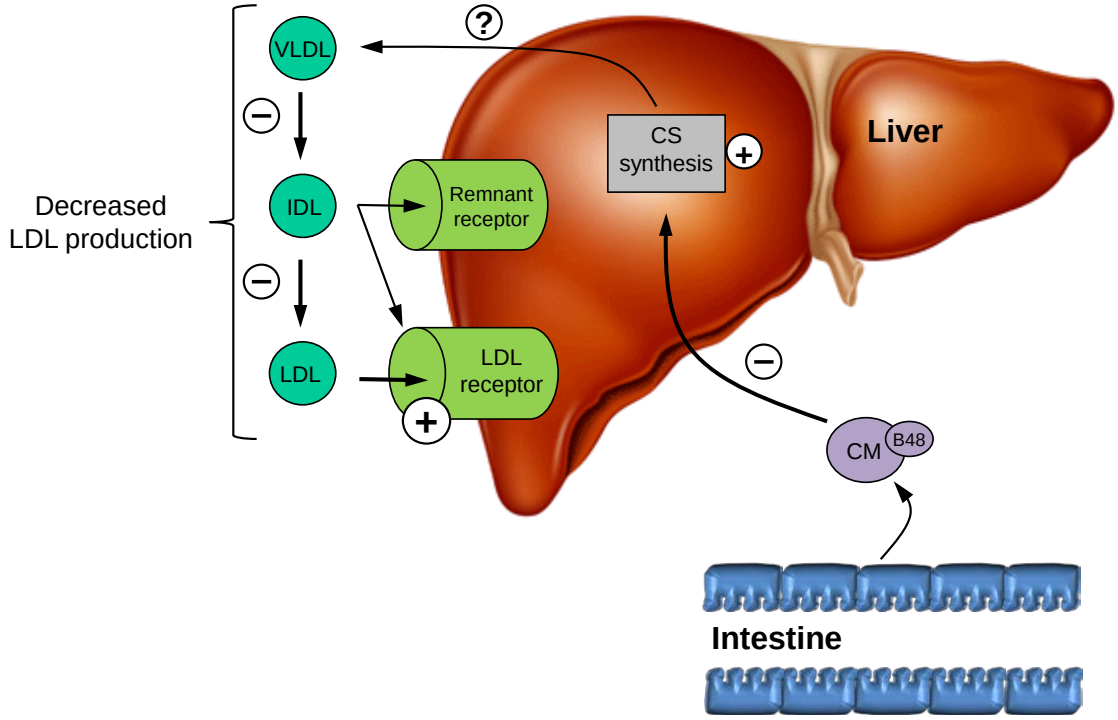

Fig. 6 Cholesterol hepatic metabolism (modified from [107]) in response to dietary phytosterol/-stanol supplementation; CM = chylomicrons; B48 = apolipoprotein B48; CS = cholesterol; VLDL = very low density lipoprotein; IDL = intermediate density lipoprotein; LDL = low density lipoprotein

A variety of studies have been performed on the effects of dietary free and esterified PS enriched in different food products on lowering plasma LDL-C and plasma total cholesterol. Two meta-analyses [4, 5] consistently confirmed a reduction of 9 – 10 % in LDL-C and TC as a result of phytosterol/-stanol intake at the recommended daily dose of about 2 – 3 g. Thereby, it has been described controversially whether the cholesterol-lowering efficiency is related to food carrier, frequency and time of intake [2, 4, 108-110].

2.1.5 SAFETY ASPECTS AND SIDE EFFECTS

Observations that increased consumption of PS under normal metabolic conditions results in elevated plasma phytosterol levels [111] and that phytosterolemia is associated with premature atherosclerosis [112, 113] provoke concerns about the safety of dietary supplementation of phytosterols/-stanols. To date, the relationship of plasma phytosterol/-stanol concentrations and enhanced risk of CHD is being discussed controversially. Epidemiological studies reported either a correlation between plasma phytosterol levels and cardiovascular risk [114-118] or no correlation [119-124]. In addition, HELSKE *et al.* [125] demonstrated that accumulation of phytosterols in stenotic aortic valves directly correlates with their plasma concentrations whereas the findings of MIETTINEN *et al.* [126] indicate that phytosterols accumulate in surgically removed carotid plaques but not disproportionately to cholesterol. Furthermore, WEINGÄRTNER *et al.* [127] demonstrated a correlation between consumption of phytosterol ester-enriched margarine with plasma and tissue phytosterol concentrations in aortic valves obtained at surgery in patients with aortic stenosis. Recently, it has been reported that elevated plasma campesterol concentrations due to long-term consumption of phytosteryl ester enriched functional foods correlates positively with an increase in retinal venular diameter in statin users independently from changes in plasma LDL-C levels [128]. It has been concluded by the authors that this correlation might serve as a biomarker for adverse effects of plant sterols on the (micro)vascular system and that further studies are required to complete the safety consideration of phytosterol/-stanol-enriched food products. So far, evidence for a direct causal relationship between plasma phytosterols and cardiovascular risk is lacking. Beyond that, it was investigated whether plasma concentrations of fat-soluble vitamins and carotenoids are affected by dietary phytosterol/-stanol supplementation. So far, it has been suggested that consumption of PS-enriched food products induces slight reduction, although not always significant, in total concentration of plasma carotenoids and fat-soluble vitamins [129-133]. Because of the fact that most of these compounds are associated with LDL particles, reduced levels of plasma TC or even LDL-C must be taken into consideration. Upon lipid-adjustment only the decrease in plasma β-carotene has been reported as being significant [5, 134, 135]. Thereby, increased intake of fruits and vegetables [136, 137] or fortified foods [138] maintains plasma carotenoid

concentrations, particularly β-carotene, while consuming PS-enriched food products. Furthermore, there are growing indications for other health benefits of dietary phytosterols including anti-inflammatory, antidiabetic, anticancer, and immune regulatory effects [139-148].

2.1.6 MICROBIAL TRANSFORMATION PRODUCTS

2.1.6.1 General aspects

Microbial biotransformtion of steroid raw material and natural sterols isolated from plants and animals is one of three large-scale industrial processes that are used worldwide for production of steroid drugs and hormones or at least their precursors [149-152]. Microbial bioconversions of phytosterols and cholesterol are carried out under aerobic conditions by a multistep enzymatic reaction including Δ^1-dehydrogenation, hydroxylations, side-chain degradation, isomerizations, reductions, and oxidations [153-161]. Thereby, side chain degradation, hydroxlation, and Δ^1-dehydrogenation are most important. Extensive studies were performed with fungi including *Rhizopus* spp., *Aspergillus* spp., *Curvularia* spp, and *Fusarium* spp. as well as bacteria including *Mycobacterium* spp., *Arthrobacter* spp., *Bacillus* spp., *Pseudomonas* spp., *Corynebacterium* spp., *Brevibacterium* spp., *Nocardia* spp., *Rhodoccus* spp., *Moraxella* spp., and *Streptomyces* spp. [153, 155-159]. The produced steroids are widely used as anti-inflammatory, immunosuppressive, progestational, diuretic, contraceptive, anabolic, and antiandrogenic agents [149, 157].

2.1.6.2 *In vitro* biotranformation of 4-desmethylsterols

A major step in the bioconversion is the degradation of the C17-side chain of phytosterols and cholesterol. Microbial side chain degradation is a process similar to β-oxidation of fatty acids starting at the terminal C27 methyl group [153, 160, 162]. Desmethylsterols like cholesterol, campesterol, and sitosterol are microbially cleavaged mainly to 17-ketosteroids, namely androst-4-ene-3,17-dione (AD), androst-1,4-diene-3,17-dione (ADD), and 9α-hydroxyandrost-4-ene-3,17-dione

(HAD) (Fig. 7), products belonging to the C_{19}-steroid family. However, single-step production of testosterone (TS) from cholesterol and phytosterols using a single strain, either *Mycobacterium* spp. NRRL B-3805 [163, 164] or *Lactobacillus bulgaricus* [165], has also been reported.

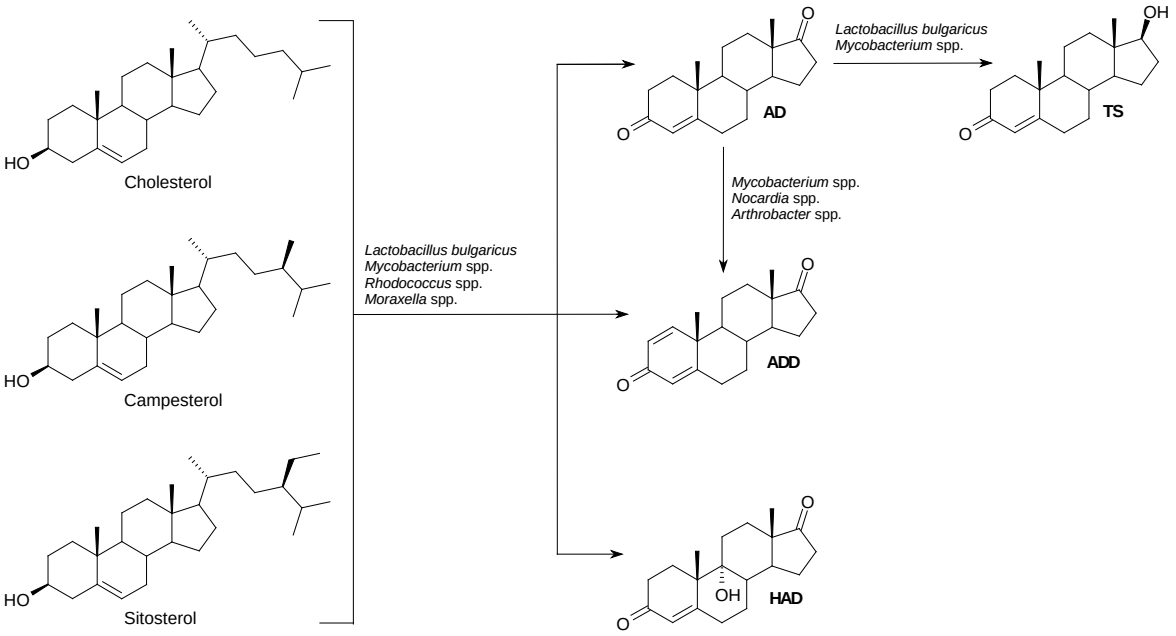

Fig. 7 Overview of microbial transformation end-products of 4-desmethylsterols with saturated side chain (modified from [149]); AD = androst-4-ene-3,17-dione; TS = testosterone; ADD = androst-1,4-diene-3,17-dione; HAD = 9α-hydroxyandrost-4-ene-3,17-dione

2.1.6.3 *In vitro* biotransformation of 4,4-dimethylsterols

Microbial biotransformations of 4,4-dimethylsterols have been described for cycloartenol and 24-methylenecycloartanol, major phytosterols present in the ferulic acid ester fraction of rice bran and rice bran oil, as well as lanosterol, the precursor in cholesterol biosynthesis [166, 167]. Analogously to desmethylsterols, steroids belonging to the C_{19}-steroid family were reported as end-products during the biotransformation of cycloartenol and 24-methylenecycloartanol using *Mycobacterium* spp. NRRL B-3805 (Fig. 8). Major metabolites were identified as 17-ketosteroids, namely androst-4,8(14)-diene-3,17-dione and androst-8(14)-ene-3,17-dione followed by 17β-hydroxyandrost-4,8(14)-diene-3-one and androst-4,8(14),15-triene-3,17-dione.

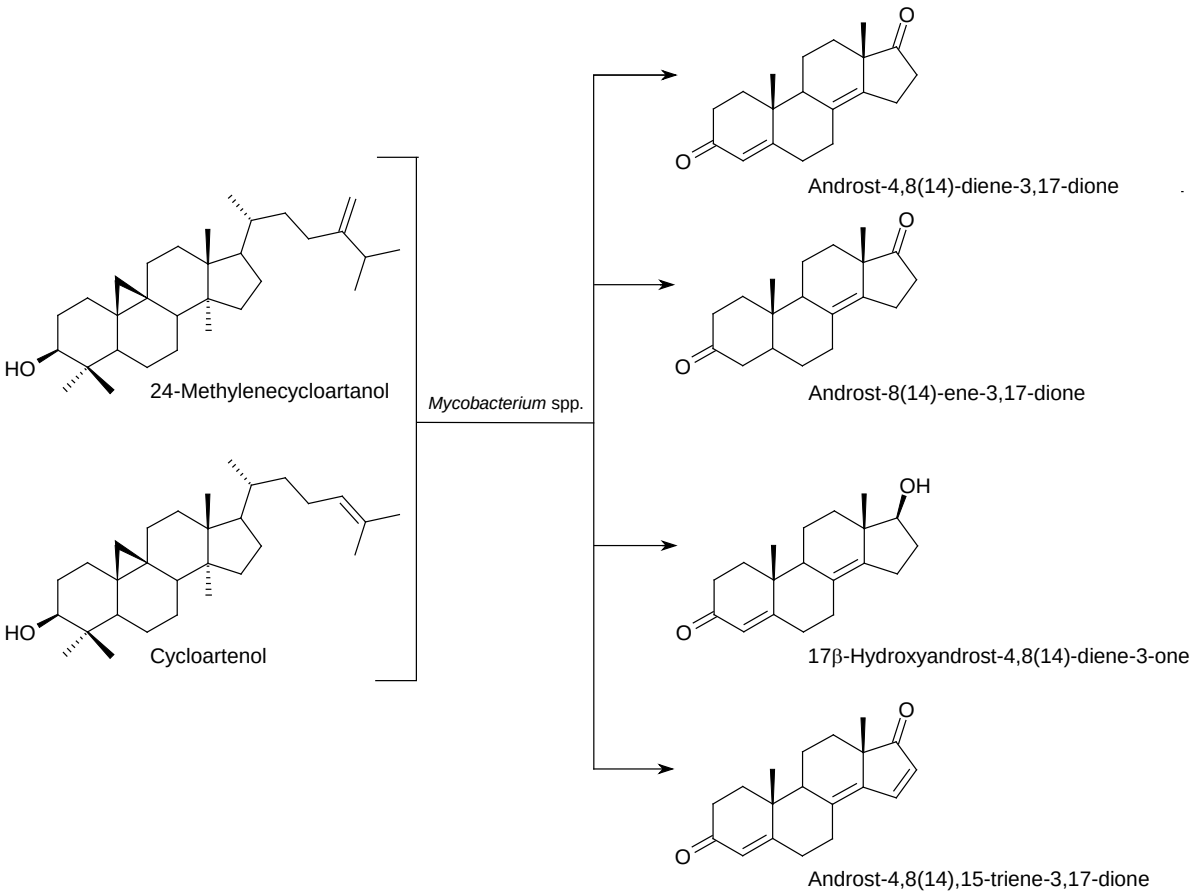

Fig. 8 Overview of microbial transformation end-products of 4,4-dimethylsterols (modified from [166, 167])

2.1.6.4 *In vivo* conversion by colonic bacteria

Microbial transformations of phytosterols during large intestine passage in humans have been reported for desmethylsterols [24-29] but not for stanols [81]. The conversion products of phytosterols identified in feces of humans correspond to those of cholesterol (Fig. 9). Metabolites belonging to the C_{19}-steroid family such as AD and ADD have only been described in rats [151]. The underlying mechanisms by which desmethylsterols, particularly cholesterol, are converted have been investigated in several studies starting in the 1930s [26, 168-177]. Generally, two distinguished metabolic pathways leading to coprostanol derivatives of cholesterol as well as campesterol and sitosterol have been described (Fig. 9): Direct stereospecific reduction of the Δ^5 double bond of the desmethylsterols leading to the respective coprostanols (1), and an indirect pathway involving the respective desmethylst-4-ene-3-ones and coprostanones as intermediates (2a-c). The corresponding coprostanols are formed due to oxidation

of the C3 hydroxyl group and isomerization of the Δ^5 double bond (2a) followed by stereospecific reduction of the Δ^4 double bond (2b), and finally reduction of the coprostanones (2c).

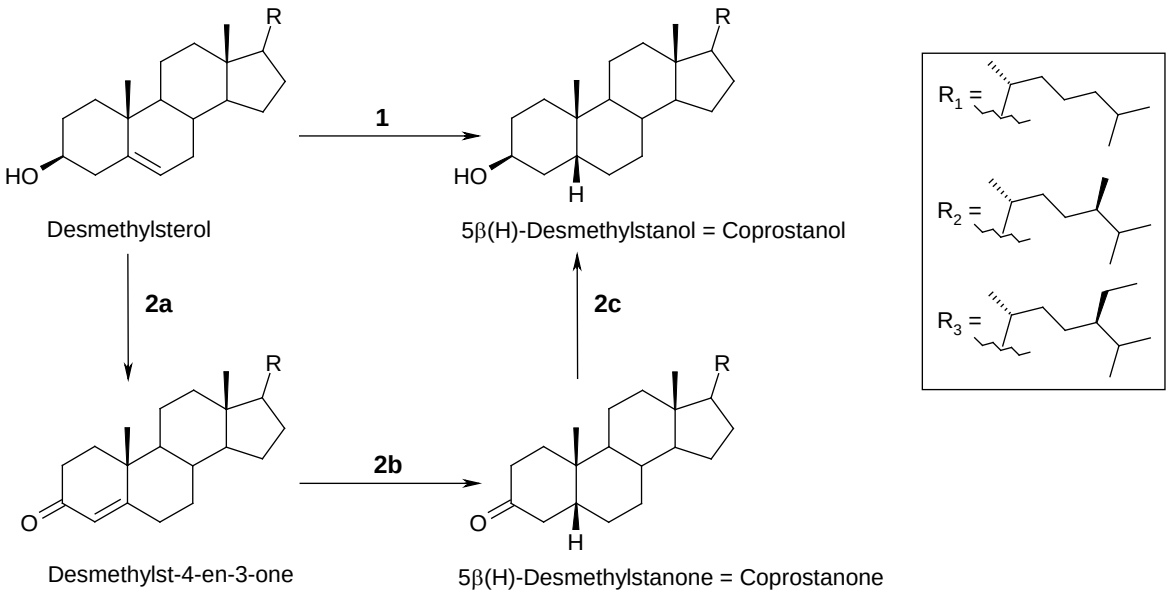

Fig. 9 Intestinal metabolic pathways of desmethylsterols (modified from [168]); 1 = direct stereospecific reduction; 2a-c = indirect conversion pathway via intermediary metabolites including oxidation and isomerization (a), stereospecific reduction (b), and reduction (c); R_1 = cholesterol; R_2 = campesterol (24-methylcholesterol); R_3 = sitosterol (24-ethylcholesterol)

2.2 PHYTOSTERYL/-STANYL ESTERS

2.2.1 STRUCTURE AND NATURAL OCCURRENCE

Derivatives of phytosterols/-stanols in which the hydroxyl group at C3 is either esterified to fatty acids (Fig. 10) or hydroxycinnamic acids, particularly ferulic acid (FA) (Fig. 11), have been described as naturally occurring conjugates. In contrast to glycosides or acylated gylcosides, phytosteryl/-stanyl esters appear to be largely excluded from membranes but rather being located intracellularly and responsible for storage and transport of phytosterols/-stanols [31, 32]. Phytosteryl/-stanyl fatty acid esters which are ubiquitous in plants but most abundant in vegetable oils typically exhibit the plant characteristic fatty acid profile. Fatty acid moieties typically range from 12 – 22 carbons with C16 and C18 being the dominant species (Fig. 10) [14, 178].

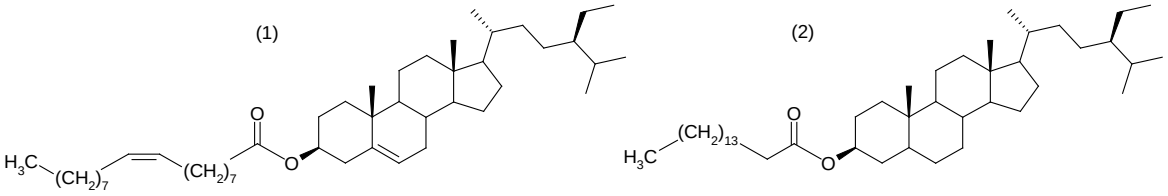

Fig. 10 Examples of naturally occurring phytosteryl/-stanyl fatty acid esters; (1) sitosteryl oleate; (2) sitostanyl palmitate

In contrast to the fatty acid esters, hydroxycinnamates of PS seem to be confined to cereals. Phytosterols/-stanols esterified with *trans*-ferulic acid and to a lesser extent *para*-coumaric acid are characteristic conjugates in grains of maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), and rice (*Oryza sativa* L.) [14, 31, 43, 44, 46]. Rice is of particular importance regarding the content as well as the composition of ferulic acid esters. In the 1950s phytosteryl/-stanyl ferulates were first isolated from rice bran oil and presumed to be a single component containing a hydroxyl group; it was named oryzanol [179]. Subsequently, oryzanol was determined to be a fraction comprising a variety of ferulic acid esters of different phytosterols/-stanols called α-, β- and γ-oryzanol [180]. Today, γ-oryzanol is the best characterized and is being used as synonym for the entire phytosteryl/-stanyl ferulates contained in rice. Major components of γ-oryzanol have been identified as *trans*-ferulic acid esters of cyclartenol and 24-methylenecycloartanol as well as campesterol, campestanol and sitosterol (Fig. 11) [43, 181-185]. In contrast to all other cereals, the dominating species in rice with a total percentage up to 76 % are 4,4-dimethylsteryl ferulates (cycloartenyl ferulate and 24-methylene-cycloartanyl ferulate) rather than desmethylsteryl ferulic acid esters [183]. Among these, lower concentrations of *trans*-ferulic acid esters of cycloartanol [182, 184], stigmasterol, Δ^7-stigmasterol, Δ^7-campesterol, Δ^7-sitosterol and sitostanol [182, 184-186], as well as *cis*-ferulic acid [182, 186] and caffeic acid esters [182] have been identified in γ-oryzanol.

The concentration of γ-oryzanol in rice and related products (rice bran and rice bran oil) is subject to large variation due to genetic and environmental conditions [187]. The contents of phytosteryl/-stanyl ferulates in European brown rice (26-63 mg/100 g) and Indian brown rice (50-72 mg/100 g) are significantly higher

compared to all other cereals like corn (4-23 mg/100 g), wheat (5-12 mg/100 g), rye (3-6 mg/100 g), triticale (5 mg/100 g) and barley (*Hordeum vulgare* L., 0.4 mg/100 g) [46, 183, 188, 189].

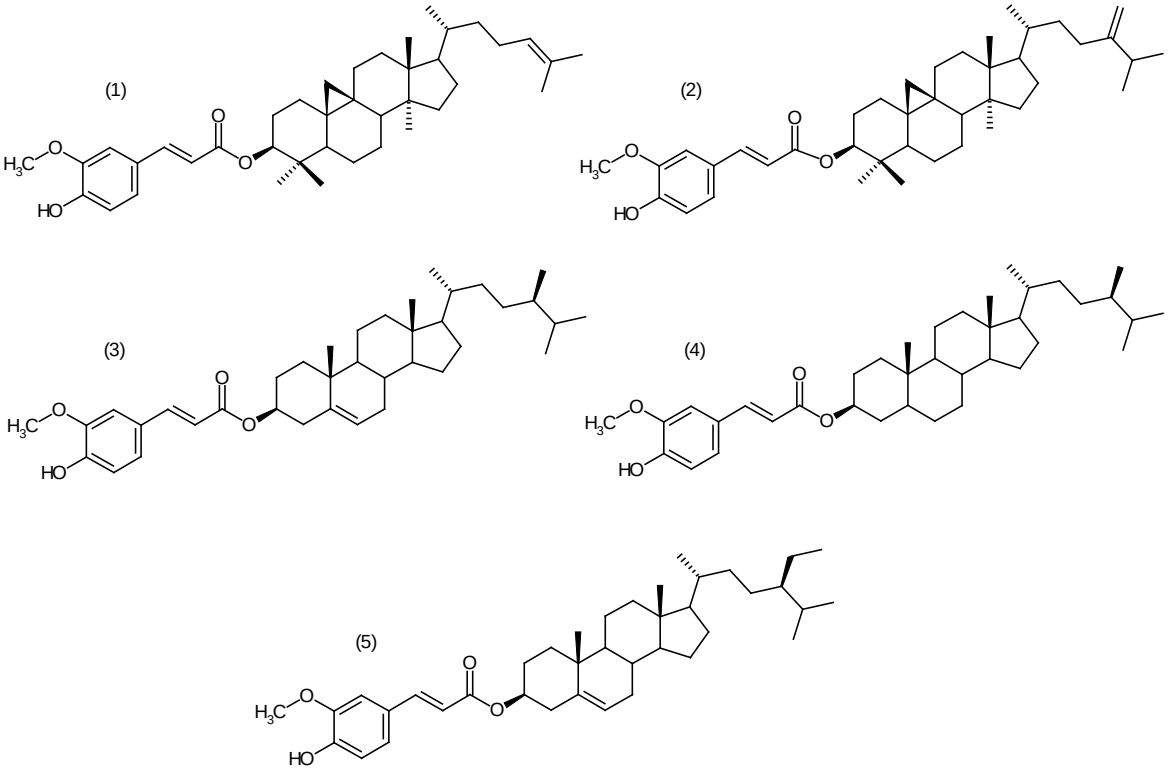

Fig. 11 Molecular structures of major ferulic acid esters naturally occurring in γ-oryzanol (*Oryza sativa* L.); (1) cycloartenyl ferulate; (2) 24-methylenecycloartanyl ferulate; (3) campesteryl ferulate; (4) campestanyl ferulate; (5) sitosteryl ferulate

Based on the fact that phytosteryl/-stanyl ferulates are primarily localized in high-fat non-endosperm tissues (e.g. aleurone layer) of cereal grains [46, 190, 191] γ-oryzanol concentrations in rice bran oil can amount up to 1.6 g/100 g [43, 192].

2.2.2 RELEVANCE IN HUMAN NUTRITION – PHYSIOLOGICAL PROPERTIES

2.2.2.1 Phytosteryl/-stanyl fatty acid esters

Since the late 1950s, after the cholesterol-lowering effects of phytosterols in humans had been demonstrated [85], commercial products such as Cytellin® (dihydro-β-sitosterol, though labelled as “sitosterol”, Eli-Lilly Co., Indianapolis, IN,

USA, marketed between 1957 and 1982) have been used for treatment of hypercholesterolemia [31, 87]. Due to the low efficacy of phytosterols administered in crystalline form, doses of 3 g phytosterols three times per day, with a maximum of even 30 g/d, were recommended for intake [87, 106]. Although MATTSON *et al.* already demonstrated at that time [193, 194] that the physical properties of phytosterols could be improved by esterfication with fatty acids without affecting their efficacy, the availabiltiy of statins led to a diminished interest in phytosterols [31, 195, 196].

Since the mid 1990s fat-soluble phytosteryl/-stanyl fatty acid esters produced at industrial-scale have been enriched in several functional foods such as spreads and dairy products for auxiliary therapeutic treatment of hypercholesterolemia. Currently, a variety of these food products are available on the EU market [197]. The cholesterol-lowering properties of phytosteryl/-stanyl fatty acid esters dosed in food products have been demonstrated in numerous studies and were excellently summarized in six meta-analyses [2-7]. Head-to-head comparisons of phytosteryl esters versus phytostanyl esters indicated roughly equivalent cholesterol-lowering efficacy both in short-term studies with hypercholesterolemic subjects [91, 131, 137, 198-200] and long-term studies with patients on statin treatment [201, 202]. The physiologically active forms are most likely the free, unesterified phytosterols and stanols due to competitive solubilization with cholesterol into dietary mixed micelles (section 2.1.4; Fig. 5). In addition, BROWN *et al.* [94] recently demonstrated *in vitro* that micellar cholesterol solubilization is not affected by intact phytosteryl fatty acid esters and that intact esters are not solubilized into micelles in a model bile, mixed-micelle system.

Considering the *in vivo* situation, it can be suggested that non-hydrolized dietary phytosteryl/-stanyl esters might be less or non effective in lowering plasma TC and LDL-C and reach the colon in intact form (Fig. 12). Therefore, the intestinal hydrolysis of these esters by digestive enzymes such as pancreatic cholesterol esterase seems to play a critical role for the cholesterol-lowering efficacy of phytosteryl/-stanyl fatty acid esters consumed with functional food products in humans. In general, it has been described both *in vitro* [14] and *in vivo* [20] that phytosteryl and phytostanyl fatty acid esters were hydrolyzed to the same extent.

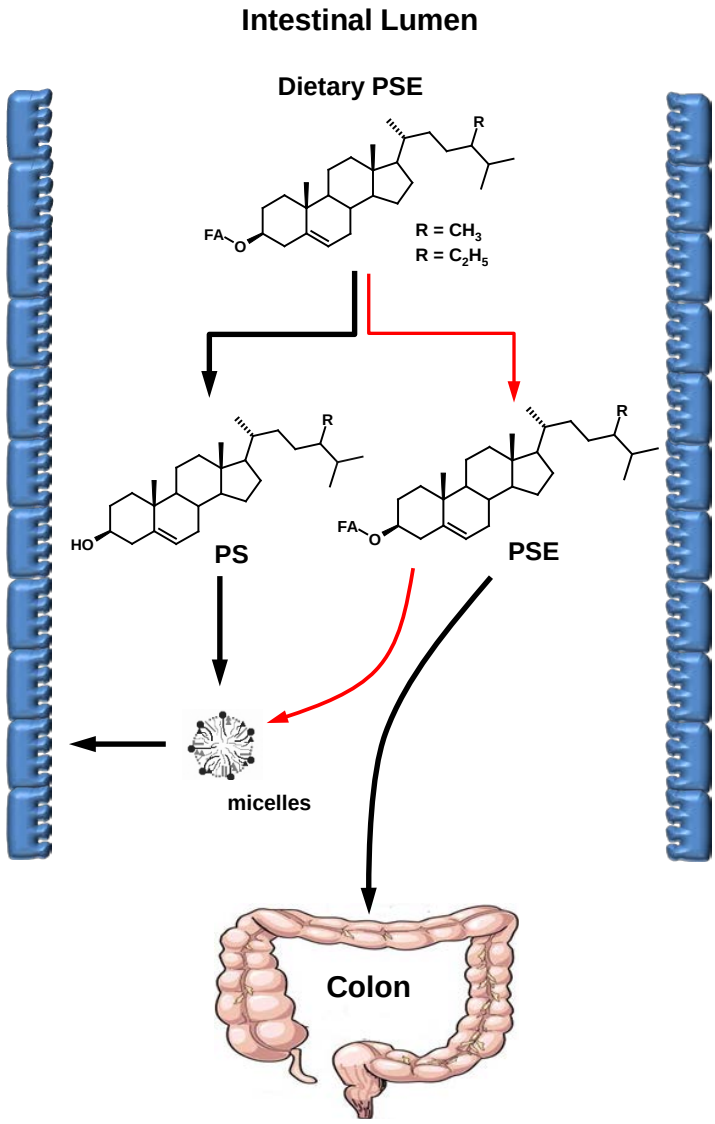

Fig. 12 Potential metabolic pathways of dietary phytosteryl/-stanyl fatty acid esters during intestinal transit; PSE = phytosteryl esters; FA = fatty acids; PS = phytosterols; Red arrows indicate both the lack of enzymatic ester hydrolysis and missing incorporation into mixed micelles

2.2.2.2 Phytosteryl/stanyl ferulic acid esters (γ-oryzanol)

γ-Oryzanol initially introduced for treatment of vegetative neurosis in Japan in the 1960s [9] exhibits both antioxidative and cholesterol-lowering properties. Reactive oxygen species (ROS) generated *in vivo* as by-products of cellular metabolic pathways (e.g. mitochondrial respiration) exhibit harmful effects to DNA, proteins, lipids and other biomolecules, especially when levels of ROS increase dramatically. The resulting oxidative stress has been suggested to be associated to the pathogenesis of several diseases such as cancer, diabetes mellitus, atherosclerosis and related CHD [203, 204]. Diet-derived antioxidants are

considered to be important in maintaining health [204]. The antioxidant potency of γ-oryzanol shown in several *in vitro* studies [205-209] was also demonstrated to be responsible for anti-inflammatory effects [210-212]. A significant reduction of serum lipid peroxide levels upon administration of a daily dose of 300 mg γ-oryzanol has been observed in menopausal women [10]. The antioxidant properties are mostly attributed to the ferulic acid moiety [213] due to the radical scavenging capabilities of the phenolic hydroxyl group followed by formation of a highly resonance-stabilized phenoxy radical (Fig. 13). This phenoxy radical is less reactive and unable to initiate or to propagate a radical chain reaction [180]. An additional impact of the phytosterol structure is indicated by different antioxidative efficiencies of the individual steryl ferulates contained in γ-oryzanol with 24-methylenecycloartanyl ferulate identified as the most active compound [206-209]. In addtion to the direct radical scavenging process, a recent study performed in Sprague-Dawley rats indicates that γ-oryzanol might be involved in the expression of antioxidant and oxidative stress related genes [214].

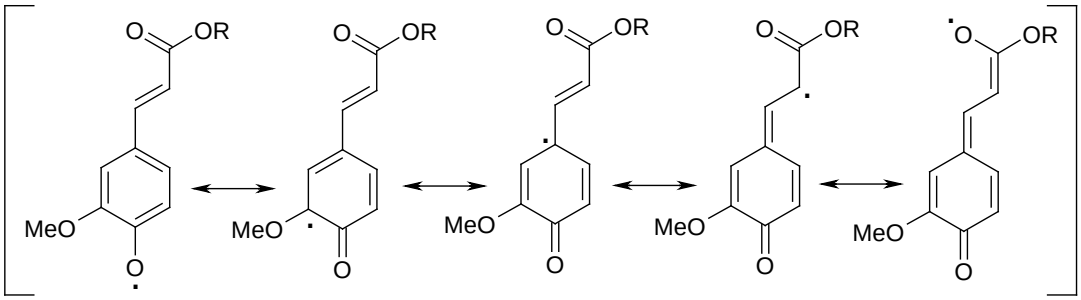

Fig. 13 Resonance-stabilized phenoxy radical of *trans*-ferulic acid; Me = methyl group; R = residue

Among these antioxidative properties, cholesterol-lowering effects were also attributed to γ-oryzanol. Siginificant reduction of plasma TC and LDL-C has been proven in several animal trials performed with rats and hamsters [215-220] as well as in human studies [8-11]. For instance, it has been reported that a daily dose of 300 mg γ-oryzanol for at least two months resulted in a reduction of serum cholesterol levels between 8 % and 12 % in hypercholesterolemic patients [9, 10]. To date, it is not fully clarified which compounds of γ-oryzanol actually are

physiologically active in terms of cholesterol-lowering, which mechanisms are involved and whether synergistic effects of different substances have to be taken into account. Analogously to phytosteryl/-stanyl fatty acid esters, the hydrolysis of steryl ferulates resulting in free phytosterols/-stanols as active form is generally assumed to be a crucial step [8]. Studies in mildly hypercholesterolemic patients [8] as well as normolipemic humans [90] indicate that the cholesterol-lowering effect is primarily referable to the desmethylsterols and not to 4,4-dimethylsterols. Furthermore, an additional impact of liberated ferulic acid on plasma cholesterol levels has been discussed [8, 220] whereas oral adminsitration of FA in rats did not result in increased fecal cholesterol excretion [220]. Thus, the cholesterol-lowering effect of ferulic acid is probably not attributed to inhibition of intestinal cholesterol absorption but rather to a previously unknown mechanism.

2.2.2 ESTER HYDROLYSIS BY DIGESTIVE ENZYMES *IN VITRO*

The intestinal hydrolysis of phytosteryl/-stanyl esters has been suggested to be crucial for the metabolic pathway and cholesterol-lowering efficiency. MOREAU & HICKS [14] were the first to report that phytosteryl/-stanyl fatty acid esters enriched in food matrices are generally accepted as substrates for hydrolysis by pancreatic cholesterol esterase (PCE; EC 3.1.1.13), the enzyme primarily responsible for cholesteryl ester hydrolysis in the GI-tract. However, there is only one study available that has been performed directly to investigate wether PCE hydrolyzes various phytosteryl/-stanyl fatty acid esters and whether hydrolysis by PCE is dependent on both the sterol and fatty acid moieties [12]. Comparing palmitates, stearates and oleates of cholesterol, sitosterol, stigmasterol and sitostanol BROWN *et al.* demonstrated that phytosteryl/-stanyl esters were hydrolyzed by PCE to a certain extent and that the rate of hydrolysis is indeed affected by both the sterol and fatty acid moieties. On average, oleic acid esters were hydrolyzed most efficiently, whereas palmitic acid and stearic acid esters were hydrolyzed statistically equally (45.8 ± 1.0 % and 41.6 ± 1.0 %, respectively, normalized to oleic acid esters). In addition, averaged hdroylsis rates of cholesteryl esters were significantly higher compared to the phytosteryl/-stanyl esters, while sitosteryl and sitostanyl esters were hydrolyzed statistically equally (58.9 ± 1.2 % and 56.4 ± 1.2 %, respectively, normalized to cholesteryl esters) but more efficiently

than stigmasteryl esters (29.3 ± 1.3 %). The following order of rates of hydrolysis has been concluded by BROWN *et al.*: cholesterol > sitosterol = sitostanol > stigmasterol (sterol moiety) as well as oleates > palmitates = stearates (acyl group effect).

Regarding phytosteryl/-stanyl ferulic acid esters it has been consistently demonstrated *in vitro* that desmethylsteryl ferulates (e.g. campesteryl ferulate, sitostanyl ferulate) were accepted as substrates by various mammalian digestive enzyme preparations employed, whereas 4,4-dimethylsteryl ferulates such as cycloartenyl ferulate were not [13-15]. First of all, sterol-specific hydrolysis of γ-oryzanol catalyzed by PCE (bovine and porcine), pancreatin (a mixture of pancreatic enzymes, containing e.g. cholesterol esterase, amylase, lipase, ribonuclease, and protease) employed as artificial pancreatic juice, and crude pancreatic acetone powders (bovine and porcine) has been shown by MILLER *et al.* [13]. Conversion rates of the ferulic acid esters have been calculated on the basis of the quantitation of the liberated phytosterols by a gas chromatography (GC)-based approach using flame ionization detection (FID). The employed pancreatin preparation was less effective than the bovine and procine PCE as well as pancreatic acetone powders. Furthermore, it has been described that hydrolysis by artificial pancreatic juice is clearly affected by the concentration of the bile salts contained. An increase of taurocholate concentration in the fluid from 0.9 to 6.0 mM resulted in increased hydrolyis rates of desmethylsteryl ferulates from approximately 2 to 15 %. However, lipases (EC 3.1.1.3) of different sources did not accept γ-oryzanol as substrate in the same study. MILLER *et al*. concluded that sterol-specific hydrolysis is potentially affected by different stuctures of the steryl moieties. While the ring structures of 4-desmethylsterols (e.g. campesterol) are identical to that of cholesterol, the 4,4-dimethylsterols (e.g. cycloartenol) differ in the presence of a methyl group at the C14 position, the dimethyl substitution at the C4 position, and the cyclopropane ring at the C9/C10 position (Fig. 14).

Subsequently, these findings have been confirmed by MOREAU & HICKS [14] who described that synthesized sitostanyl ferulate was hydrolyzed more efficiently in a time-dependent manner by PCE than γ-oryzanol. In the same study, pancreatin did not hydrolyze γ-oryzanol but sitostanyl ferulate. In contrast to MILLER *et al.* [13], the rate of hydrolysis was calculated on the basis of remaining

intact ferulates employing a normal-phase high performance liquid chromatography (NP-HPLC) methodology with evaporative light scattering detection. In a more recent study [15] diol-column HPLC with diode array detection was applied for quantitation of intact ferulic acid esters after incubation with bovine and porcine PCE.

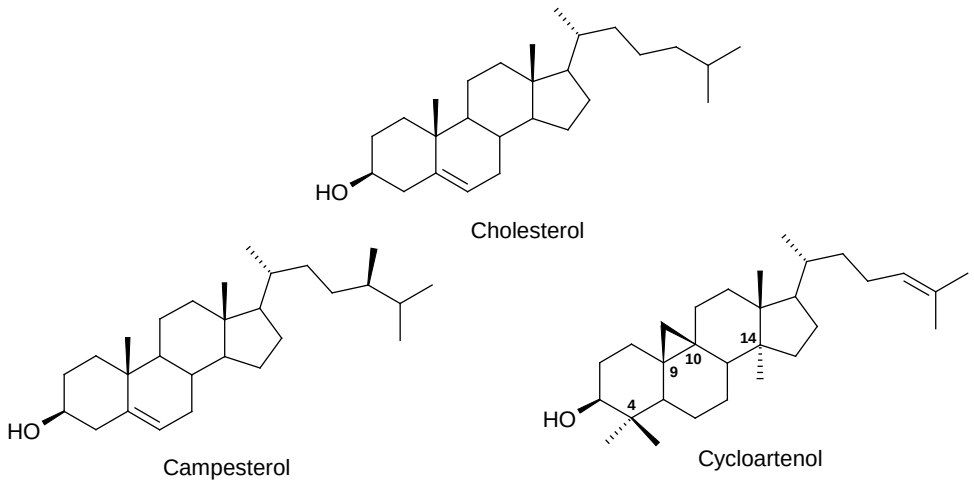

Fig. 14 Molecular structures of campesterol (4-desmethylsterol) and cycloartenol (4,4-dimethylsterol) contained in γ-oryzanol as ferulic acid esters in comparison to cholesterol

Using synthesized sitostanyl ferulate, γ-oryzanol and a mixture of desmethylstanyl/-steryl ferulates obtained from rye and wheat bran (composed of: 46 % campestanol, 32 % sitostanol, 13 % campesterol and 7 % sitosterol) as substrates, it was proven that the mixture of ferulates from rye and wheat was much more effectively hydrolyzed than γ–oryzanol. Furthermore, hydrolysis rates described for the desmethylstanyl/-steryl ferulate mixture were signifcantly higher compared to the purified sitostanyl ferulate indicating a preferred hydrolysis of desmethylsterols.

2.2.3 STUDIES ON ESTER HYDROLYSIS *IN VIVO*

So far, human trials were limited to phytosteryl/-stanyl fatty acid esters or only total fatty acid ester hydrolysis has been investigated regardless of a possible impact of varying molecular structures of respective esters contained in the fatty acid ester mixtures investigated [18-23]. Thus, data on the hydrolysis of individual phytosteryl/-stanyl esters are lacking. Except for one study [20] in which hydrolysis

rates were related to total esters, the total fatty acid ester hydrolysis has been calculated indirectly by determination of the intact ester fraction based on the quantification of free phytosterol/-stanol contents before and after saponification [18, 19, 21-23]. Human trials have either been designed as duodenal infusion [21-23] or oral administration studies were confined to ileostomy patients [18-20]. Due to the investigation of intestinal excreta, hydrolysis rates upon gastrointestinal passage ranging from 60 % [19] to 90 % [18, 20] have been reported for phytosteryl/-stanyl fatty acid esters when consumed as enriched margarine [18], as supplement to a controlled diet [20] or as fat-free pastilles with a normal fat diet [19]. Additionally, the head-to-head comparison of steryl and stanyl esters revealed comparable hydrolysis rates of 88 % and 86 %, respectively [20]. Besides an impact of the administration form indicated by the lowest hydrolysis rates observed for the fat-free pastilles, NISSINEN *et al.* [19] also demonstrated an impact of the dietary fat content. Phytostanyl fatty acid esters supplied in fat-free pastilles were hydrolyzed to a greater extent (70 %) when consumed with a normal fat diet than with a low fat diet (40 %). However, because of the lack of the colon and, therefore, an almost total absence of intestinal bacterial function, cholesterol metabolism in ileostomy patients differ to some extent from that of healthy subjects [18]. Intubation studies in humans with a normal intestinal tract described that 39 – 67 % of infused phytostanyl esters and 23 – 42 % of infused phytosteryl esters already were hydrolyzed by digestive enzymes during their 50 – 60 cm duodenojejunal transit [21-23].

A possible impact of the molecular structure on hydrolysis of phytosteryl/-stanyl fatty acid esters has been demonstrated in two animal studies [16, 17]. Sitosteryl stearate was hydrolized to a significantly higher extent (5 %) than sitostanyl stearate (1 %) when fed to male Syrian hamsters indicating that hydrolysis also in humans might be dependent on sterol/stanol moiety [16]. Feeding oleic acid and stearic acid esters mainly of phytosterols to male Sprague Dawley rats revealed a significant impact of the acyl residue; oleates were virtually absent in feces, whereas almost 80 % of the fed stearates were recovered as intact esters [17].

To date, the metabolic fate of γ-oryzanol in humans has not been investigated. In animal studies it has been demonstrated that orally adninistered γ-oryzanol was

partially hydrolyzed [221, 222]. Upon feeding labeled γ-oryzanol (^{14}C-labeling at the C3-position of ferulic acid) to rabbits and rats almost 84 – 86 % of the radioactive dose was recovered in feces and about 7 – 10 % in urine, while intact phytosteryl/stanyl ferulic acid esters were not excreted in urine. Urinary radioactivity was referred to liberated ferulic acid and its metabolites that had been identified as dihydroferulic acid, 3-(3-hydroxyphenyl)propionic acid, *meta*-coumaric acid, *meta*-hydroxyhippuric acid and hippuric acid. Besides their free form all urinary metabolites with the exception of hippuric acid were also excreted as glucuronates and sulfates indicating that conjugation reactions (phase II metabolism) are basically part of the metabolic pathway of ferulic acid [223]. Data regarding the process of absorption and hydrolysis remain unverified and conflicting. Whereas *in situ* intestinal absorption experiments in an isolated and washed mid-ileal segment of the small intestine of rabbits indicated that γ-oryzanol was not metabolized in the intestinal tract but absorbed in intact form [221], comparable experiments in rat small intestine revealed 10 – 20 % hydrolysis of γ-oryzanol in the intestinal tissue during absorption [222]. An impact of pancreatic digestive enzymes on hydrolsis was not taken into account due the lack of a connection between the isloated mid-ileal segment and pancreas during the experiments as wells as the washing step of the ileum prior to experiments.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 CHEMICALS

The following chemicals were used:

Mineral nutrients

$CaCl_2$ (anhydrous)	Sigma-Aldrich, Taufkirchen
$CoCl_2 \cdot 6\ H_2O$	Sigma-Aldrich, Taufkirchen
$CuSO_4 \cdot 5\ H_2O$	Sigma-Aldrich, Taufkirchen
$FeSO_4 \cdot 7\ H_20$	Merck, Darmstadt
CH_4N_2O	Sigma-Aldrich, Taufkirchen
KCl	Merck, Darmstadt
$MgCl_2$ (anhydrous)	Sigma-Aldrich, Taufkirchen
$MnSO_4 \cdot 7\ H_2O$	AppliChem GmbH, Darmstadt
$Mo_7(NH_4)_6O_{24} \cdot 4\ H_2O$	Merck, Darmstadt
NaCl	Merck, Darmstadt
$NaHCO_3$	Merck, Darmstadt
$Na_2HPO_4 \cdot 2\ H_2O$	Merck, Darmstadt
Na_2SO_4	Merck, Darmstadt
$ZnSO_4 \cdot 7\ H_2O$	J.T.Baker, Deventer

Reference substances

24-Ethylcoprostanol (98 %)	Chiron AS, Trondheim
3-(3-Hydroxyphenyl)propionic acid (98 %)	Alfa Aesar, Karlsruhe
3-(3-Methoxyphenyl)propionic acid (99 %)	Sigma-Aldrich, Taufkirchen
5α-Cholestane (97 %)	Sigma-Aldrich, Taufkirchen
Caffeic acid (≥ 98 %)	Sigma-Aldrich, Taufkirchen
Cholest-4-en-3-one (98 %)	Sigma-Aldrich, Taufkirchen
Cholesterol (95 %)	Sigma-Aldrich, Taufkirchen
Cholesteryl-β-D-glucuronide	Sigma-Aldrich, Taufkirchen
Cholesteryl cinnamate (Aldrich^{CPR})	Sigma-Aldrich, Taufkirchen
Cholesteryl dodecanoate (≥ 98 %)	Sigma-Aldrich, Taufkirchen
Cholesteryl palmitate (≥ 98 %)	Sigma-Aldrich, Taufkirchen
Coprostan-3-ol (98 %)	Sigma-Aldrich, Taufkirchen
Coprostan-3-one	Sigma-Aldrich, Taufkirchen
Ethyl ferulate (98 %)	Sigma-Aldrich, Taufkirchen

Hydrocaffeic acid (98%)	Sigma-Aldrich, Taufkirchen
Hydrocinnamic acid (99 %)	Sigma-Aldrich, Taufkirchen
Sodium cholesteryl sulfate	Sigma-Aldrich, Taufkircehn
Palmitic acid (99 %)	Carl Roth, Karlsruhe
Stearic acid (95 %)	Sigma-Aldrich, Taufkirchen
Syringic acid (≥ 95 %)	Sigma-Aldrich, Taufkirchen
trans-Ferulic acid (99 %)	Sigma-Aldrich, Taufkirchen
Vanillic acid (≥ 97 %)	Sigma-Aldrich, Taufkirchen

Miscellaneuos chemicals

Acetone (SupraSolv®)	Merck, Darmstadt
Dichloromethane (EMSURE®)	Merck, Darmstadt
Ethanol (96 %)	Merck, Darmstadt
Ethyl acetate (≥ 99 %)	Sigma-Aldrich, Taufkirchen
Hydrochloric acid (37 %)	Merck, Darmstadt
Methyl-*tert*-butylether (DiveronS)	Evonik Oxeno, Marl
N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS), 99:1	Sigma-Aldrich, Taufkirchen
n-Hexane (SupraSolv®)	Merck, Darmstadt
Potassium hydroxide (pellets)	Sigma-Aldrich, Taufkirchen
Propan-2-ol (EMSURE®)	Merck, Darmstadt
Pyridine (puriss. p.a.)	Sigma-Aldrich, Taufkirchen
Resazurin sodium salt	Sigma-Aldrich, Taufkirchen
Sodium taurocholate (≥ 97 %)	Sigma-Aldrich, Taufkirchen
Thioglycollate broth	Sigma-Aldrich, Taufkirchen
Tween® 80	Sigma-Aldrich, Taufkirchen

Methyl *tert*-butyl ether was distilled prior to use. If not indicated otherwise all reagents and solvents were of analytical grade and solutions were prepared with bidistilled water. Industrial gases Carbon dioxide, Helium (5.0), Hydrogen (5.0) and Nitrogen (5.0) were obtained from Westfalen AG, Münster.

3.1.2 INDUSTRIAL REFERENCE SUBSTANCES

3.1.2.1 Phytosterol/-stanol preparations

The following mixtures of phytosterols/stanols were gifts from Cognis GmbH (Illertissen, Germany): (I) plant sterols from soy bean oil (Generol® 122N; 46.9 % sitosterol, 28.5 % campesterol, 14.4 % stigmasterol, and 3.5 % brassicasterol); (II) plant stanols wood based (Reducol™ stanol powder; 91.2 % sitostanol and 8.2 % campestanol).

3.1.2.2 Phytostanyl fatty acid esters (STAEST-115)

A mixture containing fatty acid esters of sitostanol and campestanol (STAEST-115; Plant stanol ester) was provided by Raisio Life Sciences (Raisio, Finland). The composition of the mixture (Tab. 1) was determined by a gas chromatography (GC/FID)-based methodology taking into account individual response factors as described by BARNSTEINER *et al*. [224].

Tab. 1 Ester composition of STAEST-115 determined according to [224]

Phytostanyl esters		Content [g/100 g]
Campestanyl esters		
Campestanyl palmitate	(C16:0)	0.5
Campestanyl oleate	(C18:1)	5.7
Campestanyl linoleate	(C18:2)	2.1
Campestanyl linolenate	(C18:3)	1.1
Campestanyl esters (total)		9.4
Sitostanyl esters		
Sitostanyl palmitate	(C16:0)	4.1
Sitostanyl oleate	(C18:1)	54.2
Sitostanyl linoleate	(C18:2)	18.8
Sitostanyl linolenate	(C18:3)	7.5
Sitostanyl eicosanoate	(C20:0)	2.0
Sitostanyl esters (total)		86.6
Phytostanyl esters (total)	(PSA)	96.0

3.1.2.3 Phytosteryl fatty acid esters (Vegapure® 95E)

A mixture of phytosteryl fatty acid esters (Vegapure® 95E; Plant sterol ester, non-GM) was a gift from Cognis GmbH (Illertissen, Germany). The contents of the individual steryl esters in the mixture were determined according to [224] (Tab. 2). In addition to the palmitates, oleates, and linoleates of campesterol and sitosterol, the employed Vegapure® 95E further contained the respective sitostanyl esters (5.9 g/100 g), brassicasteryl linoleate (1.6 g/100 g) and unidentified phytosteryl esters (2.1 g/100 g).

Tab. 2 Ester composition of Vegapure® 95E determined according to [224]

Phytosteryl esters		Content [g/100 g]
Campesteryl esters		
Campesteryl palmitate	(C16:0)	1.2
Campesteryl oleate	(C18:1)	5.5
Campesteryl linoleate	(C18:2)	11.6
Campesteryl esters (total)		18.3
Sitosteryl esters		
Sitosteryl palmitate	(C16:0)	4.6
Sitosteryl oleate	(C18:1)	23.0
Sitosteryl linoleate	(C18:2)	43.5
Sitosteryl esters (total)		71.1
Phytosteryl esters (total)	(PSE)	89.4

3.1.2.4 Phytosteryl/-stanyl ferulic acid esters (γ-Oryzanol)

γ-Oryzanol (purity: 99.8 %), a mixture of phytosteryl/-stanyl ferulic acid esters obtained from rice bran was purchased from Henry Lamotte Oils GmbH (Bremen, Germany). The contents of the individual ferulic acid esters (Tab. 3) were determined on the basis of area-% of the intact esters.

Tab. 3 Ester composition of γ-oryzanol determined by GC/FID

Phytosteryl/-stanyl ferulates		Content [g/100 g]
Desmethylsteryl/-stanyl ferulates		
Campesteryl ferulate		8.3
Campestanyl ferulate		1.0
Sitosteryl ferulate		2.8
Desmethylsteryl/-stanyl ferulates (total)		12.1
Dimethylsteryl/-stanyl ferulates		
Cycloartanyl ferulate		1.3
Cycloartenyl ferulate		37.7
24-Methylenecycloartanyl ferulate		42.9
Dimethylsteryl/-stanyl ferulates (total)		81.9
Phytosteryl/-stanyl ferulates (total)	(PSF)	94.0

In order to compensate for the thermal degradation of intact phytosteryl/-stanyl ferulates the contents were adjusted by taking into account the quantitative distribution of free sterols/stanols determined by GC/FID after alkaline hydrolysis of the employed γ-oryzanol.

3.1.3 SUBSTRATES

As examples for commercially available functional food products, skimmed milk drinking yogurts enriched with complex mixtures of phytosteryl/-stanyl esters were employed as food carriers of the target substrates in the human study. The compositions of the respective phytosteryl/-stanyl ester fractions were determined by means of GC/FID by use of the “fast extraction method” developed by BARNSTEINER *et al.* [224].

3.1.3.1 Emmi Benecol®

Emmi Benecol® enriched with phytostanyl fatty acid esters (produced by Emmi, Switzerland; distributed in Germany by Emmi Deutschland GmbH, Essen)

was purchased in local supermarkets. The following information was included in the list of ingredients per daily dose (65 mL): plant stanyl ester, corresponding to 2 g plant stanols; the fat (without stanol) was declared as 1.4 g, thereof 0.1 g saturated, 0.9 g monounsaturated and 0.4 g polyunsaturated fatty acids. The composition of the contained phytostanyl fatty acid ester fraction was analyzed according to [224] (Tab. 4).

Tab. 4 Ester composition of Emmi Benecol® determined according to [224]

Phytostanyl esters		Content	
		[g/daily dose]	[mmol/daily dose]
Campestanyl esters			
Campestanyl palmitate	(C16:0)	0.046	0.071
Campestanyl oleate	(C18:1)	0.514	0.770
Campestanyl linoleate	(C18:2)	0.176	0.264
Campestanyl linolenate	(C18:3)	0.072	0.108
Campestanyl esters (total)		0.808	1.213
Sitostanyl esters			
Sitostanyl palmitate	(C16:0)	0.124	0.189
Sitostanyl oleate	(C18:1)	1.573	2.309
Sitostanyl linoleate	(C18:2)	0.520	0.766
Sitostanyl linolenate	(C18:3)	0.182	0.269
Sitostanyl eicosanoate	(C20:0)	0.046	0.064
Sitostanyl esters (total)		2.445	3.597
Phytostanyl esters (total)	(PSA)	3.253	4.810

3.1.3.2 Becel pro.activ®

Becel pro.activ® enriched with phytosteryl fatty acid esters (produced by Unilever, Germany) was purchased in local supermarkets. The following information was included in the list of ingredients per daily dose (100 g): 3.4 % phytosteryl esters, corresponding to 2 g phytosterols; the fat (without 2 g sterols) was declared as 1.5 g, thereof 0.2 g saturated, 0.4 g monounsaturated and 0.9 g polyunsaturated fatty acids. The composition of the contained phytosteryl fatty acid ester fraction was analyzed according to [224, 225] (Tab. 5). The discrepancy

between the total content of phytosteryl esters labelled (3.4 g) and the respective content presented in Tab. 5 (2.75 g) is due to the presence of phytostanyl esters (0.4 g/daily dose), trace amounts of brassicasteryl fatty acid esters (0.1 g/daily dose) and unidentified esters (0.05 g/daily dose).

Tab. 5 Ester composition of Becel pro.activ® determined according to [224, 225]

Phytosteryl esters		Content	
		[g/daily dose]	[mmol/daily dose]
Campesteryl esters			
Campesteryl palmitate	(C16:0)	0.030	0.047
Campesteryl oleate	(C18:1)	0.170	0.256
Campesteryl linoleate	(C18:2)	0.290	0.437
Campesteryl esters (total)		0.490	0.740
Sitosteryl esters			
Sitosteryl palmitate	(C16:0)	0.140	0.214
Sitosteryl oleate	(C18:1)	0.840	1.237
Sitosteryl linoleate	(C18:2)	1.280	1.890
Sitosteryl esters (total)		2.260	3.341
Phytosteryl esters (total)	(PSE)	2.750	4.081

3.1.3.3 γ-Oryzanol-enriched skimmed milk drinking yogurt

A skimmed milk drinking yogurt enriched witch phytosteryl/-stanyl ferulic acid esters was prepared in-house using γ-oryzanol and a commercial non-enriched skimmed milk drinking yogurt (GOLDHAND Vertriebsgesellschaft mbH, Germany). γ-Oryzanol (58 g) and yogurt (0.1 % fat, 1042 g) were homogenized using an ULTRA TURRAX® directly before each treatment period. The obtained preparation contained per daily dose (65 g): 3.4 g γ-oryzanol and 0.07 g fat (without γ-oryzanol). The contents of individual phytosteryl/-stanyl ferulates in the preparation based on the composition of the employed γ-oryzanol (section 3.1.2.4) are shown in Tab. 6; they have been confirmed by GC/FID according to [224].

Tab. 6 Ester composition of γ-oryzanol-enriched skimmed milk drinking yogurt

Phytosteryl/-stanyl ferulates		Content	
		[g/daily dose]	[mmol/daily dose]
Desmethylsteryl/-stanyl ferulates			
Campesteryl ferulate		0.280	0.485
Campestanyl ferulate		0.033	0.058
Sitosteryl ferulate		0.096	0.164
Desmethylsteryl/-stanyl ferulates (total)		0.409	0.707
Dimethylsteryl/-stanyl ferulates			
Cycloartanyl ferulate		0.043	0.072
Cycloartenyl ferulate		1.283	2.129
24-Methylenecycloartanyl ferulate		1.460	2.367
Dimethylsteryl/-stanyl ferulates (total)		2.787	4.568
Phytosteryl/-stanyl ferulates (total)	(PSF)	3.196	5.275

3.2 METHODS

3.2.1 GAS CHROMATOGRAPHY (GC/FID)

Gas chromatographic analysis was performed on an Agilent Technologies instrument 6890N (Böblingen, Germany) equipped with a flame ionization detector (GC/FID).

GC conditions

Column:	Rtx®-200MS (Restek, Bad Homburg, Germany) 30 m × 0.25 mm i.d. fused silica capillary column coated with a 0.1 µm film of trifluoropropylmethylpolysiloxane
Injector type:	Split / Splitless; autosampler 7683 Series
Split flow:	11.2 mL/min
Split ratio:	1:7.5
Injection volume:	1 µL
Carrier gas:	Hydrogen
Flow modus:	1.5 mL, constant flow
Make-up gas / flow:	Nitrogen, 25 mL/min

Injector temperature:	280 °C
Detector temperature:	360 °C
Temperature programm	100 °C (2 min) to 310 °C (2 min) at 15 °C/min up to 340 °C (3 min) at 1.5 °C/min
Data acquisition:	ChemStation (Agilent Technologies, Germany)

3.2.2 GAS CHROMATOGRAPHY – MASS SPECTROMETRY (GC/MS)

Gas chromatography – mass spectrometry (GC/MS) was performed on a Finnigan Trace GC equipped with Finnigan Trace DSQ mass spectrometer (Thermo Fisher Scientific, Austin, TX).

GC conditions

Column:	Rtx®-200MS (Restek, Bad Homburg, Germany) 30 m × 0.25 mm i.d. fused silica capillary column coated with a 0.1 µm film of trifluoropropylmethylpolysiloxane
Injector type:	Split / Splitless; TriPlus autosampler
Split flow:	12 mL/min
Split ratio:	1:8
Injection volume:	1 µL
Carrier gas:	Helium
Flow modus:	1.0 mL, constant flow
Injector temperature:	280 °C
Temperature programm	100 °C (2 min) to 310 °C (2 min) at 15 °C/min up to 340 °C (3 min) at 1.5 °C/min

MS conditions

Interface temperature:	320 °C
Ionization method:	EI+, 70 eV
Source temperature:	250 °C
Scan range:	40 – 700 Da (Full Scan Mode), Single Ion Monitoring
Scan interval:	0.4 s (Full Scan Mode)
Data acquisition:	Xcalibur 1.4 SR1 (Thermo Fisher Scientific, Austin, TX)

3.2.3 *IN VITRO* TRANSFORMATION STUDIES

3.2.3.1 Isolation of 4,4-dimethylsteryl ferulic acid esters by recrystallization from γ-oryzanol

Isolation of cycloartenyl ferulate and 24-methylencycyloartanyl ferulate from γ-oryzanol was performed according to a method described by Yoshida et al. [226] by a six-fold recrystallization procedure of 300 g γ-oryzanol. Within the first two steps the dimethylsteryl ferulates were completely separated from the desmethylsteryl ferulates using a mixture of ethyl acetate / ethanol (3/1, v/v). The following four recrystallization steps using ethyl acetate were performed for the enrichment of cycloartenyl ferulate (CF, 90 %, GC-purity; yield: 5 % of γ-oryzanol employed).

3.2.3.2 Liquid-liquid extraction of dimethylsterols after alkaline hydrolsis

A dimethylsterol fraction consisting of cycloartenol (90 %) and 24-methylene-cycloartanol (10 %) was prepared by alkaline hydrolysis of the respective ferulic acid ester mixture obtained according to section 3.2.3.1 followed by liquid-liquid extraction. After saponification of the dimethylsteryl ferulates (5 g) using ethanolic potassium hydroxide (100 mL, 3 M KOH, 90 % EtOH) at 80 °C for 2 h and neutralization with hydrochloric acid (50 mL, 6 M HCl) cycloartenol and 24-methylenecycloartanol were extracted with dichloromethane (20 mL, 4-fold). After removal of the combined organic phases, the mixture of dimethylsterols (yield: 67 %) was obtained as white coloured crystalline powder.

3.2.3.3 Preparation of the inoculum

Basis for the *in vitro* fermentation studies was a “one-batch”-procedure described for experiments focussing on the fermentability of dietary fibre [227]. The employed inoculum was composed of fresh human feces mixed with a carbonate-phophate buffer complex supplemented with trace elements and urea hereinafter referred to as nutritive buffer (NB). Additionally, thioglycollate broth (THB), a more complex medium especially used for propagation of anaerobic

bacteria, was employed for preparation of the inoculum. Prior to use all solutions were autoclaved. To ensure anaerobic conditions media, were flushed with nitrogen (N_2) and carbon dioxide (CO_2) during cooling after sterilization and directly before usage.

Fresh feces samples were collected by the volunteers in insulated and oxygen (O_2)-free bottles previously autoclaved and flushed with N_2 and CO_2. The inoculum was produced in the insulated bottle by adding five parts of the nutritive buffer tempered at 37 °C to one part of weighed feces (v/m). After vigorous agitation, the suspended slurry was filtered through two layers of surgical gauze. The filtrate was further diluted to a ratio of feces /NB or THB (1:50) by adding either ten parts of the nutritive buffer or thioglycollate broth both tempered at 37 °C to one part of the filtrate. During preparation of the inoculum including the final filtration continous flushing with CO_2 maintained anaerobiosis and ensured a constant pH. To define and control anaerobic conditions a solution of the redox indicator resazurin (1 mL/L, 0.1 %) was added additionally. Before the fermentation experiments were performed, the inoculum was stored overnight in a warmed water bath (37 °C) and continously flushed with CO_2 to give the microbiota the opportunity for adaption to the conditions.

3.2.3.4 *In vitro* fermentation procedure

Fermentations were conducted in sterilized 50 mL polypropylene centrifuge tubes (VWR International, Darmstadt). The substrate (10 mg) was weighed into the tube and 10 mL of the respective inoculum was added after suspension of the substrate using autoclaved Tween®80 solution (1 mL, 1 %). Oxygen was removed by a constant flow of N_2 for 5 min. The centrifuge tubes were sealed with screw caps, placed horizontally in a shaking water bath (JULABO, Germany) and incubated at 37 °C by 100 rpm for a period of five days. Fermentation of each substrate in inoculated media was performed in duplicate. Furthermore, the following blanks were used:

Blank I: Inoculum + NB or THB + Tween®80
Blank II: NB or THB + substrate + Tween®80

3.2.3.5 Sample preparation

3.2.3.5.1 Liquid-liquid extraction

As illustrated in Fig. 15, extraction was performed at two different pH values depending on the type of metabolites: A) extraction of free and esterified sterols using methyl *tert*-butyl ether (MTBE) at the existing pH 6-7 and B) extraction of acidic compounds, e.g. ferulic acid (FA) and further metabolites using ethyl acetate (EtOAc), after acidification of the fermentation batch to pH ≤ 2. In analogy to BRAUNE et al. [228], the purification step with bidistilled water (H_2O) was not conducted for the extraction of acidic compounds because of their moderate water solubility.

Accelerated separation of the organic and the aqueous phases after each extraction or purification step was achieved by centrifugation at 4000 rpm for 5 min. In order to balance losses during extraction, reference substances were used to determine recoveries. Cholesteryl dodecanoate (CD) for extraction of neutral esterified and free sterols as well as syringic acid (SGA) for extraction at acidic pH were selected. The samples were stored at + 4 °C until further analysis.

3.2.3.5.2 Derivatization

Aliquots of the extracts obtained according to Fig. 15 were dried completely under a gentle stream of N_2 upon addition of the respective internal standards. Silylation of the residue to trimethylsilyl (TMS) ethers was performed by adding 100 µL BSTFA + TMCS (99:1) and 20 µL pyridine followed by incubation at 80 °C for 20 min. After removal of the derivatization reagents under a gentle stream of N_2, the silylated residue was redissolved in 1 mL MTBE and analyzed by GC/FID and GC/MS.

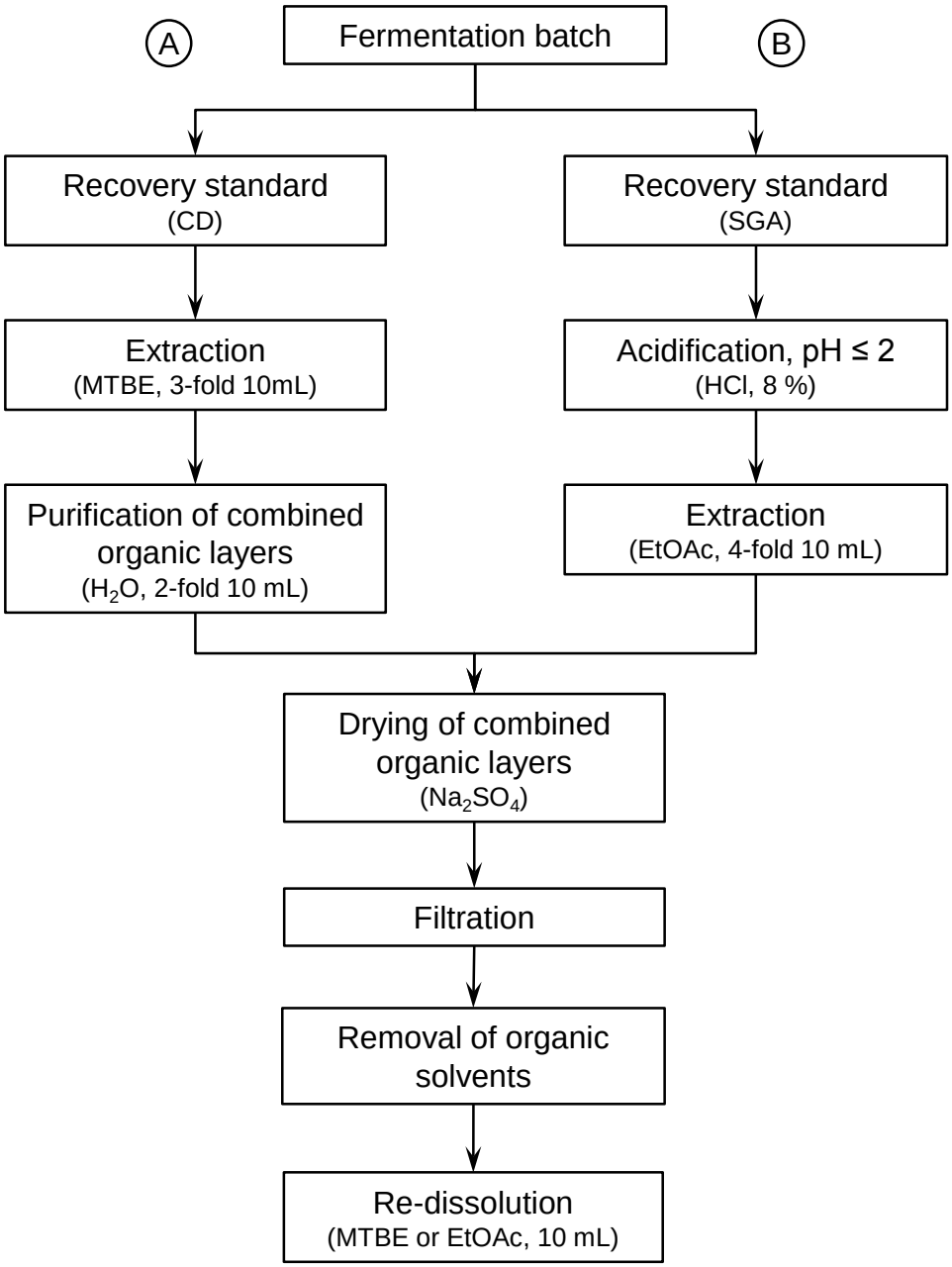

Fig. 15 Liquid-liquid extraction of batches within the *in vitro* fermentation experiments; CD = cholesteryl dodecanoate; SGA = syringic acid; MTBE = methyl *tert*-butyl ether; HCl = hydrochloric acid; H_2O = bidistilled water; EtOAc = ethyl acetate; Na_2SO_4 = sodium sulfate (anhydrous)

3.2.4 HUMAN STUDY

3.2.4.1 ANALYSIS OF FECES SAMPLES

3.2.4.1.1 Determination of analytical validation data (GC/FID)

Analytical validation data were determined on the basis of the linear regression methodology according to DIN 32645 (2008-11) [229]. Triplicate linear regression analysis for the individual phytosteryl/-stanyl esters and ester-derived phytosterols/-stanols were performed in coordinate ratios of peak areas (component / internal Standard (IS)) and amounts (component / IS). Validation parameters in terms of residual standard deviation, standard deviation of the method, coefficient of variation, limits of detection, and limits of quantitation were calculated using the equations (Eq.) listed below.

Residual standard deviation ($s_{y,x}$)

$$s_{y,x} = \sqrt{\frac{\sum_{i=1}^{n} (y_i - Y_i)^2}{n-2}} \qquad \text{(Eq. 1)}$$

Y_i = measured peak-area ratios; y_i = calculated peak-area ratios; n = number of measurements; $\sum (y_i - Y_i)^2$ = residual sum of squares.

Standard deviation of the method (s_x)

$$s_x = \frac{s_{y,x}}{m} \qquad \text{(Eq. 2)}$$

$s_{y,x}$ = residual standard deviation; m = slope of the regression line.

Coefficient of variation (CV)

$$CV_x[\%] = \frac{s_x}{x_x} \cdot 100 \qquad \text{(Eq. 3)}$$

s_x = standard deviation of the method; x_x = mean of amount ratios.

Limits of detection (LOD)

$$LOD = \frac{s_{y,x}}{m} \cdot t_{f,a} \cdot \sqrt{\frac{1}{N} + \frac{1}{\hat{N}} + \frac{\bar{x}^2}{Q_{xx}}} \qquad \text{(Eq. 4)}$$

$s_{y,x}$ = residual standard deviation; m = slope of the regression line; $t_{f,a}$ = t-value (f = N-2, a = 0.05); N = number of calibration standards; $\hat{N}$ = number of parallel determinations; Q_{xx} = deviance; $\bar{x}$ = mid-working range

Limits of quantitation (LOQ)

$$LOQ = k \cdot \frac{s_{y,x}}{m} \cdot t_{f,a} \cdot \sqrt{\frac{1}{N} + \frac{1}{\hat{N}} + \frac{(3 \cdot LOD - \bar{x})^2}{Q_{xx}}} \qquad \text{(Eq. 5)}$$

$s_{y,x}$ = residual standard deviation; m = slope of the regression line; $t_{f,a}$ = t-value (f = N-2, a = 0.05); N = number of calibration standards; $\hat{N}$ = number of parallel determinations; Q_{xx} = deviance; LOD = limit of detection; $\bar{x}$: mid-working range

3.2.4.1.2 Isolation procedure

The main steps of sample preparation are illustrated in Fig. 16. For each of the subjects the daily feces samples collected during the treatment / collecting period were pooled, weighed, and homogenized with water by magnetic stirring at 500 rpm for 2 hours. Three aliquots (25 g each) of the feces homogenate were lyophilized, ground and stored deep-frozen at -35 °C until analysis.

Each aliquot was analyzed in triplicate: 100 mg lyophilized feces were weighed into 15 mL centrifuge tubes containing 375 µg 5α-cholestane (5αC = IS_1) (375 µL from a solution of 5αC in *n*-hexane / MTBE (3/2, v/v); 1 µg/µL), and either 225 µg cholesteryl palmitate (CP = IS_2) (225 µL from a solution of CP in *n*-hexane / MTBE (3/2, v/v); 1 µg/µL) or 750 µg cholesteryl cinnamate (CC = IS_3) (250 µL from a solution of CC in *n*-hexane / MTBE (3/2, v/v); 3 µg/µL) as internal standards. Fecal lipids were extracted three times with 5 mL of *n*-hexane / MTBE (3/2, v/v). After membrane filtration (0.45 µm), 1 mL of the filtrate was evaporated to dryness by a gentle stream of N_2 at room temperature. The residue was derivatized by adding 300 µL BSTFA + TMCS (99:1) and 200 µL pyridine followed

by incubation at 80 °C for 20 min. After drying under N_2 the resulting TMS ethers were re-dissolved in 1 mL *n*-hexane / MTBE (3/2, v/v) and subjected to gas chromatography.

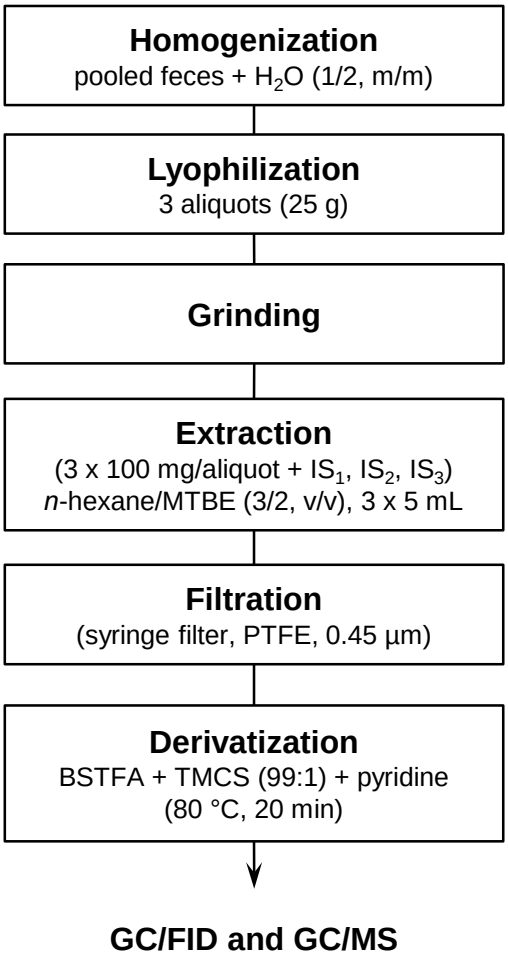

Fig. 16 Preparation of feces samples obtained within the human study

3.2.4.1.3 Identification of intact esters, sterols/stanols and metabolites by GC/MS

Identification of the compounds was carried out by comparing their mass fragmentation pattern with those of reference substances, an in-house database containing fatty acid esters synthesized at laboratory scale according to a patent application [230] and literature data [181, 224, 225, 231-234].

3.2.4.1.4 Quantitations of intact esters and sterols/stanols by GC/FID

The concentrations of individual intact esters and liberated phytosterols/-stanols were calculated from calibration functions using the respective internal standard (IS_1, IS_2, IS_3). Linear regression analysis was performed in coordinate ratios of peak areas (component / IS) and amounts (component / IS). Calibrations allowing the selective quantitation of individual phytosteryl/-stanyl esters were performed at concentrations ranging from 70 µg to 20 mg STAEST-115/100 mg feces for stanyl fatty acid esters, 70 µg to 4.0 mg Vegapure®95E/100 mg feces for steryl fatty acid esters and 175 µg to 7.0 mg γ-oryzanol/100 mg feces for steryl/stanyl ferulates. Calibrations for the individual esters were done in triplicate.

Calibrations allowing the quantitation of the individual liberated desmethylsterols/-stanols were performed in triplicate at concentrations ranging from 34.5 µg to 3.0 mg Reducol™/100 mg feces (campestanol and sitostanol), 3.75 µg to 4.0 mg Generol® 122N/100 mg feces (campesterol and sitosterol) and 18.75 µg to 2.25 mg dimethylsterol preparation/100 mg feces (cycloartanol, cycloartenol, and 24-methylenecycloartanol).

Microbial transformation products (secondary metabolites) of sitosterol and campesterol were calculated using relative response factors (RRF) (Eq. 6) compared to 5αC according to equation 7. The RRF were determined in triplicate using 24-ethylcoprostanol and the corresponding fecal cholesterol metabolites as references at three concentration levels, respectively.

Relative response factor (RRF)

$$RRF(x) = \frac{RF(x)}{RF(IS)_{.}} = \frac{\left(\frac{(c_x)_{standard\ solution}}{(Peak\ area_x)_{standard\ solution}}\right)}{\left(\frac{(c_{IS})_{standard\ solution.}}{(Peak\ area_{IS})_{standard\ solution.}}\right)} \qquad \text{(Eq. 6)}$$

RF = response factor; x = analyt; IS = internal standard; c_x = concentration of analyt in standard solution; c_{IS} = concentration of internal standard in standard solution

Contents of secondary metabolites in feces $c(x)_{feces}$

$$c(x)_{feces} = RRF(x) \cdot Peak\ area(x)_{feces} \cdot \frac{c(IS)_{feces}}{Peak\ area(IS)_{feces}} \qquad \text{(Eq. 7)}$$

RRF = relative response factor; x = analyt; IS = internal standard; c_x = concentration of analyt in feces sample; c_{IS} = concentration of internal standard in feces sample

RRF to 5α-cholestane used for the quantitation of secondary metabolites were calculated as 0.9690 (24-methylcoprostanol), 1.0427 (24-methylcoprostanone), 1.1278 (24-methylcholest-4-en-3-one) for campesterol metabolites as well as 1.0916 (24-ethylcoprostanol), 1.1746 (24-ethylcoprostanone) and 1.2705 (25-ethylcholest-4-en-3-one) for sitosterol metabolites.

3.2.4.1.5 Contents of fecal phytosterols/-stanols after alkaline hydrolysis

Lyophilized feces were hydrolyzed under alkaline conditions according to [25]. Aliquots of 100 mg were weighed into 4 mL screw capped vials containing 375 µg 5α-cholestane (375 µL from a stock solution of 5αC in *n*-hexane / MTBE (3/2, v/v); 1 µg/µL) as internal standard. After saponification with ethanolic KOH (2 mL, 3 M KOH, 90 % EtOH) at 80 °C for 60 min and neutralization with HCl (1.0 mL, 6 M) lipids were extracted three times with 5 mL of *n*-hexane/MTBE (3/2, v/v). Analysis of each feces sample was done in triplicate. Sample derivatization was performed as described for the isolation of intact phytosteryl/-stanyl esters and steroids (section 3.1.4.1.2).

3.2.4.1.6 Determination of fecal cholesterol excretion

Total cholesterol excretion was determined by simultaneous analysis of intact esters and liberated phytosterols/-stanols via GC/FID, using RRF compared to 5α-cholestane. Total cholesterol excretion is described as sum of cholesterol and the corresponding fecal metabolites (coprostanol, coprostanone and cholest-4-en-3-one). Relative response factors and contents were determined according to

equations 6 & 7 (section 3.2.4.1.4). RRF to 5αC used for the quantitation were calculated as 0.9475 (cholesterol), 0.8463 (coprostanol), 0.9107 (coprostanone), and 0.9851 (cholest-4-en-3-one).

3.2.4.2 Analysis of plasma samples

3.2.4.2.1 Analytical validation data (GC/MS)

In analogy to section 3.2.4.1.1, analytical validation data were determined on the basis of the linear regression methodology. Triplicate linear regression analysis for the individual phytosterols/-stanols were performed in coordinate ratios of peak areas (phytosterols/-stanols / IS) and amounts (phytosterols/-stanols / IS) using selected ion monitoring (SIM) mode. Determinations were carried out by monitoring *m/z* 372 $[M]^+$ for 5α-cholestane, *m/z* 472 $[M]^+$ for campesterol, *m/z* 486 $[M]^+$ for sitosterol, *m/z* 474 $[M]^+$ for campestanol, *m/z* 488 $[M]^+$ for sitostanol, *m/z* 408 $[M\text{-}TMSOH]^+$ for cycloartenol, *m/z* 410 $[M\text{-}TMSOH]^+$ for cycloartanol, and *m/z* 422 $[M\text{-}TMSOH]^+$ for 24-methylenecycloartanol.

3.2.4.2.2 Lipids and lipoproteins

Plasma total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and plasma triacylglycerols (TG) were analyzed with a Roche MODULAR DPE system using established enzymatic methods (Roche Diagnostics GmbH, Germany). The following enzymatic kits were used: (I) TC (Cat.No. 11875523216), (II) LDL-C (Cat.No. 05230438190), (III) HDL-C (Cat.No. 04713265190) and (IV) TG (Cat.No. 1876040216), respectively.

3.2.4.2.3 Phytosterol/-stanol levels (GC/MS)

Saponification, lipid extraction and quantitative determination by means of GC/MS were performed as combination of two methods [235, 236] with minor modifications. Briefly, 6.25 µg of 5α-cholestane (125 µL from a stock solution of 5αC in *n*-hexane/MTBE (3/2, v/v); 50 µg/mL) was added as internal standard to

1 mL of plasma. After saponification with ethanolic KOH (3 mL, 3 M KOH, 90 % EtOH) at 100 °C for 60 min and neutralization with HCl (1.5 mL, 6 M), sterols were extracted three times with 2 mL of *n*-hexane / MTBE (3/2, v/v). The combined organic phases were dried completely under a gentle stream of N_2 and the residual sterols/stanols were derivatized to TMS ethers by adding 600 µL BSTFA+TMCS (99:1) and 400 µL pyridine followed by incubation at 80 °C for 20 min. Analogously to section 3.2.4.2.1, quantitation using SIM mode was carried out by monitoring *m/z* 372 $[M]^+$ for 5α-cholestane, *m/z* 472 $[M]^+$ for campesterol, *m/z* 486 $[M]^+$ for sitosterol, *m/z* 474 $[M]^+$ for campestanol, *m/z* 488 $[M]^+$ for sitostanol, *m/z* 408 $[M\text{-}TMSOH]^+$ for cycloartenol, *m/z* 410 $[M\text{-}TMSOH]^+$ for cycloartanol, and *m/z* 422 $[M\text{-}TMSOH]^+$ for 24-methylenecycloartanol.

The concentrations of the phytosterols/-stanols were calculated from calibration curves using 5αC as internal standard. Linear regression analysis was performed in coordinate ratios of peak areas (phytosterol/-stanol / IS) and amounts (phytosterol/-stanol / IS).

3.2.4.3 Screening of urine samples

24-hour urine samples of three individuals obtained within the Benecol®-trial as well as the pro.activ®-trial were analyzed regarding sterols/stanols and related metabolites after acidic hydrolysis. Two aliquots (50 mL each) of urine samples were transferred into a 100 mL round-bottom flasks using a volumetric pipette, respectively. After adding 6.25 µg 5α-cholestane (125 µL from a solution of 5αC in *n*-hexane / MTBE (3/2, v/v); 0.05 µg/µL) and 10 mL HCl (6 M), samples were heated to boiling point and refluxed for one hour. After cooling to room temperature samples were adjusted to a neutral pH (6.5 – 7.5) by addition of KOH solution (10 mL, 6 M). Uric lipids were extracted three times with 25 mL of *n*-hexane / MTBE (3/2, v/v). Combined organic phases were dried with sodium sulftate (anhydrous), filtered and finally removed using a rotary evaporator. The residue was derivatized by adding 150 µL BSTFA + TMCS (99:1) and 100 µL pyridine followed by incubation at 80 °C for 20 min. After drying under N_2 the resulting TMS ethers were re-dissolved in 250 µL *n*-hexane / MTBE (3/2, v/v) and subjected to gas chromatography.

3.2.4.4 Statistical evaluation

Statistical analyses were performed with IBM® SPSS® Statistics (version 19.0). Normal distribution and homogeneity of variance were tested with the Kolmogorov-Smirnov-Lilliefors-test and Levene's test, respectively. Contents of intact phytosteryl/-stanyl esters in feces were analyzed using ANOVA and post-hoc Tukey-HSD-test if recoveries have been ascertained significantly different. Additionally, separate comparison was performed on the two groups of ferulic acid esters (desmethylsteryl and dimethylsteryl ferulates) by Student's unpaired *t*-test. Plasma samples were compared either with repeated-measures ANOVA including Bonferroni multiple comparisons or pairwise Friedman-test depending on data distribution. Differences were considered significant at $P \leq 0.05$ and *P*-values < 0.1 were considered as indicators for a statistical trend. Unless otherwise indicated, data were expressed as mean ± standard error of the mean (SEM).

4 TRANSFORMATION OF STERYL/-STANYL ESTERS BY HUMAN COLONIC MICROBIOTA *IN VITRO*

4.1 INTRODUCTION

Microbial transformations of food components play an important role in the gastrointestinal (GI) digestion of the diet by humans. Especially during large intestine transit fermentative processes are of decisive importance. The colon is the part of the GI tract that exhibits the highest biodiversity of microbiota, including more than 500 different species and with 10^9 – 10^{12} colony-forming units / g feces the most dense population of microorganisms [237]. Obligate anaerobes including species of the genera *Bacteroides, Clostridium, Eubacterium, Peptostreptococcus, Ruminococcus, Fusobacterium, Bifidobacterium, Atopobium, and Peptococcus* are predominant. The total amount of facultative anaerobes e.g., *Lactobacillus* spp*., Enterobactericeae* spp., *Enterococcus* spp., *and Streptococcus* spp. is about one-thousandth the size of the obligate anaerobes [237-239].

The most important substrates for fermentations by colonic microbiota *in vivo* are non-digestible food components like soluble (pectins, fructans, glucans, alginates) and insoluble dietary fibers (cellulose, lignans, resistant starch). Also undigested dietary proteins, endogenous proteins from intestinal and bacterial secretions and mucins (glycoproteins) produced by the epithelia are accepted as substrates [240, 241]. Microbial transformations of phytosterols during large intestinal transit have been reported to be identical to those reported for cholesterol [24-29]. Although orally administered phytosteryl/-stanyl esters, if not hydrolyzed by digestive enzymes, might reach the colon in intact form, conversion of these esters or hydrolysis by the colon microbiota have not been reported. Especially the 4,4-dimethylsteryl ferulic acid esters of γ-oryzanol which were not accepted as substrates by PCE [161, 213, 214] are of interest due to the activity of human epithelial and gut bacteria-derived feruloyl esterases in de-esterification of dietary fibers during digestion [242].

The use of a representative microbial population is a fundamental prerequisite for the simulation of colonic fermentation processes *in vitro*. Since MOORE *et al.* [243] reported a constant composition of the colonic microflora from the

ascendening colon to the rectum in individuals who experienced sudden death, freshly voided feces is assumed to be the best non-invasive way to obtain a population comparable to the gut microbiota. Several studies employing human feces as inoculum have been reported for the *in vitro* fermentation of carbohydrates and dietary fibers [227, 244-249] under different more or less sophisticated conditions. A well-functioning and simple methodology called “one-batch”-procedure in which the anaerobic environment of the large intestine is simulated in a single reaction vessel has been reported by BARRY *et al.* [227]. On the other hand, instrumental setups made up of multistage, continuous phases were employed to simulate different regions of the digestive tract [250, 251].

To date, fermentation studies of phytosteryl/-stanyl esters involving human feces as source of mibrobiota under anaerobic conditions have not been described. Thus, the objective of this study was to investigate *in vitro* whether the colonic microflora is capable to hydrolyze phytosteryl/-stanyl esters and whether intact esters and liberated acid and sterol moieties are subject to bacterial transformations. On the basis of the methodology described by BARRY *et al.* [227], fermentation experiments of varying phytosteryl/-stanyl esters were performed with two different culture media and investigated by means of GC/FID and GC/MS.

4.2 RESULTS AND DISCUSSION

4.2.1 SELECTION OF SUITABLE FECES DONORS

Due to varying colonic microflora, the metabolic activity might be different in individuals. For instance, the conversion of cholesterol to coprostanol (Fig. 9) is obviously dependent on the microbial colonization of the large intestine [28, 252]. Due to the rate of conversion, expressed as percentage coprostanol of the sum of cholesterol and coprostanol, calculated on the basis of fecal contents of coprostanol and cholesterol (Eq. 8) determined in 622 healthy humans, BENNO *et al.* [252] differentiated the subjects into three categories: “Non-Converter” (conversion rates < 5 %), “Low-Converter” (conversion rates 5 up to < 40 %) and “High-Converter” (conversion rates > 40 %). For the purpose of this study, suitable

feces donors should be characterized as “High-Converter” indicating a microflora that accepts at least free sterols as substrates for microbial transformations [28].

$$\text{conversion rate}\,[\%] = \frac{\text{coprostanol}_{\text{feces}}}{(\text{coprostanol} + \text{cholesterol})_{\text{feces}}} \cdot 100 \qquad \text{(Eq. 8)}$$

Contents of coprostanol and cholesterol were determined in the feces of three volunteers to calculate the conversion rates and to indentify suitable donors. Conversion rates calculated on the basis of the peak area ratios obtained by GC/FID (Fig. 17) were 27 % (subject 1), 77 % (subject 2) and 94 % (subject 3). Accordingly, the feces of subject 2 and 3 characterized as “High-Converter” were employed as mixture in equal shares for fermentation experiments. The selected male subjects, both 31 years of age, consumed an unspecific western diet and had not taken antibiotics during the six months prior to the experiments.

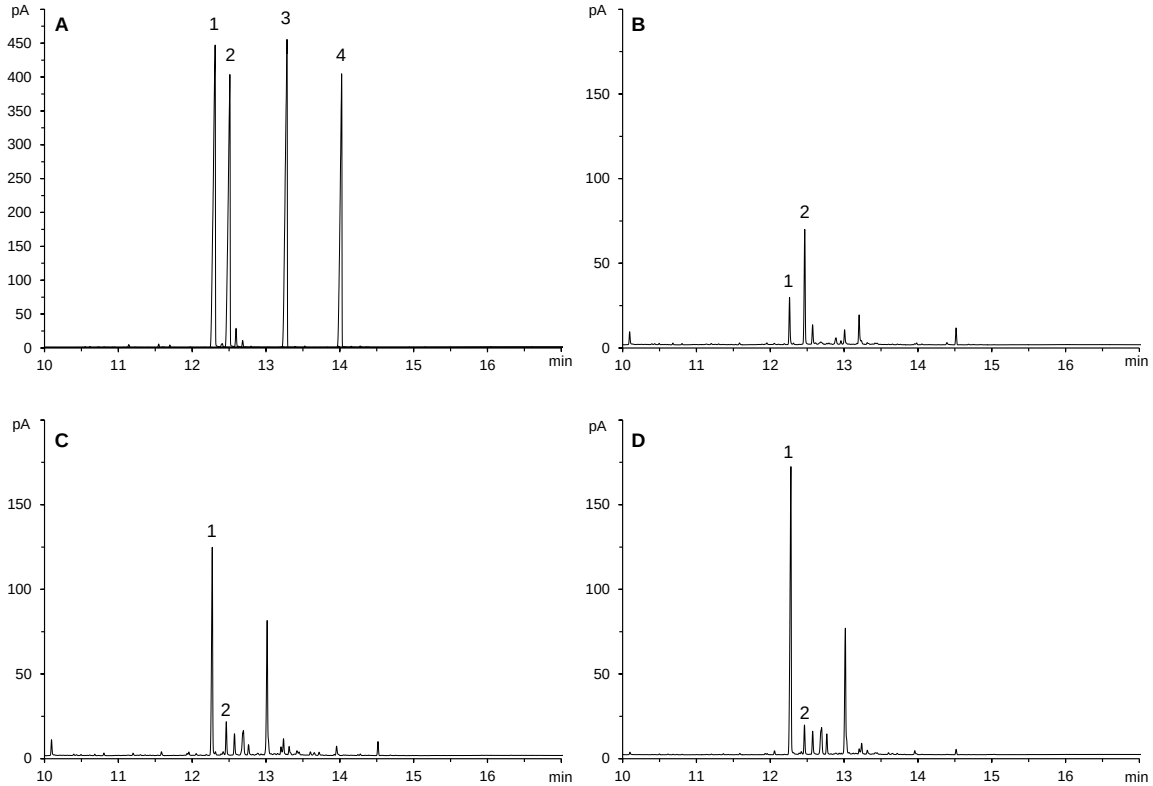

Fig. 17 GC/FID analysis of fecal steroid fraction of three subjects consuming an unspecified western diet for calculation of cholesterol conversion rates; (A) External reference solution: 1 = coprostanol; 2 = cholesterol; 3 = coprostanone; 4 = cholest-4-ene-3-one; (B) Subject 1; (C) Subject 2; (D) Subject 3.

4.2.2 ADJUSTMENT OF FERMENTATION CONDITIONS

Preliminary studies using cholesterol and ferulic acid as substrates indicated that the conditions described by BARRY *et al.* [227] might to be suitable for the fermentation of ferulic acid but not of desmethylsterols. Thus, the setup was adapted and tested in further fermentation experiments resulting in an optimized methodology (section 3.2.3.3 and 3.2.3.4).

Period of fermentation

Upon incubation for 24 h, BARRY *et al.* [227] reported that pectin and soy bean dietary fibers were highly degraded (> 90 %), whereas cellulose and maize bran degradabilities were less than 10 %. The transit time of the diet within the colon and accordingly the period of microbial degradation *in vivo* averaged 54 – 56 h [238]; this is more than two times higher than the incubation time described by BARRY *et al.* [227]. Additionally, prolonged incubations up to 120 h [150] or even 140 h [154] have been described for the aerobic transformation of phytosterols by *Mycobacterium* ssp. Thus, the incubation time for the fermentation experiments including steryl esters as substrates was extended to 5 days.

Substrate concentration

BARRY *et al.* [227] described substrate concentrations of about 1.0 % (100 mg substrate / 10 mL inoculum), whereas bioconversion of sterols were usually performed with 0.1 % substrate in the medium [150, 163]. To avoid inhibitory effects caused by too high contents of the substrates in the medium, concentrations were set to 0.1 % (10 mg substrate / 10 mL inoculum).

Substrate addition

Hydrophobic and powdered substances added to aqueous fermentation media are prone to agglutination or sedimentation and are difficult to disperse leading to a diminished bioavailability of the substrate. Thus, the overall biotransformation rate of steroids in aqueous media was considered to depend not only on the activity of the microorganisms, but also on the dissolution rate of the solid substrate. Solubilization of the substrates can be improved by at least two concepts: On the one hand, hydrophobic substrates were added to the aqueous fermentation media in the form of saturated solutions in water-miscible organic solvents like dimethylformamide [253], methanol [254], acetone [255] and

propane-1,2-diol [256]. In order to prevent bacteriotoxic conditions, the amount of co-solvent added usually has to be kept below 1.5 –5 % (v/v) [149]. On the other hand, the addition of substrate suspensions in surface-active agents such as Tween® 80 [257], Triton® X-100 [258] and lecithin [255] was applied.

Surface-active substances tested in the present study were Tween® 80 and sodium taurocholate representative for bile salts. Addition of 1 mL of substrates suspensions in form of solutions containing 0.1 % of the respective surface active agents to fermentation media clearly demonstrated more pronounced emulsifying properties of Tween® 80 compared to sodium taurocholate. Acetone and propan-2-ol were applied as organic solvents. It was possible to dissolve 10 mg of the substrates in 200 µL of both of the solvents which is equivalent to a solvent concentration of 2 % in the media. Nevertheless, substrate suspension using Tween® 80 was preferred in the following experiments due to the immediate precipitation of the substrates employed as organic solutions upon addition to the fermentation media.

Inoculum preparation

The inoculum reported by BARRY *et al.* [227] made up of one part of feces and five parts of a nutritive buffer (1/5, m/v) was forty-fold higher concentrated than the inoculum (1/200, m/v) used in experiments focussing on the fermentability of dehydrodiferulic acids [228]. To investigate an impact of the inoculum concentration, fermentation experiments employing inocula composed either of one part feces and fifty parts NB (1/50, m/v) or one part and one hundred parts (1/100, m/v) were performed. Due to incomplete metabolization of ferulic acid upon incubation using the latter one the inoculum containing one part feces and fifty parts of the nutrtive buffer (1/50, m/v) was chosen.

Nutritive medium

The nutritive medium that had been used for the production of the inoculum was a simple carbonate – phosphate buffer solution enriched in urea and trace elements [227]. Colonic microorganisms capable to metabolize sterols and possibly steryl esters probably have very specific growth requirements. Thus, thioglycollate broth, an all-purpose medium for the cultivation and isolation of fastidious anaerobic microorganisms commonly used in sterility tests, was applied

as a second medium for the preparation of the inoculum. The reduction of the redox potential in the media due to the contained sodium thioglycolate (0.5 g/L) and L-cysteine (0.5 g/L) ensured anaerobic conditions. In order to verify anaerobicity during the fermentation procedure resazurin was added to the thioglycollate broth as redox indicator. Resazurin is colorless in its reduced state and would turn pink due to oxidation within an aerobic environment. During the whole fermentation procedure (5 d) no color shift of the thioglycollate broth was observed in blank II, indicating maintenance of absolute anaerobicity.

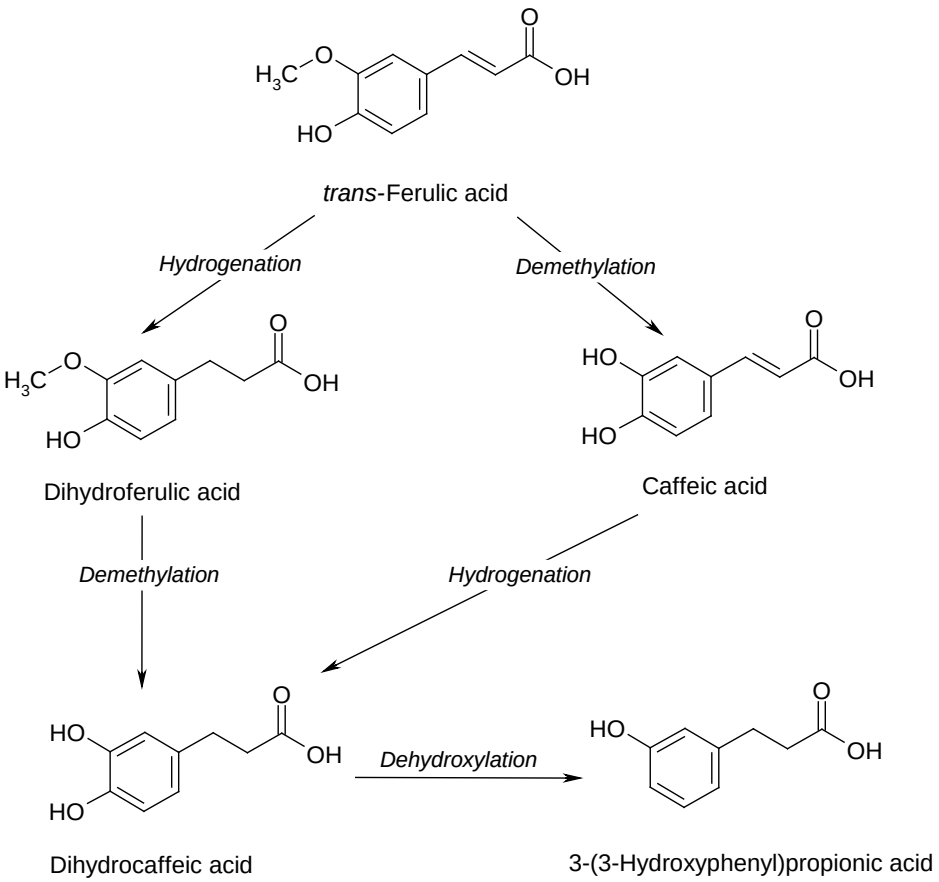

Fig. 18 Metabolic pathway of *trans*-ferulic acid conversion by human intestinal microbiota *in vitro* (adapted and modified from [228])

4.2.3 VERIFICATION OF SETUP – FERMENTATION OF *TRANS*-FERULIC ACID

Fermentaion experiments applying *trans*-ferulic acid also a potential hydrolysis product of phytosteryl/-stanyl ferulates as substrate were performed due to the evidenced microbial conversion pathway of FA (Fig. 18) obtained under similar conditions [228]. Transformation products of dehydrodiferulic acids-derived ferulic acid have been identified as dihydroferulic acid, caffeic acid (CA), dihydrocaffeic acid (HCA) and 3-(3-hydroxyphenyl)propionic acid (HPA). These metabolites were also expected in the present *in vitro* fermentation experiments as a proof of the functionality of the experimental setup.

Tab. 7 Recovery (Rec) of the extraction standard within *in vitro* fermentation of *trans*-ferulic acid (FA) by human fecal microbiota using nutritive buffer (NB) and thioglycollate broth (THB)

Sample	$Area_{IS}$ [pA · s]	$Area_{ExS}$ [pA · s]	$A = \frac{Area_{ExS\ (100\ \%)}}{Area_{IS}}$	$B = \frac{Area_{ExS}}{Area_{IS}}$	$Rec = \frac{B}{A}$
ExS (100 %)[a]	*49.82*	*222.43*	*4.46*		[%]
Blank NB I a	49.38	229.57		4.65	104.2
Blank NB I b	43.62	196.32		4.50	100.9
Blank NB II a	53.65	235.15		4.38	98.3
Blank NB II b	53.53	221.98		4.15	93.0
FA NB a	46.94	204.22		4.35	97.5
FA NB b	46.15	200.85		4.35	97.6
Blank THB I a	45.33	205.22		4.53	101.5
Blank THB I b	47.16	214.81		4.55	102.1
Blank THB II a	53.92	232.70		4.32	96.8
Blank THB II b	53.45	229.71		4.30	96.4
FA THB a	47.04	205.92		4.38	98.2
FA THB b	49.55	212.81		4.29	96.3
Mean	49.14	215.77		4.40	98.6
SD	3.52	13.03		0.13	3.0

[a] Mean value obtained by eight-fold analysis of an external standard solution of ExS;
ExS = extraction standard (syringic acid); IS = internal standard (vanillic acid); SD = standard deviation

The reocvery rate of the extraction standard (ExS; syringic acid) illustrated in Tab. 7 was determined in both fermentation media to compensate for losses during the extraction at the acidic pH value (Fig. 15 B). The reovery rate was

calculated on the basis of the area-ratios of the ExS and the IS (vanillic acid), analyzed in incubated samples (B), as well as the respective area-ratio (A) determined upon eight-fold analysis of a reference solution corresponding to a complete extraction ($ExS_{100\,\%}$). As illustrated in Tab. 7, syringic acid was recovered to almost 98.6 ± 3.0 %. Thus, it could be assumed that ferulic acid and related metabolites were also measured quantitatively. In order to estimate transformation rates of ferulic acid under the employed conditions, calibrations allowing the quantitation of FA, CA, HCA and HPA were performed in triplicate at concentrations ranging from 1.0 µg to 150.0 µg /mL, respectively.

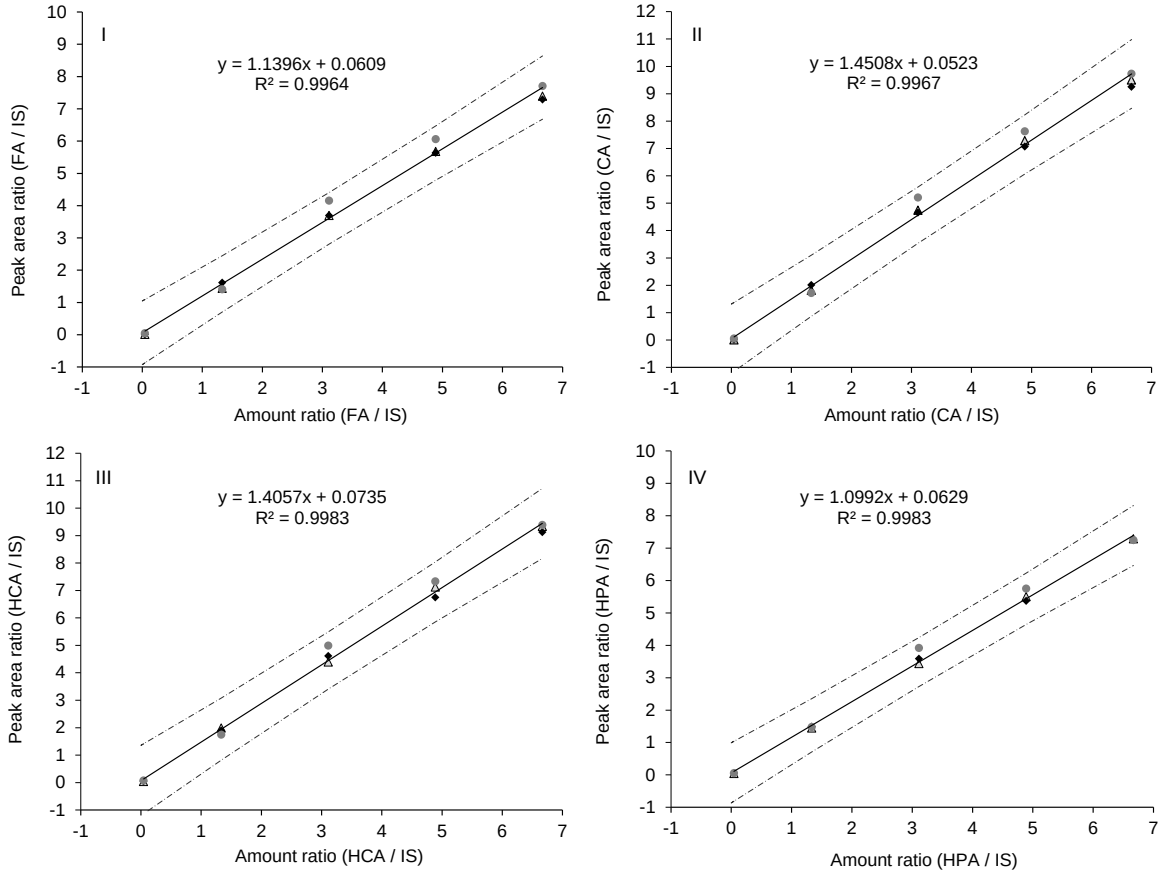

Fig. 19 Linear regression analysis performed in triplicate of *trans*-ferulic acid (FA) (I), caffeic acid (CA) (II), dihydrocaffeic acid (HCA) (III), and 3-(3-hydroxyphenyl)propionic acid (HPA) (IV); IS = internal standard (vanillic acid); dashed lines represent confidence interval (CI, 95 %)

On the basis of linear regression analysis performed in coordinate ratios of peak areas (component / IS) and amounts (component / IS) shown in Fig. 19, analytical validation data (Tab. 8) were calculated according to equations 1 – 5 (section 3.2.4.1.1).

Tab. 8 Relative retention times (RRT), limits of detection (LOD), limits of quantitation (LOQ) and coefficients of variation (CV) of *trans*-ferulic acid and expected microbial conversion products determined on the basis of the linear regression methodology according to [229]

Acid	RRT[a]	LOD[b]	LOQ[b]	CV
	[min]	[µg/mL]		[%]
3-(3-Hydroxyphenyl)propionic acid	0.951[a]	4.44	12.61	5.5
Dihydrocaffeic acid	1.160[a]	4.72	13.35	5.8
trans-Ferulic acid	1.362[a]	6.21	17.26	7.6
Caffeic acid	1.387[a]	5.79	16.18	7.1

[a] Relative retention time compared to vanillic acid (IS)

[b] LOD and LOQ related to 1 mL injection solution (section 3.2.3.5.2)

4.2.3.1 Nutritive buffer

Results obtained upon fermentation of *trans*-ferulic acid using the inoculated nutritive buffer medium are illustrated in Fig. 20.

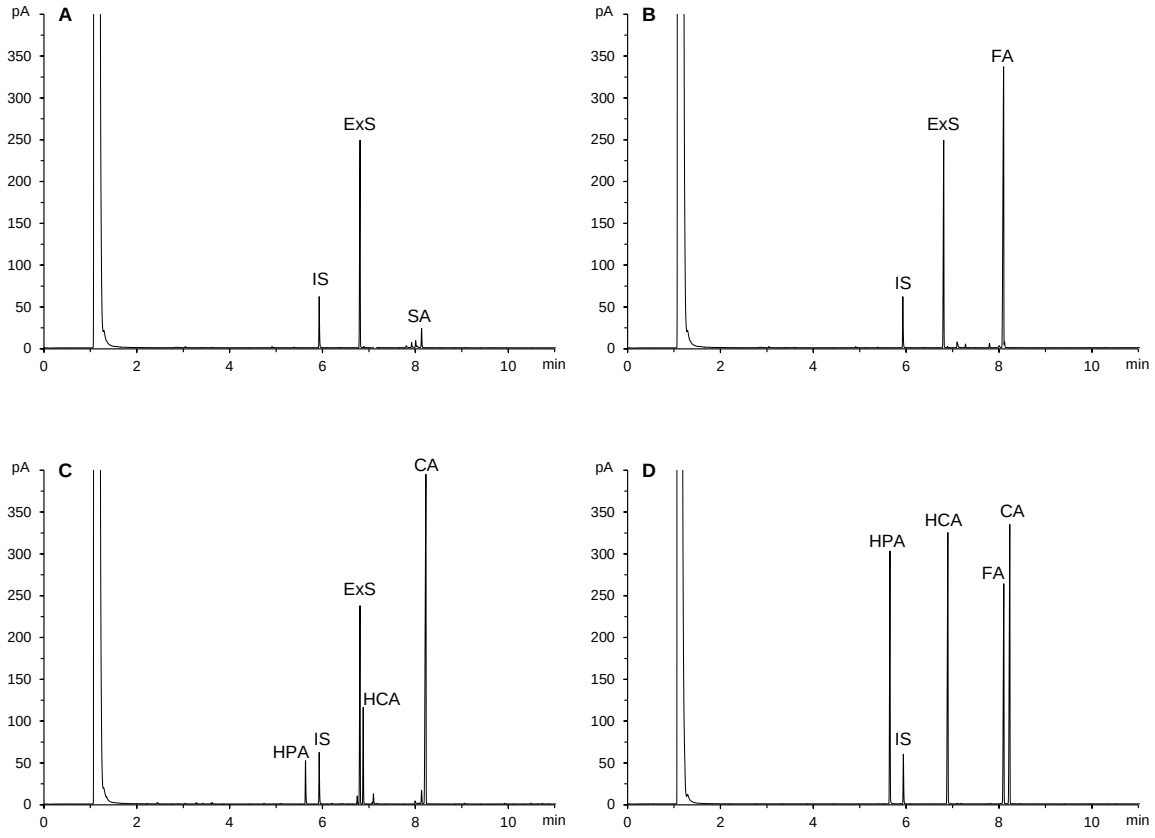

Fig. 20 GC/FID analysis of microbial conversion products obtained upon *in vitro* fermentation of *trans*-ferulic acid (FA) in nutritive buffer medium inoculated with human feces; (A) Blank I; (B) Blank II; (C) Incubated sample; (D) External reference solution; IS = internal standard (vanillic acid); ExS = extraction standard (syringic acid); SA = stearic acid; HPA = 3-(3-hydroxyphenyl)-propionic acid; HCA = dihydrocaffeic acid; CA = caffeic acid

As shown in the chromatograms of the respective blanks neither FA nor the expected metabolites were detectable within the limits of detection (Tab. 8) in the inoculated medium (blank I; Fig. 20 A);there was also no indication that *trans*-ferulic acid was chemically degraded by components contained in the nutritive buffer (blank II; Fig. 20 B).

A potentially interfering natural component of human feces revealing a RRT of 1.368 was identified in blank I as stearic acid (SA) on the basis of the relative retention time and the mass spectrometric fragmentation pattern (Fig. 21) compared to a reference. The molecular ion $[M]^+$ as well as fragment ions derived from McLafferty rearrangement (*m/z* 132) and β-cleavage (*m/z* 145), described as characteristic fragements of saturated fatty acid methyl ester [259], were used for identification.

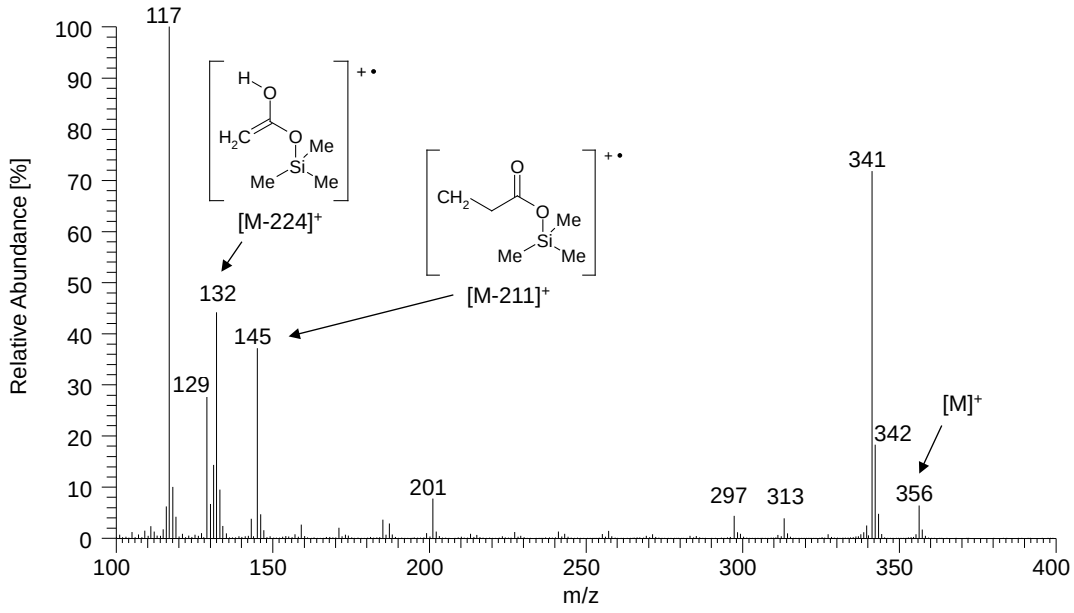

Fig. 21 GC/EI-MS full scan spectrum of silylated stearic acid identified in inoculated nutritive buffer (blank I); M = molecular ion

As illustrated in chromatogram C (Fig. 20), *trans*-ferulic acid was not detectable within LOD in the incubated nutritive buffer indicating an almost complete degradation of FA by human colonic bacteria. The identification of the microbial conversion products was performed by comparison of relative retention times (Fig. 20 D; Tab. 8) and mass spectra to those of reference substances. GC/EI-MS spectra of silylated phenolcarboxylic acids (Fig. 22) are commonly

characterized by abundant molecular ions $[M]^+$ and the fragment $[M\text{-}CH_3]^+$ facilitating the identification. Thus, caffeeic acid, dihydrocaffeic acid as well as 3-(3-hydroxy-phenyl)propionic acid were unambiguously identified. These metabolites are the same as those described by BRAUNE *et al.* [228] (Fig. 18), demonstrating the functionality of the experimental setup used in this study.

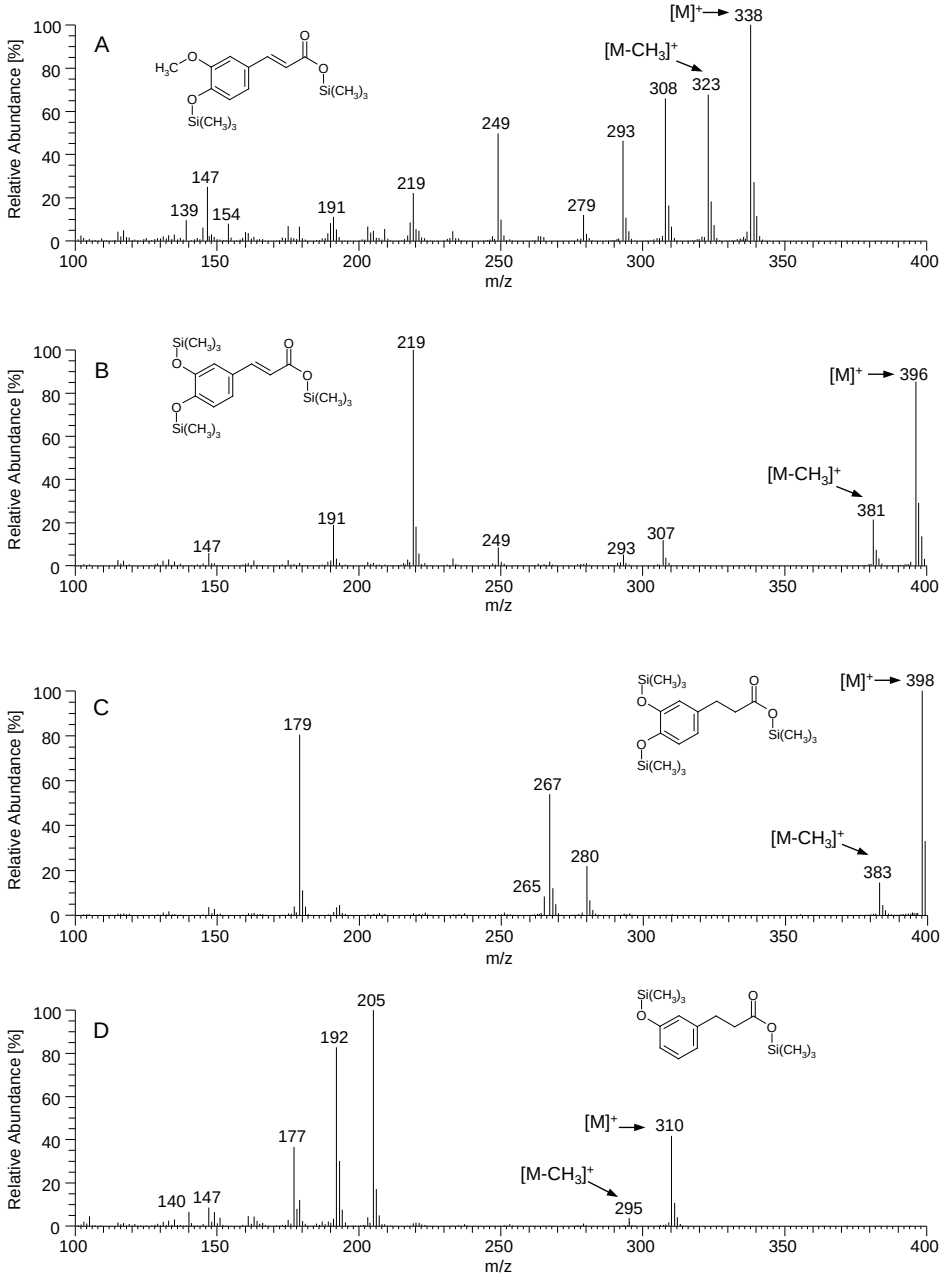

Fig. 22 Molecular structures and GC/EI-MS full scan spectra of silylated *trans*-ferulic acid (A), caffeic acid (B), dihydrocaffeic acid (C), and 3-(3-hydroxyphenyl)propionic acid; M = molecular ion

Solely dihydroferulic acid as an intermediate product of the verified metabolic pathway of *trans*-ferulic acid (Fig. 18) has not been detected. This might be explained by the fact that in contrast to BRAUNE et al. [228] in this study the incubated samples were not investigated in a time-dependent manner but only after 120 h of incubation.

The total conversion rate of *trans*-ferulic acid and the respective proportion of each metabolite identified was determined by quantifying the indivdual conversion products using linear regression analysis (section 4.2.3). The amounts calculated for the individual transformation products were converted to ferulic acid-equivalents considering the molecular weights of HPA (166 g/mol), HCA (182 g/mol), and CA (180 g/mol) as well as FA (194 g/mol). In total, almost 85 % of the *trans*-ferulic acid initially employed as substrate were recovered as the metabolites described. It can be assumed that the missing 15 % probably were degraded to low molecular substances which were not accessible via the methodology employed. Although the total conversion rate of *trans*-ferulic acid remained constant in repeated incubations, the proportions of individual metabolites ranged between 9 – 16 % for 3-(3-hydroxphenyl)propionic acid, 14 – 25 % for dihydrocaffeic acid, and 59 – 77 % for caffeic acid- indicating varying microbial activity.

4.2.3.2 Thioglycolate broth

In analogy to the fermentation experiments performed with the nutritive buffer medium, *trans*-ferulic acid or potential metabolites were not detectable in the inoculated THB (blank I; Fig. 23 A) nor was FA degraded by medium components (blank II; Fig. 23 B). In the freshly prepared inoculated thioglycollate broth not only feces-derived stearic acid but also palmitic acid (RRT = 1.205) has been identified (blank I; Fig. 23 A) on the basis of relative retention times and mass spectromteric fragmentation pattern as previously described for stearic acid. Surprisingly, with the exception of HPA the metabolites which have been identified upon fermentation using nutritive buffer medium were unverifiable within their limits of detection (Tab. 8), although FA again was almost degraded completely. Instead, two new microbial conversion products exhibiting relative retention times of 0.552

and 0.875, respectively, have been detected (Fig. 23 C). The mass spectrometric fragmentation patterns of the two unknown metabolites illustrated in Fig. 24 are characterized by abundant molecular ions $[M]^+$ as well as the demethylated fragment $[M\text{-}CH_3]^+$, respectively. Furthermore, both mass spectra revealed a base peak fragment $[M\text{-}118]^+$, a fragment also observed for dihydrocaffeic acid and

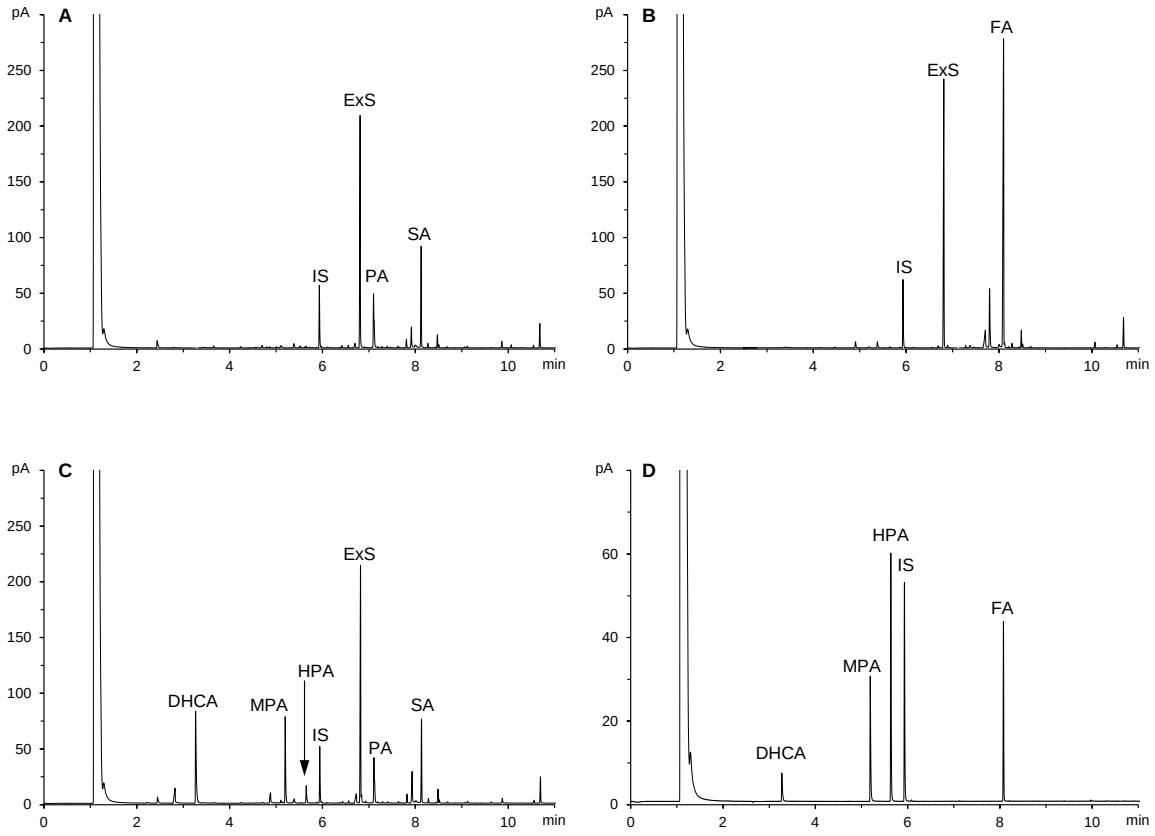

Fig. 23 GC/FID analysis of microbial conversion products obtained upon *in vitro* fermentation of *trans*-ferulic acid (FA) in thioglycollate broth inoculated with human feces; (A) Blank I; (B) Blank II; (C) Incubated sample; (D) External reference solution; IS = internal standard (vanillic acid); ExS = extraction standard (syringic acid); PA = palmitic acid; SA = stearic acid; DHCA = dihydrocinnamic acid; MPA = 3-(3-methoxyphenyl)propionic acid; HPA = 3-(3-hydroxy phenyl)propionic acid

3-(3-hydroxyphenyl)propionic acid (Fig. 22 A, D), indicating phenylpropionic acids. Base peak fragments at *m/z* 134 and *m/z* 104 are presumably attributed to α-cleavage of TMSOH followed by decarbonylation. Thus, these metabolites were tentativley identified as dihydrocinnamic acid (DHCA; RRT = 0.552) and 3-(3-methoxyphenyl)propionic acid (MPA; RRT = 0.875). Finally, identification has been confirmed by comparison of the RRT (Fig. 23 D) and mass spectrometric fragmentation pattern to those of reference substances.

Since no calibrations were performed for DHCA and MPA, the respective amounts were calculated using RRF (Eq. 6) compared to vanillic acid according to Eq. 7. Relative response factors of 5.58 for DHCA and 1.57 for MPA were determined in triplicate at a concentration of 22.5 µg/mL for each metabolite and IS, respectively.

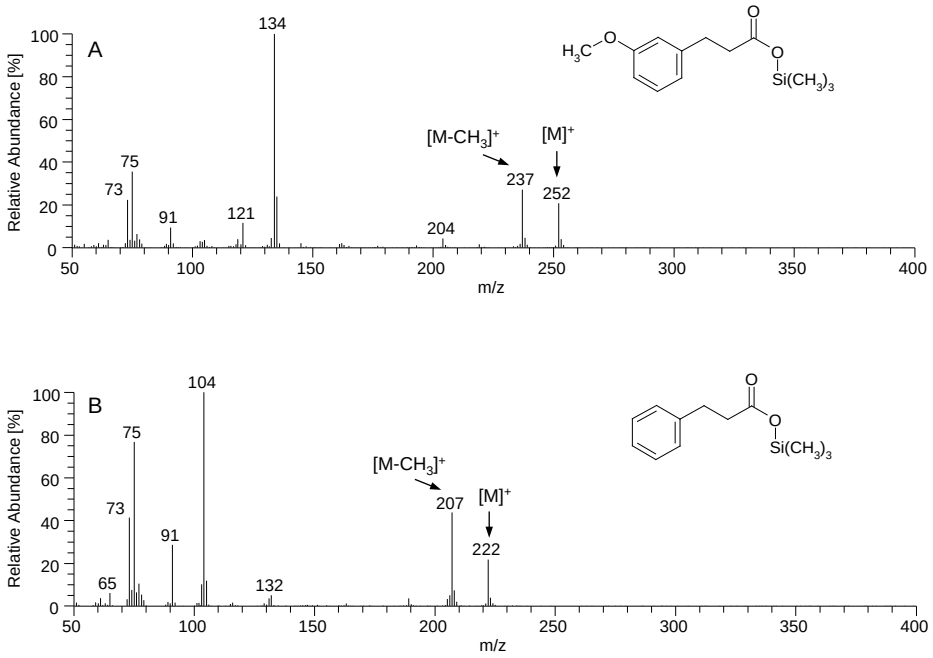

Fig. 24 Molecular structures and GC/EI-MS full scan spectra of two new metabolites of *trans*-ferulic acid identified upon incubation with human fecal suspensions; (A) = 3-(3-methoxyphenyl)-propionic acid; (B) = dihydrocinnamic acid; M = molecular ion

Upon translation of the calculated amounts into ferulic acid-equivalents, the proportions of dihydrocinnamic acid (150 g/mol) and 3-(3-methoxyphenyl)propionic acid (180 g/mol) of the total identified conversion products ranged in repeated fermentation experiments between 74 – 81 % and 17 – 23 %, repectively. The portion of 3-(3-hydroxyphenyl)propionic acid (2 – 3 %) has been determined as described in section 4.2.3.1. In total, more than 90 % of the *trans*-ferulic acid initially employed as substrate were recovered in form of these three metabolites.

Considering that hydrogenation, demethylation and dehydroxlation are the major metabolic steps of the bioconversion of hydroxycinnamic acids in ruminants as well as in humans [260] and the reaction sequence in buffered human fecal suspensions observed by BRAUNE et al. [228] (Fig. 18), a potential metabolic pathway shown in Fig. 25 can be inferred from the identified metabolites for the *in*

vitro biotransformation of *trans*-ferulic acid by human colonic microbiota cultivated in thioglycollate broth.

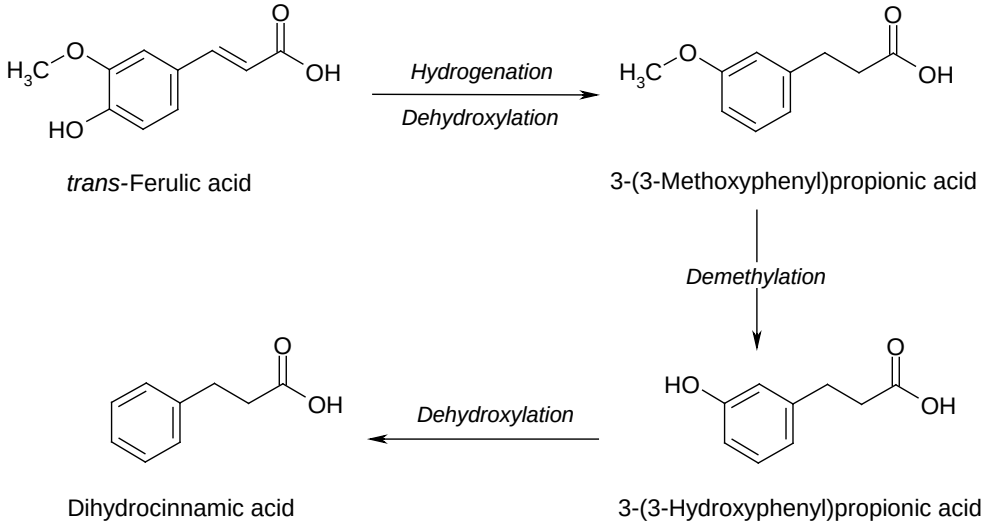

Fig. 25 Proposed metabolic pathway of *trans*-ferulic acid upon *in vitro* incubation with human fecal suspension using thioglycollate broth as nutritive medium

Trans-ferulic acid is initially converted to MPA via hydrogenation of the aliphatic side chain and elimination of the phenolic hydroxyl group. Based on demethylation of 3-(3-methoxyphenyl)propionic acid leading to HPA and subsequent dehydroxylation, dihydrocinnamic acid has been produced as the main metabolite upon *in vitro* fermentation of *trans*-FA in human feces continaining thioglycollate broth. Due to the fact that these experiments were not performed in a time-dependent manner it cannot be ruled out that the metabolites identified in THB were probably made up of dihydroferulic acid, caffeic acid and dihydrocaffeic acid formed as intermediates comparable to Fig. 18. Particularly, the formation of MPA could also result from a stepwise transformation of *trans*-FA via intermediately produced dihydroferulic acid.

In summary, it has been demonstrated upon *in vitro* fermentation experiments of *trans*-ferulic acid employing two different media (NB and THB) inoculated with freshly voided human feces as source of microbiota that metabolization appears to be affected by the media used. This might be attributable either to varying biological activities of the microorganisms or to a different composition of the microflora cultivated in the two media.

4.2.4 FERMENTATION OF ESTERS EXHIBITING DIFFERENT MOLECULAR STRUCTURES

To investigate whether phytosteryl/-stanyl esters *in vivo* might be subject to microbial transformations or ester hydrolysis when entering the colon in intact form, fermentation experiments employing the developed *in vitro* setup were conducted with esters exhibiting different molecular structures. In addition to cholesteryl cinnamate (CC) as an internal standard for the qantitation of intact esters, 5αC was used for the determination of hydrolysis rates and of sterol-related microbial conversion products, considering liberated sterols as biomarkers of hydrolysis. As shown in the chromatogramm of blank I (inoculated thioglycollate broth, Fig. 26 A), identification of liberated sterols is not influenced by feces-derived steroids, e.g. cholesterol and its conversion products (Fig. 9), due to their negligible concentrations. The recovery rate, determined as decribed for syringic acid (section 4.2.3), of cholesteryl dodecanoate (ExS) related to both cholesteryl cinnamate and 5α-cholestane averaged to 94.6 ± 3.4 %, thus demonstrating an almost quantitative extraction of intact steryl esters and steroids, respectively.

As representative for dimethysteryl ferulic acid esters, the dominating compounds of γ-oryzanol, purified **cycloartenyl ferulate** (90 %, 10 % 24-methylenecycloartanyl ferulate (MCF)) obtained according to section 3.2.3.1 was used as substrate for the *in vitro* fermentation procedure. Results obtained in experiments using inoculated THB are illustrated in Fig. 26. Chemical alterations of cycloartenyl ferulate, e.g. ester hydrolysis, solely induced by the thioglycollate broth can be excluded. As shown in the chromatogram of the blank II (substrate + THB + Tween®80) (Fig. 26 B) besides CF, RRT = 1.288 related to cholesteryl cinnamate, residual MCF (RRT = 1.326) and the respective standard substances, no further peaks have been detected.

Liberated cycloartenol (RRT = 1.215, related to 5αC) (Fig. 26 C) verifiable within a LOD of 0.15 µg/mL was used as marker substance for ester hydrolysis. In analogy to the blank II, free cycloartenol was not detectable in fecal suspensions upon fermentation of cycloartenyl ferulate using inoculated THB (Fig. 26 D). Due to the lack of free dimethylsterols or potential microbial transformation products

and the remaining unchanged substrate, it can be concluded that neither hydrolysis nor transformation of the intact ferulic acid ester had taken place.

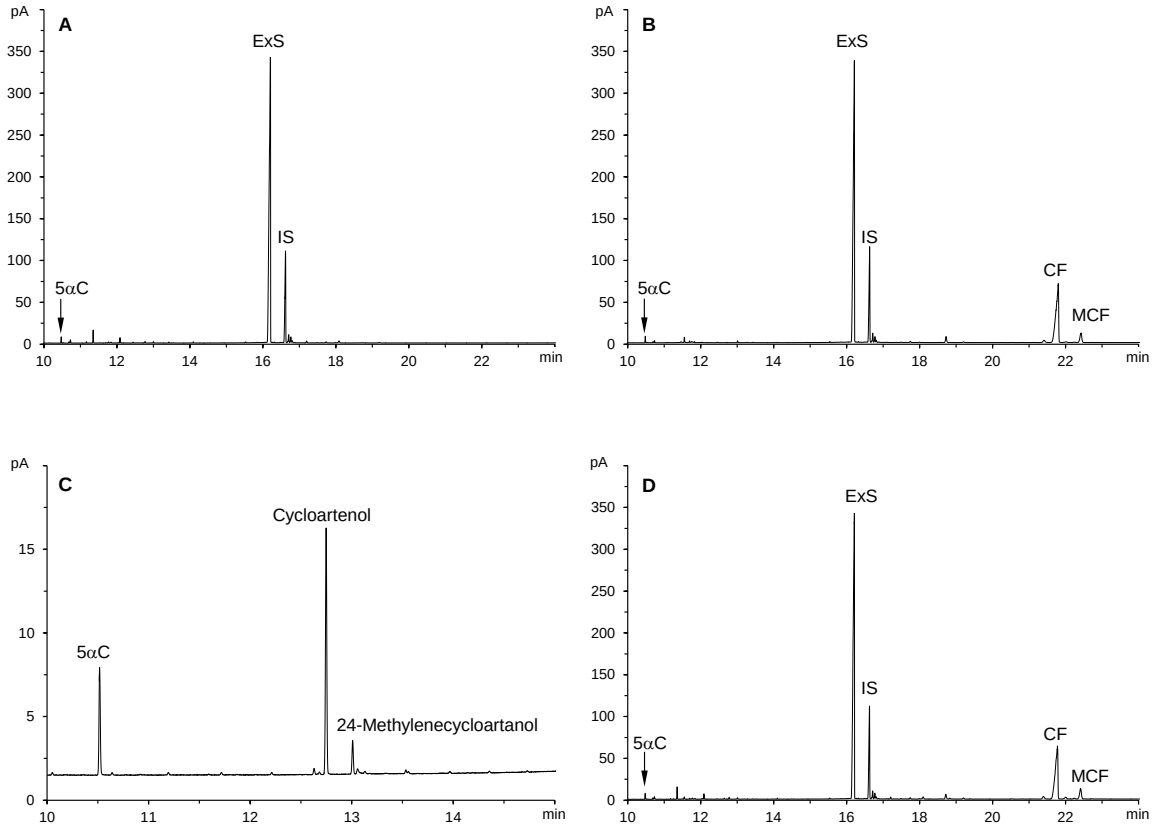

Fig. 26 GC/FID analysis of samples upon *in vitro* fermentation of cycloartenyl ferulate in thioglycollate broth (THB); (A) Blank I; (B) Blank II; (C) External reference solution of dimethylsterols; (D) Inoculated THB; IS = internal standard (cholesteryl cinnamate); ExS = extraction standard (cholesteryl dodecanoate); 5αC = 5α-cholestane; CF = cycloartenyl ferulate; MCF = 24-methylenecycloartanyl ferulate

Similar results were observerd in the fermentation experiments using the inoculated nutritive buffer medium (not illustrated). An explanation for non-hydrolysis might be a steric hindrance of the active center of hydrolytic enzymes based on the structural characteristics of dimethylsteryl ferulates. When investigating the enzyme-catalyzed hydrolysis of γ-oryzanol, MILLER et al. [13] also assumed that the sterol-specifity observed for PCE (hydrolysis confined to desmethylsteryl ferulates) is probably attributed to the presence of an additionial methyl group at the C14 position, the dimethyl substitution at C4 and the

cyclopropane structure at the C9/C10 position in the ring structure of 4,4-dimethylsterols (Fig. 11).

In order to examine the potential impact of the mentioned structural characteristics of 4,4.dimethylsterols on the hydroylsis of ferulic acid esters by colonic microbiota, a comparable desmethylsteryl ester has to be used. Due to the lack of commercial desmethylsteryl ferulates, **cholesteryl cinnamate** (Fig. 27) was employed as substrate. CC has also been used as internal standard

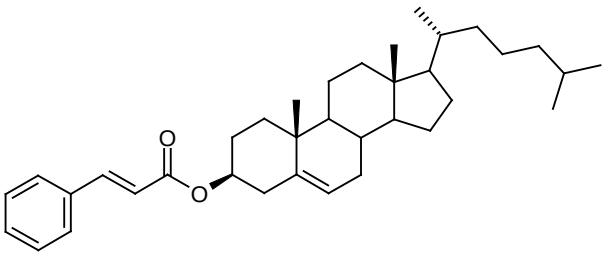

Fig. 27 Molecular structure of cholesteryl cinnamate

within the fermentation experiments of cycloartenyl ferulate. Consequently, in this experiments no internal standard was added making the exact calculation of the recovery rate of the ExS impossible. By comparing the absolute peak areas of cholesterol dodecanoate obtained in this experiments to those used for the determination of the recovery rate which were in the same order of magnitude, a recovery rate of almost 95 % has been assumed. Thus, it is also warranted that upon hydrolysis liberated cholesterol revealing a RRT of 1.122 related to 5αC (Fig. 28) should be extracted quantitatively.

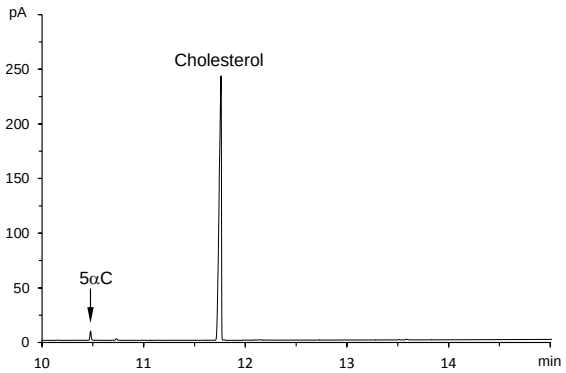

Fig. 28 GC/FID analysis of external reference solution of cholesterol; 5αC = 5α-cholestane

Comparable to CF, cholesteryl cinnamate was not degraded chemically by the respective media used as shown in Fig. 29 A for the incubation with pure THB. Representative for the experiments in the inoculated media, the chromatogramm obtained within the more complex thioglycollate broth is illustrated in Fig. 29 B. Comparing the two chromatograms, it is recognizable that neither substrate-derived cholesterol nor its bacterial transformation products could be detected and that cholesteryl cinnamate remains unchanged upon *in vitro* fermentation with human fecal suspension. Owing to these results, it can be excluded that hydrolysis of dimethylsteryl ferulic acid esters as well as desmethylsteryl cinnamic acid esters by colonic microbiota is solely inhibited by the contained sterol moiety. It is also unlikely that steric hindrance of microbial esterases is exclusively caused by the acid moiety due to the structural similarity of *trans*-ferulic acid and *trans*-cinnamic acid. Presumably, inhibition of hydrolytic enzymes of bacterial origin is based on both the acid and sterol moiety.

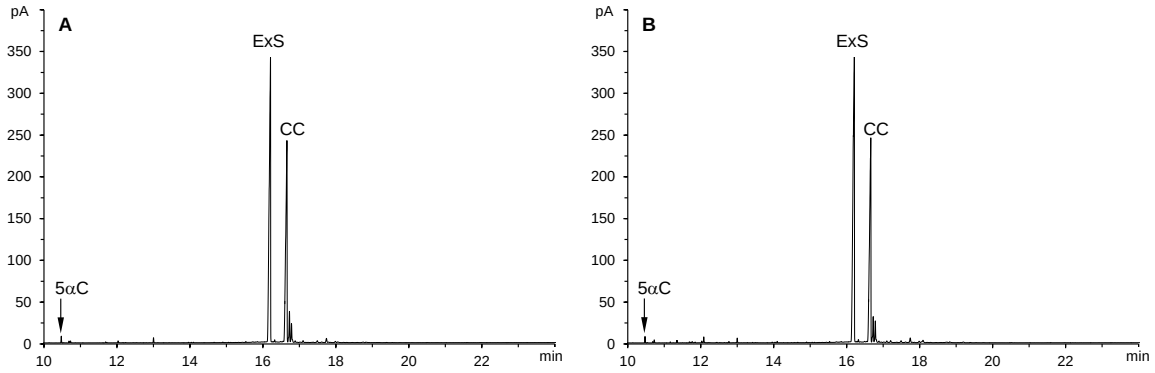

Fig. 29 GC/FID analysis of samples upon *in vitro* fermentation of cholesteryl cinnamate in thioglycollate broth (THB); (A) Blank II; (B) Inoculated THB; ExS = extraction standard (cholesteryl dodecanoate); 5αC = 5α-cholestane; CC = cholesteryl cinnamate

In order to prove whether hydrolysis of steryl esters by gut microbiota is affected synergistically by the sterol and acid residues, **cholesteryl palmitate**, a desmethylsteryl fatty acid ester, was used as a substrate for fermentation in THB (Fig. 30) and nutritive buffer (not illustrated). Comparing the chromatograms of the blank II (Fig. 30 A) and the inoculated substrate suspension (Fig. 30 B) within the fermentation in thioglycollate broth, no cholesterol could be detected as a proof of hydrolysis upon 5 days of incubation. Furthermore no shift in the ratios of

cholesteryl palmitate to the IS and ExS has been observed. In conclusion, CP was obviously not degraded by the gut microbiota under the employed *in vitro* fermentation conditions. Considering that steryl fatty acid esters *in vivo* usually do not reach the colon in intact form based on the efficient hydrolysis by digestive enzymes already within their small intestine passage, the gut microorganisms might not be equipped with hydrolytic enzymes accepting steryl esters as substrates.

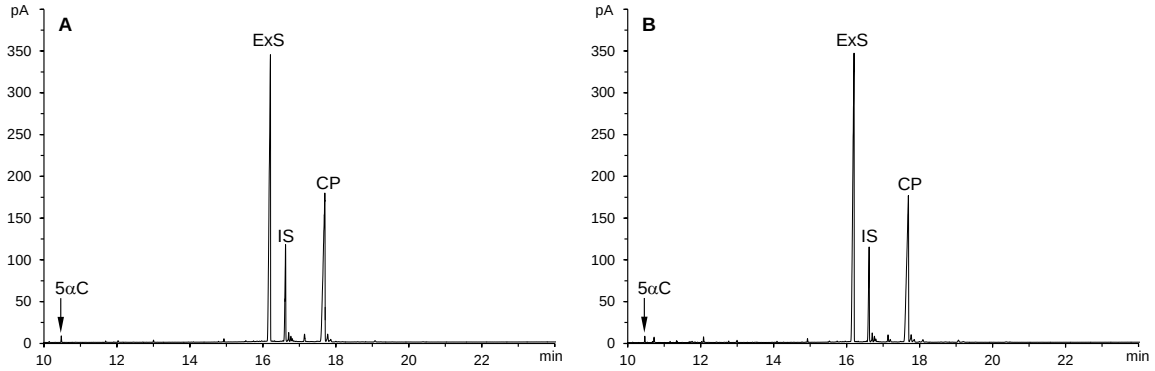

Fig. 30 GC/FID analysis of samples upon *in vitro* fermentation of cholesteryl palmitate in thioglycollate broth (THB); (A) Blank II; (B) Inoculated THB; ExS = extraction standard (cholesteryl dodecanoate); 5αC = 5α-cholestane; CP = cholesteryl palmitate

Finally, employing **ethyl ferulate** (EF) as substrate it was investigated whether the fecal microbiota used showed hydolytic activity, in general. Ethyl ferulate was chosen due to CHEESON *et. al* [260] who described that besides dehydrodimers of ferulic acid also methyl ferulate was metabolized under anaerobic conditions with buffered suspensions of human fecal microorgansims upon *in vitro* incubations for periods up to 72 h. Ethyl ferulate not only exhibits a lower structural complexity, but also is less lipohilic than the previously employed steryl esters. Thus, a potential impact on metabolism based on the hydrophobicity of the substrate used could be expected. Ferulic acid and the respective metabolites identified upon fermentation of *trans*-ferulic acid using inoculated NB and THB (sections 4.2.3.1 & 4.2.3.2) were taken as biomarkers for hydrolysis of ethyl ferulate. Liquid-liquid extraction of fermentation batches was performed after adjustment of a pH ≤ 2 (Fig. 15 B). The mean recovery rate of ExS (syringic acid) amounted to 98 % (Tab. 7).

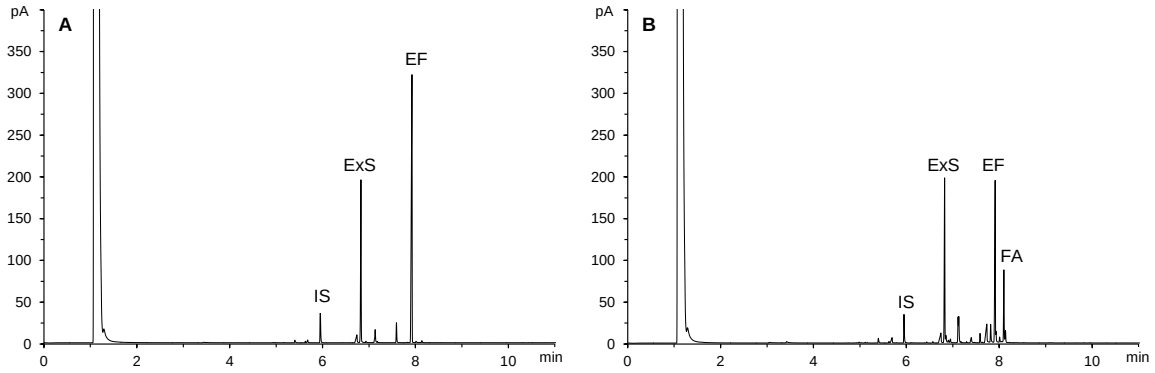

Fig. 31 GC/FID analysis of microbial conversion products obtained upon *in vitro* fermentation of ethyl ferulate (EF) in nutritive buffer medium inoculated with human feces; (A) Blank II; (B) Incubated sample; IS = internal standard (vanillic acid); ExS = extraction standard (syringic acid); FA = ferulic acid

Results obtained for the incubation of EF with inoculated NB are illustrated in Fig. 31. As indicated by the blank II (Fig. 31 A), hydrolysis of ethyl ferulate due to chemical reaction conditions can be excluded because there were no peaks detectable besides the substrate, vanillic acid (IS) and syringic acid (ExS). Upon fermentation for 5 days, ferulic acid has been identified in the inoculated batches demonstrating a least hydrolysis of ethyl ferulate to a certain degree by colon microorganisms *in vitro* (Fig. 31 B).

Respective chromatograms resulted from experiments using thioglycollate broth are given in Fig. 32. Comparable to the fermentations in NB it can be ruled out that ethyl ferulate was subjected to chemical alterations (Fig. 32 A). As illustrated in Fig. 32 B, ethyl ferulate was hydrolyzed almost completely by the gut microbiota cultivated in thioglyocollate broth. Metabolites identified as MPA and DHCA were identical to the conversion products with the exception of HPA detected for the fermentation of *trans*-ferulic acid using inoculated THB (section 4.2.3.2). In addition to the known metabolites, a prominent peak (X) was detected at a RRT of 0.410 related to vanillic acid. It can be assumed this peak is probably another aromatic acid related to the microbial transformation of ethyl ferulate or intermediately formed FA, DHCA or MPA. A conclusive identification of this substance could not be achieved during the present study. Based on the RRF of dihydrocinnamic acid (5.58) and 3-(3-methoxyphenyl) propionic acid (1.57) and determination of the concentrations, the proportions of DHCA and MPA in repeated incubation experiments were 57 – 59 % and 41 – 43 %, respectively.

However, compared to the proportions of DHCA and MPA determined upon *trans*-ferulic acid fermentations using analogous conditions (section 4.2.3.2) the ratio of the two metabolites shifted for in favour of MPA in these experiments. Due to the hypothesis that dihydrocinnamic acid might be the end-product of microbial conversion under these conditions (Fig. 25), the diminished amount of DHCA after fermentation of EF is presumably attributed to ester hydrolysis representing the rate-determining step of the metabolization. Thus, the microbial converison of *trans*-FA using inoculated THB (Fig. 25) does not proceed before ferulic acid is liberated from respective esters. An increasing amount of the intermediate MPA also appears to be a result from prolonged ester hydrolysis.

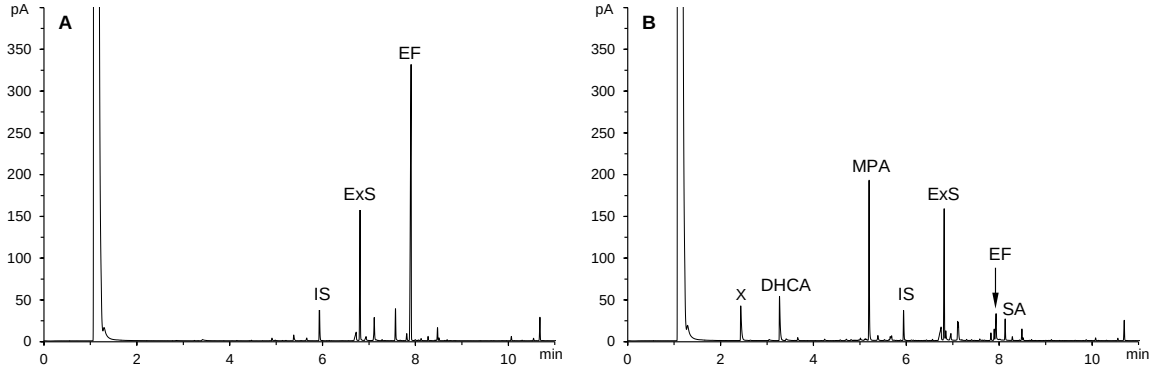

Fig. 32 GC/FID analysis of microbial conversion products obtained upon *in vitro* fermentation of ethyl ferulate (EF) in thioglycollate broth inoculated with human feces; (A) Blank II; (B) Incubated sample; IS = internal standard (vanillic acid); ExS = extraction standard (syringic acid); DHCA = dihydrocinnamic acid; MPA = 3-(3-methoxyphenyl)propionic acid; SA = stearic acid; X = not identified

In analogy to the *in vitro* fermentation experiments employing *trans*-ferulic acid as substrate (section 4.2.3), the metabolic fate of ethyl ferulate also differs in the two media used. Using inoculated nutritive buffer, EF was only partially hydrolyzed and the liberated ferulic acid was not subjected to microbial transformations whereas hydrolysis upon incubation with inoculated THB was virtually complete and liberated FA was not detectable due to conversion to the mentioned metabolites. Varying biological activities of the microorganisms or different compositions of the microflora cultivated in the two distinguished media (NB and THB) may be the reasons for these differences.

4.3 CONCLUSIONS

In conclusion, the presented *in vitro* fermentation studies using freshly voided human feces as source of microbiota indicate that colonic microorgansims do not accept steryl esters of any kind as substrates for *in vitro* metabolization in terms of ester hydrolysis. Whilst the suitability of the experimental setup has been verified, only ethyl ferulate exhibiting a considerably simpler molecular structure than steryl esters was hydrolyzed by the gut microbiota upon incubation in the two media. It can be assumed that the more complex steryl esters do not fit to the active center of the currently unspecified esterases. However, the inability of enzymes to hydrolyze steryl esters is probably not the only explanation. Although all substrates have been provided as suspensions containing Tween® 80 as emulsifying agent, their bioavailability might still be influenced by the hydrophobicity of the respective substrates. It cannot be ruled out that due to the higher hydrophobicity of the steryl esters compared to ethyl ferulate the accessibility in the aqueous fermentation media might be diminished, with the result that enzyme-substrate complexes are not formed. In the end, it has been proven that metabolization of substrates by human colonic microbiota is dependent on the conditions applied at least *in vitro*. Furthermore, it has to be taken into consideration that *in vitro* studies usually cannot imitate exactly the *in vivo* situation. For example, to date only 30 – 40 % of the bacterial species present in human colonic environment could be cultivated using varying selective media [28].

5 HUMAN STUDY: METABOLIC FATE OF INDIVIDUAL PHYTOSTERYL/-STANYL ESTERS UPON DIGESTION

5.1 INTRODUCTION

Increased plasma TC and LDL-C levels are established risk factors for coronary heart diseases. The cholesterol-lowering properties of phytosterols/–stanols and their fatty acid esters in humans have been described in several studies [2-7]. Similar effects on serum cholesterol have been reported for phytosteryl/-stanyl ferulic acid esters in animals [215, 220] and humans [8-11] using γ-oryzanol, a ferulate mixture obtained from rice bran. Several functional foods (e.g. skimmed milk drinking yogurts, margarines) enriched with fatty acid esters of phytosterols and –stanols are currently available on the EU market [197].

The cholesterol-lowering action of these products is closely associated with the intestinal and hepatic metabolism shared by cholesterol and phytosterols/-stanols [33, 69, 86]. The inhibition of intestinal cholesterol absorption by competitive incorporation into dietary mixed micelles appears to play a major role [93-95]. Recently published *in vitro* data demonstrated that micellar cholesterol solubilization is not affected by intact phytosteryl fatty acid esters and that intact esters are not solubilized into micelles in a model bile, mixed-micelle system [94]. Therefore, intestinal hydrolysis of these esters by digestive enzymes seems to be a crucial step for the cholesterol-lowering effects of dietary phytosteryl/-stanyl esters.

In vitro data indicate that the rate of intestinal hydrolysis, e.g. by pancreatic cholesterol esterase, depends on both the phytosterol/-stanol structure and the acid moiety [12]. Feeding stearates of sitosterol, sitostanol and stigmasterol to hamsters confirmed differences in hydrolysis rates depending on the sterol/stanol moiety [16]; the results obtained in rats fed either phytosteryl/stanyl oleates or stearates indicate an impact of the acyl residue [17]. *In vitro* studies focussing on the enzymatic hydrolysis of phytosteryl/-stanyl ferulic acid esters by different digestive enzymes consistently demonstrate that solely desmethylsteryl ferulates (e.g. campesteryl ferulate) are accepted as substrates; 4,4-dimethylsteryl ferulic

acid esters such as cycloartenyl ferulate were not hydrolyzed by the enzymes employed [13-15].

In human trials involving duodenal infusion [21-23] or oral administration of phytosteryl/-stanyl fatty acid esters [18-20], the total fatty acid ester hydrolysis has either been calculated indirectly by determination of the intact ester fraction based on the quantification of free phytosterol/-stanol contents before and after saponification [18, 19, 21-23] or the data reported referred to total esters [20]. Data on the hydrolysis of individual phytosteryl/-stanyl esters and a consideration of the impact of their molecular structures are lacking. In addition, oral administration trials have been confined to ileostomy patients [18-20].

Therefore, the objective of the human study was to investigate the metabolic fate of individual phytosteryl/-stanyl fatty acid and ferulic acid esters upon consumption and digestion by healthy human subjects. A GC-based methodology developed for the quantification of individual intact phytosteryl/-stanyl esters in enriched foods [224] was adapted to the analysis of feces. In combination with an efficient isolation procedure, this approach was used to follow a randomized human trial and thus to allow the simultaneous quantification of intact esters, liberated phytosterols/-stanols and their metabolites. As examples for commercially available functional food products, skimmed milk drinking yogurts enriched with complex mixtures of phytosteryl/-stanyl esters (section 3.1.3) were employed as food carriers of the target substrates.

5.2 STUDY POPULATION

Fifteen male subjects (age: 28 ± 3 y (range: 22 - 33 y); BMI [kg/m^2]: 23.6 ± 1.6 (range: 21.6 - 25.7)) volunteered to participate in the study. All candidates gave written informed consent to participate; the consent form and the study design were approved by the ethics committee of the Faculty of Medicine of the Technische Universität München (#2943/10). According to personal history, all participants were non-smokers, non-vegetarians and free of chronic metabolic diseases. They reported not to have used antibiotics or statins for at least six months before the beginning of the study.

5.3 EXPERIMENTAL PROTOCOL

A randomized, single-blind three group cross-over clinical study lasting for seven weeks was performed. Healthy volunteers were given three skimmed milk drinking yogurts enriched with different phytosteryl/-stanyl ester mixtures. The participants were asked to consume the yogurts once a day for a period of three days besides their habitual diet; they were also advised not to consume other phytosterol-enriched products. Each treatment period, including sample collection, lasted for 6 days; wash-out periods of fifteen days were used between the interventions (Fig. 33).

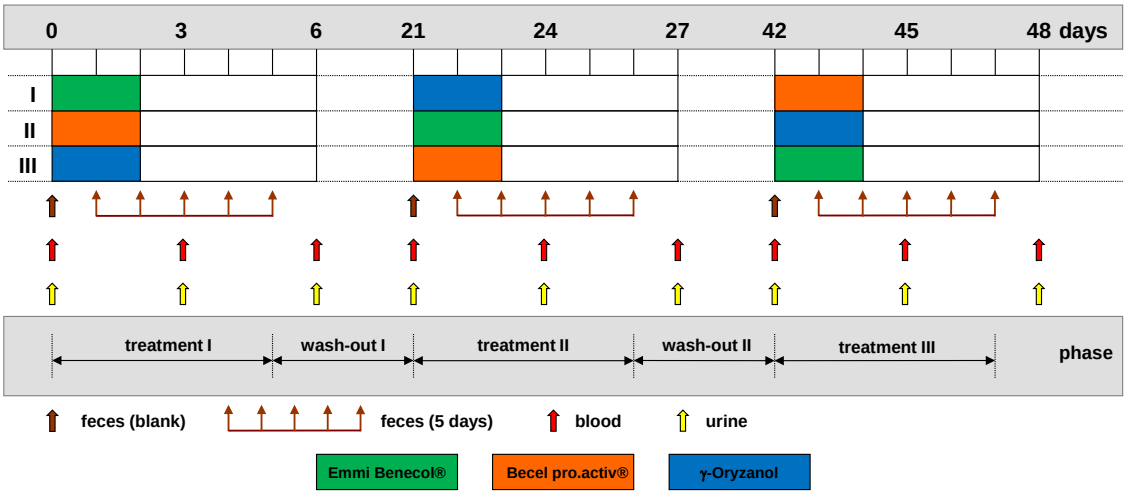

Fig. 33 Study design

5.4 SAMPLE COLLECTION

5.4.1 FECES SAMPLES

The entire daily feces was collected by the participants in plastic containers equipped with lockable sterile freezer bags at baseline and during 5 days of each treatment period starting with the second day of application (day 1, 22, 42) (Fig. 33). The individual daily feces samples were delivered to the laboratory, deep-frozen and stored at -35 °C until sample preparation.

5.4.2 PLASMA SAMPLES

During each of the three intervention periods overnight fasting blood samples (S-Monovette® EDTA K_2-Gel, SARSTEDT, Germany) were taken from subjects at baseline (day 0, 21, 42), at the end of consumption (day 3, 24, 45) and after six days (day 6, day 27, day 48) (Fig. 33). Plasma was separated from blood by centrifugation at 3000 g for 10 min at 20 °C (Eppendorf centrifuge 5702R, Germany) and stored at -35 °C until further analysis.

5.4.3 URINE SAMPLES

Urine samples for a period of 24 hours were collected by the volunteers on days 0, 3 and 6 of each treatment period (Fig. 33) using UriSet 24 (SARSTEDT, Germany) a complete set for standardized 24-hour urine collection. Aliquots of 200 mL were taken out of the collective sample by the subjects and when received in the laboratory deep-frozen and stored at -35 °C until further analysis.

5.5 RESULTS AND DISCUSSION

5.5.1 FECES ANALYSIS

The employed capillary gas chromatographic separation allowed the simultaneous determination of individual intact phytosteryl/-stanyl fatty acid and ferulic acid esters, of phytosterols and –stanols liberated upon hydrolysis and of fecal metabolites in a single GC run. Typical chromatograms obtained from the Benecol®-, the pro.activ®- and the γ-oryzanol-intervention, respectively, are shown in Fig. 34. The two regions of interest - (I) liberated sterols/stanols and microbial transformation products and (II) intact phytosteryl/-stanyl esters - are depicted at enlarged scale. Identifications were based on GC/MS; peak assignments and relative retentions times of fecal intact esters are given in Tab. 9.

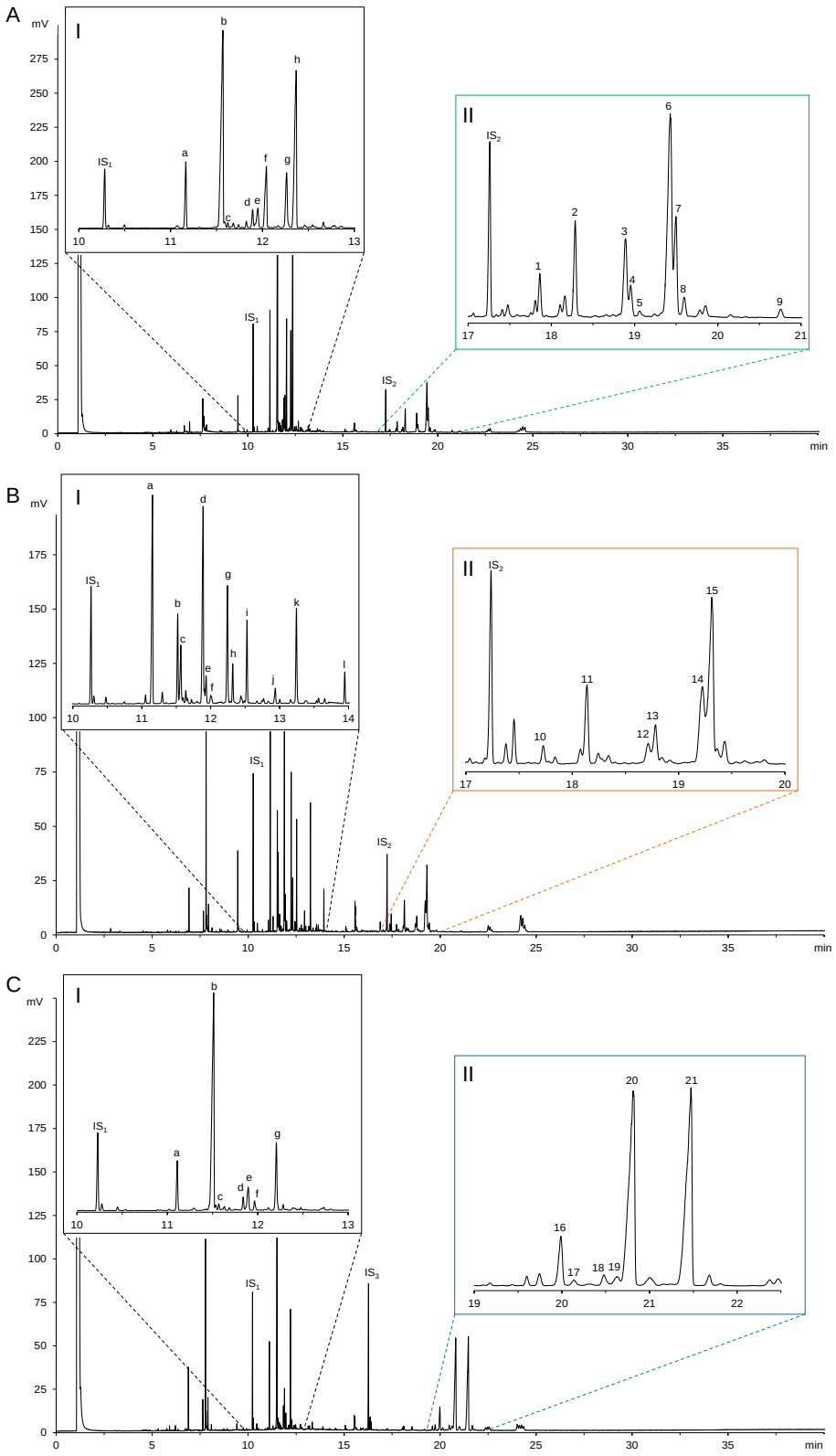

Fig. 34 Capillary gas chromatographic separations of intact phytosteryl/-stanyl esters, upon digestion released sterols/stanols and microbial transformation products in human feces after oral administration of skimmed milk drinking yogurts enriched with phytosteryl/-stanyl esters; (A) Benecol®, (B) pro.activ® and (C) γ-Oryzanol; (I) Steroids: a = coprostanol; b = cholesterol; c = 24-methylcoprostanol; d = 24-ethylcoprostanol; e = campesterol; f = campestanol; g = sitosterol; h = sitostanol; i = coprostanone; j = 24-methylcoprostanone; k = 24-ethyl-coprostanone; l = 24-ethylcholest-4-en-3-one; (II) Intact phytosteryl/-stanyl esters; IS_1 = 5α-cholestane, IS_2 = cholesteryl palmitate and IS_3 = cholesteryl cinnamate; peak numbering according to Tab. 9

5.5.1.1 GC/FID calibration and validation data

GC/FID calibration for quantitation of individual intact esters in feces was performed on the basis of linear regression analysis in coordinate ratios of peak areas (individual intact ester / IS) and amounts (individual intact ester / IS). As examples for the three types of phytosteryl/stanyl esters consumed with the skimmed milk drinking jogurts, linear regression analysis of sitostanyl-C18:1 (Benecol®), sitosteryl-C18:2 (pro.activ®) as well as 24-methylenecycloartenyl ferulate (γ-oryzanol) representing major compounds, are illustrated in Fig. 35.

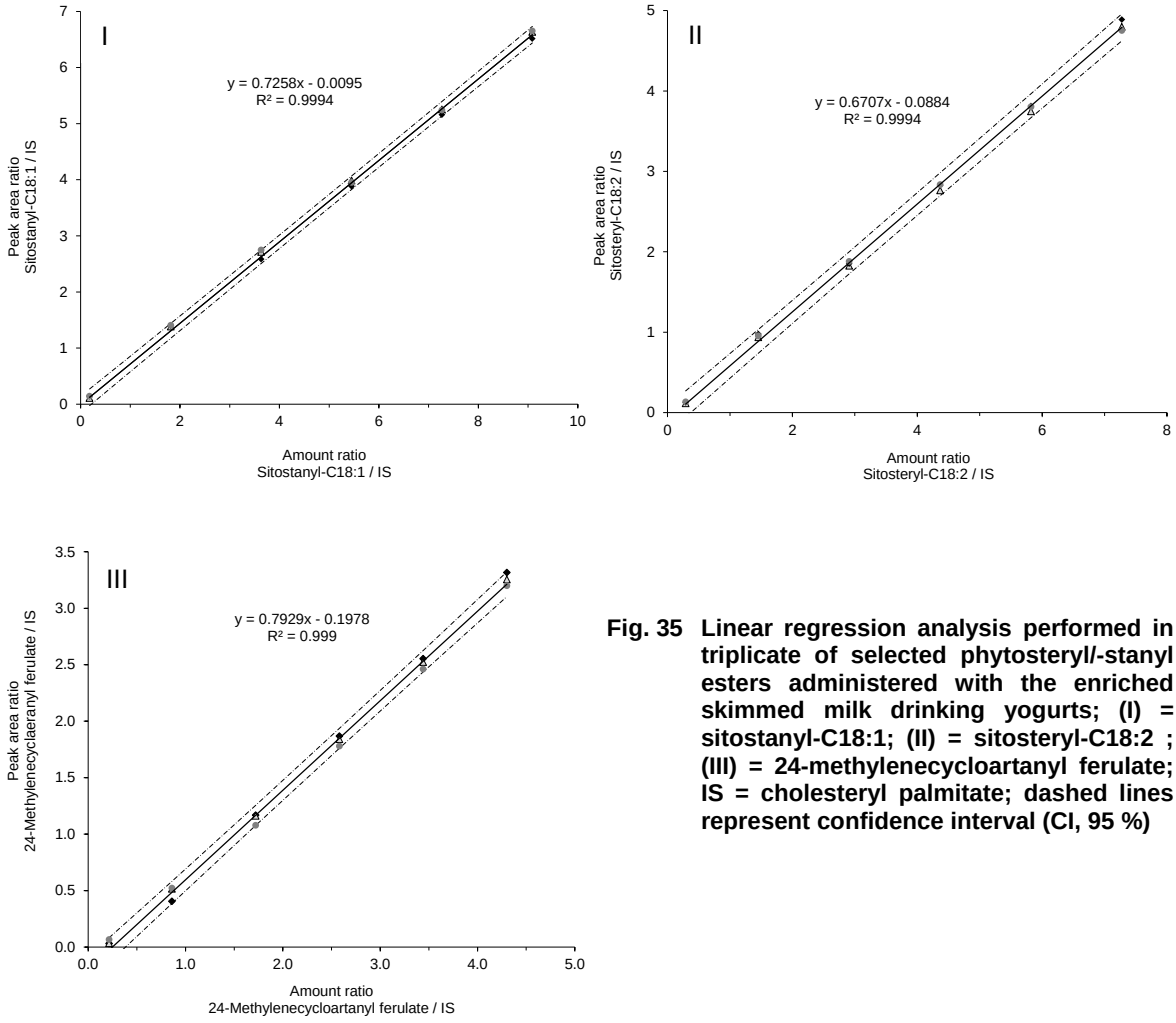

Fig. 35 Linear regression analysis performed in triplicate of selected phytosteryl/-stanyl esters administered with the enriched skimmed milk drinking yogurts; (I) = sitostanyl-C18:1; (II) = sitosteryl-C18:2 ; (III) = 24-methylenecycloartanyl ferulate; IS = cholesteryl palmitate; dashed lines represent confidence interval (CI, 95 %)

Across all calibrated esters the coefficients of correlation ranged between 0.9954 (sitostanyl-C20:0) and 0.9999 (cycloartenyl ferulate). On the basis of

calibrations performed in triplicate, analytical validation data (Tab. 9) for individual intact esters were calculated according to equations 1 – 5 (section 3.2.4.1.1).

Tab. 9 Relative retention times (RRT), limits of detection (LOD), limits of quantitation (LOQ) and coefficients of variation (CV) of individual phytosteryl/-stanyl esters determined on the basis of the linear regression methodology according to [229]

No[a]	Phytosteryl/-stanyl ester	RRT	LOD	LOQ	CV
		[min]	[µg/100 mg dry feces]		[%]
Stanyl fatty acid ester[b]					
1	Campestanyl-C16:0	1.045[e]	0.50	1.37	4.6
3	Campestanyl-C18:1	1.096[e]	1.50	3.90	2.3
4	Campestanyl-C18:2	1.100[e]	1.96	5.54	5.4
5	Campestanyl-C18:3	1.106[e]	2.11	5.27	5.5
2	Sitostanyl-C16:0	1.060[e]	0.72	2.07	2.8
6	Sitostanyl-C18:1	1.128[e]	2.75	6.89	2.8
7	Sitostanyl-C18:2	1.131[e]	4.83	10.26	5.3
8	Sitostanyl-C18:3	1.138[e]	9.70	24.06	4.8
9	Sitostanyl-C20:0	1.206[e]	4.41	11.30	5.0
Steryl fatty acid ester[c]					
10	Campesteryl-C16:0	1.029[e]	0.99	2.70	4.8
12	Campesteryl-C18:1	1.090[e]	1.00	2.70	5.3
13	Campesteryl-C18:2	1.094[e]	1.94	5.28	3.2
11	Sitosteryl-C16:0	1.053[e]	0.44	0.83	5.8
14	Sitosteryl-C18:1	1.117[e]	1.81	3.54	6.6
15	Sitosteryl-C18:2	1.123[e]	4.17	8.39	3.5
Steryl/stanyl ferulic acid ester[d]					
16	Campesteryl ferulate	1.235[f]	15.34	43.66	3.6
17	Campestanyl ferulate	1.244[f]	2.57	7.08	4.0
19	Sitosteryl ferulate	1.274[f]	6.93	18.70	4.2
18	Cycloartanyl ferulate	1.266[f]	2.64	7.48	4.0
20	Cycloartenyl ferulate	1.288[f]	70.67	199.12	3.4
21	24-Methylenecycloartanyl ferulate	1.326[f]	83.29	236.50	4.7

[a] Peak numbers correspond to Fig. 34

[b] Phytostanyl fatty acid ester mixture (STAEST-115)

[c] Phytosteryl fatty acid ester mixture (Vegapure® 95E)

[d] Phytosteryl/-stanyl ferulic acid ester mixture (γ-orzanol)

[e] Relative retention time compared to cholesteryl palmitate (IS_2)

[f] Relative retention time compared to cholesteryl cinnamate (IS_3)

The corresponding analytical characteristics for the ester-derived phytosterols/-stanols were determined in the same way (Tab. 10). Due to the lack of commercial reference substances, the LODs and LOQs calculated for campesterol and sitosterol also served as the basis for the RRF-based quantitation of the corresponding fecal metabolites 24-methylcoprostanol, 24-methylcoprostanone, 24-methylcholest-4-en-3-one and 24-ethylcoprostanol, 24-ethylcoprostanone as well as 24-ethylcholest-4-en-3-one. Similarly, analytical characteristics determined for cycloartenol were used for cycloartanol.

Tab. 10 Relative retention times (RRT) compared to 5α-cholestane, coefficients of correlation (R^2), limits of detection (LOD), limits of quantitation (LOQ) and coefficient of variation (CV) of liberated phytosterols/-stanols sterols determined on the basis of the linear regression methodology according to [229] by means of GC/FID

Phytosterol/-stanol	RRT	R^2	LOD	LOQ	CV
	[min]		[µg/100 mg dry feces]		[%]
Campesterol	1.162	0.9973	0.17	0.40	5.7
Campestanol	1.170	0.9967	0.11	0.31	6.7
Sitosterol	1.192	0.9972	0.16	0.29	5.8
Sitostanol	1.200	0.9979	0.11	0.28	5.5
Cycloartenol	1.258	0.9981	0.15	0.36	7.6
24-methylenecycloartanol	1.290	0.9940	0.24	0.64	7.9

5.5.1.2 Repeatability and reproducibilty of determinations

The combination of the isolation procedure (Fig. 16) with the GC/FID analysis resulted in high recovery rates (92.9 ± 5.6 %, 99.5 ± 5.0 % and 98.1 ± 2.2 %) of the internal standards (5αC, cholesteryl palmitate and cholesteryl cinnamate) across all subjects in the three study periods. Based on the analysis of feces of one of the participants in the Benecol®-exposure, data on the repeatability and reproducibility of the employed method are presented in Tab. 11. The high repeatability is reflected by very low standard deviations of the results obtained by triplicate analysis of the three feces aliquots at each point of analysis. The reproducibility of the results was demonstrated by repeated analysis of the feces in a time-dependent manner. Relative standard deviations calculated across the three time points of analysis ranged between 3.3 % (campestanyl-C16:0) and 7.3 % (sitostanyl-C20:0) for intact phytostanyl esters and between 5.6 %

(campestanol) and 6.0 % (sitostanol) for liberated phytostanols. They demonstrate a well functioning and robust analytical methodology. In addition, changes in the composition of fecal intact esters and liberated sterols due to microbial activity during storage of the samples can be excluded.

Tab. 11 Reproducibility and repeatability data on the analysis of individual intact stanyl esters and liberated phytostanols in feces upon consumption of Emmi Benecol® drinking yogurt

Stanyl fatty acid ester	Point of analysis		
	Day 1	Day 28	Day 56
	[mmol/dry weight][a]		
Campestanyl-C16:0	0.143 ± 0.004	0.141 ± 0.004	0.137 ± 0.004
Campestanyl-C18:1	0.433 ± 0.013	0.412 ± 0.012	0.431 ± 0.012
Campestanyl-C18:2	0.119 ± 0.004	0.126 ± 0.005	0.131 ± 0.005
Campestanyl-C18:3	0.045 ± 0.001	0.043 ± 0.002	0.045 ± 0.002
Sitostanyl-C16:0	0.380 ± 0.011	0.364 ± 0.011	0.366 ± 0.014
Sitostanyl-C18:1	1.514 ± 0.048	1.395 ± 0.034	1.517 ± 0.041
Sitostanyl-C18:2	0.450 ± 0.014	0.450 ± 0.011	0.469 ± 0.015
Sitostanyl-C18:3	0.153 ± 0.004	0.140 ± 0.003	0.147 ± 0.004
Sitostanyl-C20:0	0.089 ± 0.002	0.085 ± 0.004	0.092 ± 0.006
Campestanol	2.085 ± 0.066	2.061 ± 0.071	2.064 ± 0.052
Sitostanol	6.630 ± 0.220	6.511 ± 0.245	6.574 ± 0.200

[a] Values represent mean ± SD; three aliquots of combined feces of one subject were worked up in triplicate on each time point, respectively

5.5.1.3 Subject compliance and acquisition of feces data

Fourteen of the intially included fifteen participants completed the study and demonstrated excellent compliance with the study protocoll based on substrate consumption and especially collection and delivery of feces samples. Across the three intervention periods all volunteers had daily bowel movements. Individual data on daily feces weights and the corresponding dry matter contents during the three treatment periods are illustrated in (Fig. 36). None of the volunteers reported to have diarrhea (medically defined as more than three passages of loose or liquid stools per day [261]). For the entire study population the mean weights of daily feces of 145.7 ± 50.6 g (Benecol®), 140.1 ± 49.3 g (pro.activ®) and 139.5 ± 58.9 g (γ-oryzanol) as well as the corresponding feces dry weights of 25.5 ± 4.6 %,

25.3 ± 5.4 % and 26.0 ± 4.8 % were within ranges reported for healthy humans [262, 263].

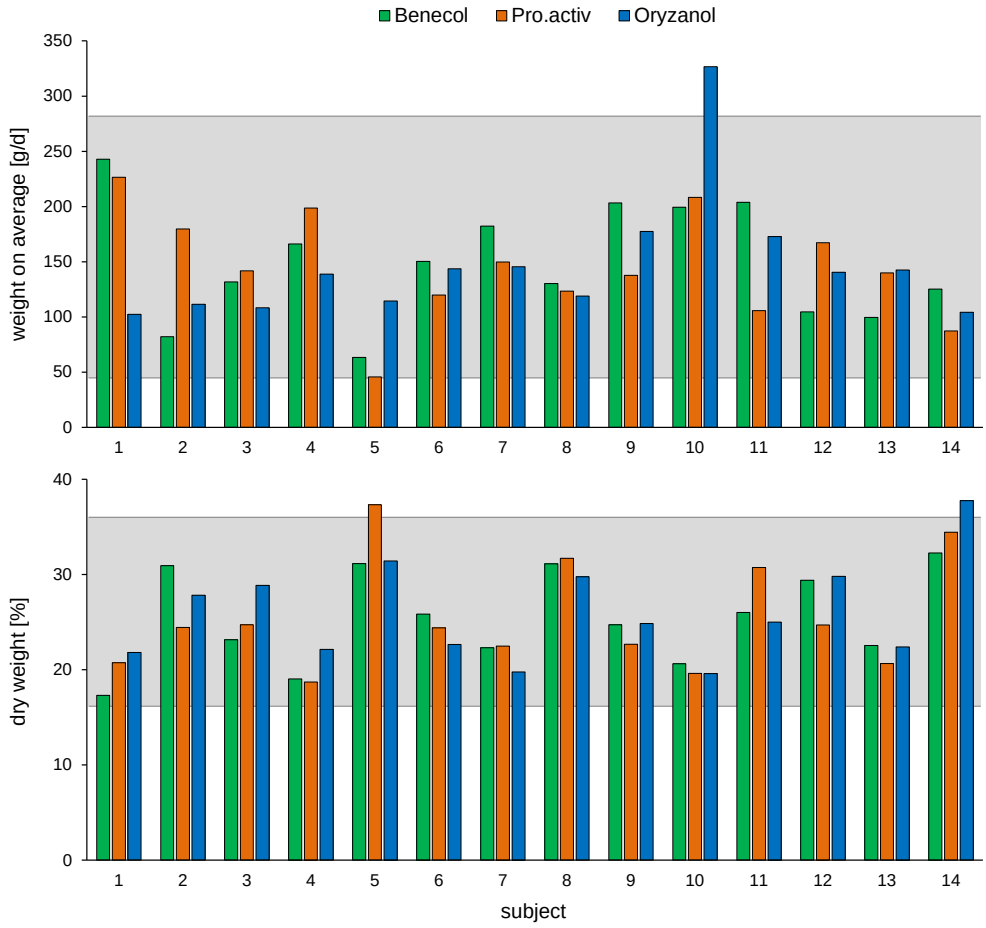

Fig. 36 Individual data on averaged daily weights and dry matter contents of feces excreted by fourteen subjects during the three intervention periods; physiological ranges according to [262, 263] are shaded in gray

5.5.1.4 Simultaneous determination of intact esters and liberated phytosterols/-stanols

A comparison of the amounts of consumed phytosteryl/-stanyl esters with the amounts of intact esters and liberated sterols/stanols recovered in the feces is shown in Tab. 12. In the Benecol®-trial average hydrolysis rates, calculated on the basis of the intact esters recovered in the feces, were 73.2 ± 3.3 % for campestanyl fatty acid esters and 72.6 ± 3.4 % for sitostanyl fatty acid esters. In the pro.activ®-trial the hydrolysis rates calculated for campesteryl and sitosteryl fatty acid esters amounted to 80.2 ± 3.3 % and 79.4 ± 3.7 %, respectively. Average total recoveries, i.e. the sum of intact esters and liberated phytosterols/-

stanols in feces, ranged from 68.2 ± 4.5 % for campesteryl fatty acid esters to 77.0 ± 4.9 % for sitostanyl fatty acid esters. These hydrolysis rates are only slightly lower than those calculated on the same basis for total phytostanyl fatty acid esters (86 %) and phytosteryl fatty acid esters (88 %) after small intestine transit for seven ileostomy patients [20].

Tab. 12 Amounts of consumed esters (ConEs) and recoveries (Rec) of intact esters and liberated phytosterols and -stanols in human feces upon consumption of enriched skimmed milk yogurt drinks (n = 14)

Substrate	ConEs	Feces			
		Intact esters	Free sterol/stanol	Total	Rec
	[mmol]	[mmol/dry weight][a]			[%]
Benecol®					
Campestanyl esters	3.637	0.974 ± 0.122	1.788 ± 0.150	2.762 ± 0.193	75.9
Sitostanyl esters	10.788	2.953 ± 0.789	5.349 ± 0.450	8.302 ± 0.908	77.0
Pro.activ®					
Campesteryl esters	2.220	0.439 ± 0.072	1.076 ± 0.057[b]	1.515 ± 0.092	68.2
Sitosteryl esters	10.025	2.061 ± 0.373	5.588 ± 0.246[c]	7.649 ± 0.447	76.3
γ-Oryzanol					
Campesteryl ferulate	1.456	1.082 ± 0.060	0.269 ± 0.020[b]	1.351 ± 0.050	92.8
Campestanyl ferulate	0.173	0.146 ± 0.007	0.032 ± 0.007	0.178 ± 0.010	102.8
Sitosteryl ferulate	0.491	0.362 ± 0.021	0.154 ± 0.018[c]	0.516 ± 0.028	105.1
Cycloartanyl ferulate	0.215	0.181 ± 0.010	n.d.[d]	0.181 ± 0.010	84.2
Cycloartenyl ferulate	6.386	5.362 ± 0.287	n.d.[d]	5.362 ± 0.287	84.0
24-Methylenecycloartanyl ferulate	7.100	5.899 ± 0.329	n.d.[d]	5.899 ± 0.329	83.1

[a] Values represent mean ± SEM; content in feces samples related to dry matter; the combined feces samples (5 days) of individual subjects were divided into three aliquots and worked up in triplicate

[b] Value represents the sum of campesterol and fecal metabolites (24-methylcoprostanol, 24-methylcoprostanone and 24-methylcholest-4-en-3-one)

[c] Value represents the sum of sitosterol and fecal metabolites (24-ethylcoprostanol, 24-ethylcoprostanone and 24-ethylcholest-4-en-3-one)

[d] Below limits of detection determined according to DIN 32645 (2008-11) [229]; LOD [µg/100 mg dry feces]: cycloartanol/cycloartenol = 0.15 , 24-methylenecycloartanol = 0.24

In all other available consumption studies, hydrolysis rates have been derived based on the amounts of excreted phytosterols/-stanols before and after saponification of the esterified fraction. Administration of phytostanyl fatty acid esters-enriched margarine in eleven colectomized patients revealed 90 % hydrolysis [18]. Almost 60 % of sitostanyl and campestanyl fatty acid esters

supplied in fat-free pastilles were hydrolyzed during gastrointestinal passage in nine colectomized patients when consumed with a normal fat diet [19]. Intubation studies in humans with a normal intestinal tract described hydrolysis rates of 39 – 67 % for stanyl esters and 23 – 42 % for steryl esters during their 50 – 60 cm duodenojejunal transit [21-23].

Using γ-oryzanol as substrate, the present study provides for the first time data on the metabolic fate of phytosteryl/-stanyl ferulic acid esters upon human digestion. Almost 80 % of the consumed ferulates were recovered in intact form in the feces (Tab. 12). The hydrolysis rates determined for the desmethylsteryl/-stanyl ferulic acid esters (campesteryl ferulate: 25.7 ± 3.2 %, campestanyl ferulate: 16.2 ± 2.7 %, sitosteryl ferulate: 26.3 ± 3.4 %) were significantly lower than those of the fatty acid esters. The average total recovery (intact esters and sterols/stanols) for these substrates was 100 %. For dimethylsteryl ferulates, no hydrolysis products could be detected; the recovery rates of the intact dimethylsteryl ferulates were approximately 84 %. This is comparable to results obtained after feeding labelled γ-oryzanol to rabbits and rats as well as to *in situ* experiments using isolated rat ileum [221, 222]; upon oral administration about 10 % of the radioactive dose referable to ferulic acid and its metabolites was excreted in urine and almost 85 % was recovered in feces. The data are also in agreement with hydrolysis rates for γ-oryzanol (exhibiting a composition comparable to the substrate employed in the present study) by cholesterol esterases from bovine and porcine between 5 – 10 % [15]. In an earlier *in vitro* study employing the same bovine cholesterol esterase, hydrolysis rates ranged from 34 to 56 % [14]; however, in this study no information on the composition of the γ-oryzanol used as substrate was provided.

Individual data of the fourteen subjects regarding the recoveries of phytosteryl/-stanyl fatty acid esters and liberated sterols/stanols in the feces are presented in Tab. 13 and Tab. 14. The data demonstrate large inter-individual variability of the hydrolysis rates. For campestanyl esters the hydrolysis rates ranged from 43 % (subject 7) to 88 % (subject 3) and for campesteryl esters from 53 % (subject 10) to 96 % (subject 5). It is noteworthy that the same subjects also exhibited the lowest and highest hydrolysis rates, respectively, for the sitostanyl and sitosteryl esters. This might be explained by both subject-dependent

differences in the activities of digestive enzymes as well as by effects of the habitual and non-controlled diets of the volunteers on the bioavailability of the employed steryl/stanyl fatty acid esters. The range of hydrolysis rates reported for ileostomy patients (75 – 96 %) despite controlled dietary conditions confirm the dependence on the individual digestive system [20]. An impact of the type of administration, in particular the dietary fat content, was indicated by a study performed in eight colectomized patients in which phytostanyl fatty acid esters supplied in fat-free pastilles were hydrolyzed to a greater extent (70 %) when consumed with a normal fat diet rather than with a low fat diet (40 %) [19].

In a previous study, hydrolysis rates calculated by comparing the amounts of intact esters recovered in the ileostomy excreta to those consumed were only marginally lower than hydrolysis rates calculated by comparison of the amounts of recovered intact esters to the sum of esterified and free sterols/stanols in the excreta [20]. In contrast, in the present study the hydrolysis rates calculated on the basis of the latter method were considerably lower than those calculated by comparing the amounts of intact esters recovered in the feces to those consumed (for phytostanyl fatty acid esters: 65 % vs. 86 %; for phytosteryl fatty acid esters: 73 % vs. 80 %).

Surprisingly, the employed quantitation revealed that the amounts of liberated phytosterols and -stanols actually determined in the feces did not necessarily correlate to the amounts expected from the hydrolysis rates calculated on the basis of recovered intact esters(Tab. 13 and Tab. 14). On average, only 68.5 % of the amounts of sterols/stanols expected from the amounts of remaining intact esters were found; among the subjects the levels ranged, for example, from 27.7 % to 99.4 % for campestanol and from 32.0 % to 99.4 % for campesterol.

As a result of these two non-correlating sources of inter-individual variability, the total recoveries, i.e. the sum of intact esters and liberated phytosterols/-stanols in feces, also exhibited strong inter-individual variability, ranging from 32.8 to 99.9 % in the Benecol®-exposure and from 36.9 to 102.5 % in the pro.activ®-trial. In consequence, the overall recoveries of fecal intact esters and released phytosterols/-stanols in healthy humans did not reflect the amount of respective esters consumed (Tab. 13 and Tab. 14).

Tab. 13 Individual data on fecal recovered intact esters and liberated phytostanols within the Benecol®-trial

Subject	Campestanyl esters						Sitostanyl esters					
	Intact esters		Stanol		Total		Intact esters		Stanol		Total	
	[mmol]	[%]	[mmol]	[%]	[mmol]	[%]	[mmol]	[%]	[mmol]	[%]	[mmol]	[%]
Substrate Feces[a]	3.637						10.788					
1	0.694 ± 0.067	19.1[b]	2.077 ± 0.102	70.6[c]	2.771 ± 0.122	76.2[b]	2.506 ± 0.079	23.2[b]	6.547 ± 0.335	79.1[c]	9.053 ± 0.344	83.9[b]
2	0.551 ± 0.018	15.1	1.242 ± 0.052	40.2	1.193 ± 0.055	32.8	1.801 ± 0.056	16.7	3.968 ± 0.178	44.2	5.769 ± 0.187	53.5
3	0.443 ± 0.017	12.2	1.610 ± 0.064	50.4	2.053 ± 0.066	56.4	1.407 ± 0.055	13.0	4.883 ± 0.195	52.1	6.290 ± 0.203	58.3
4	0.744 ± 0.021	20.5	2.285 ± 0.066	79.0	3.029 ± 0.069	83.3	2.607 ± 0.078	24.2	7.300 ± 0.220	89.2	9.907 ± 0.233	91.8
5	0.552 ± 0.010	15.2	0.854 ± 0.021	27.7	1.406 ± 0.023	38.7	1.590 ± 0.032	14.7	2.412 ± 0.054	26.2	4.002 ± 0.063	37.1
6	1.060 ± 0.055	29.1	2.318 ± 0.131	89.9	3.378 ± 0.142	92.9	3.584 ± 0.179	33.2	6.224 ± 0.354	86.4	9.808 ± 0.397	90.9
7	2.063 ± 0.053	56.7	1.195 ± 0.053	75.9	3.258 ± 0.075	89.6	6.417 ± 0.148	59.5	2.953 ± 0.121	67.6	9.370 ± 0.191	86.9
8	0.997 ± 0.066	27.4	2.492 ± 0.093	84.4	3.489 ± 0.114	95.9	2.748 ± 0.168	25.5	7.297 ± 0.299	90.8	10.045 ± 0.343	93.1
9	1.298 ± 0.095	35.7	1.666 ± 0.151	71.2	2.964 ± 0.178	81.5	4.661 ± 0.331	43.2	5.418 ± 0.465	88.4	10.079 ± 0.571	93.4
10	1.747 ± 0.036	48.0	0.992 ± 0.053	52.3	2.739 ± 0.064	75.3	4.569 ± 0.103	42.4	3.602 ± 0.134	57.9	8.171 ± 0.169	75.7
11	1.102 ± 0.021	30.3	2.520 ± 0.035	99.4	3.622 ± 0.041	99.6	3.060 ± 0.087	28.4	7.720 ± 0.139	99.9	10.780 ± 0.164	99.9
12	0.640 ± 0.018	17.6	1.883 ± 0.045	62.8	2.523 ± 0.048	69.4	1.559 ± 0.047	14.5	6.101 ± 0.127	66.1	7.660 ± 0.135	71.0
13	0.716 ± 0.017	19.7	1.479 ± 0.044	50.6	2.195 ± 0.047	60.4	2.093 ± 0.048	19.4	3.953 ± 0.113	45.5	6.046 ± 0.123	56.0
14	1.032 ± 0.031	28.4	2.413 ± 0.112	92.6	3.445 ± 0.116	94.7	2.736 ± 0.094	25.4	6.503 ± 0.244	80.8	9.239 ± 0.261	85.6

[a] Values represent mean ± SD; content in feces samples related to dry matter; the combined feces samples of individual subjects were divided into three aliquots and worked up in triplicate

[b] Recovery related to consumed esters

[c] Recovery related to amount of hydrolyzed esters

Tab. 14 Individual data on fecal recovered intact esters and liberated phytosterols within the pro.activ®-trial

Subject	Campesteryl esters						Sitosteryl esters					
	Intact esters		Sterol[b]		Total		Intact esters		Sterol[c]		Total	
	[mmol]	[%]	[mmol]	[%]	[mmol]	[%]	[mmol]	[%]	[mmol]	[%]	[mmol]	[%]
Substrate	2.220						10.025					
Feces[a]												
1	0.452 ± 0.011	20.4[d]	1.115 ± 0.049	63.1[e]	1.567 ± 0.050	70.6[d]	2.211 ± 0.044	22.1[d]	4.857 ± 0.218	62.2[e]	7.068 ± 0.222	70.5[d]
2	0.715 ± 0.017	32.2	1.496 ± 0.018	99.4	2.211 ± 0.025	99.6	3.514 ± 0.069	35.1	6.744 ± 0.057	103.6	10.258 ± 0.089	102.3
3	0.417 ± 0.014	18.8	0.577 ± 0.054	32.0	0.994 ± 0.056	44.8	0.786 ± 0.024	7.8	2.917 ± 0.071	31.6	3.703 ± 0.075	36.9
4	0.222 ± 0.013	10.0	1.469 ± 0.106	73.5	1.691 ± 0.107	76.2	0.964 ± 0.068	9.6	8.235 ± 0.421	90.9	9.199 ± 0.426	91.8
5	0.096 ± 0.004	4.3	0.849 ± 0.007	40.0	0.945 ± 0.008	42.6	0.376 ± 0.008	3.8	4.789 ± 0.034	49.6	5.165 ± 0.035	51.5
6	0.469 ± 0.017	21.1	1.232 ± 0.060	70.4	1.701 ± 0.062	76.6	2.316 ± 0.081	23.1	5.220 ± 0.238	67.7	7.536 ± 0.251	75.2
7	0.844 ± 0.012	38.0	0.938 ± 0.036	68.2	1.782 ± 0.038	80.3	4.197 ± 0.044	41.9	3.984 ± 0.149	68.4	8.181 ± 0.155	81.6
8	0.125 ± 0.008	5.6	1.091 ± 0.027	52.1	1.216 ± 0.028	54.8	0.928 ± 0.036	9.3	6.028 ± 0.114	66.3	6.956 ± 0.120	69.4
9	0.548 ± 0.015	24.7	1.475 ± 0.042	88.2	2.023 ± 0.045	91.1	2.536 ± 0.069	25.3	7.738 ± 0.211	103.3	10.274 ± 0.222	102.5
10	1.048 ± 0.026	47.2	0.602 ± 0.030	51.4	1.650 ± 0.040	74.3	5.235 ± 0.147	52.2	2.701 ± 0.101	56.4	7.936 ± 0.178	79.2
11	0.103 ± 0.008	4.6	1.087 ± 0.012	51.3	1.190 ± 0.014	53.6	0.553 ± 0.011	5.5	9.390 ± 0.052	99.1	9.943 ± 0.053	99.2
12	0.406 ± 0.015	18.3	1.260 ± 0.052	69.5	1.666 ± 0.054	75.0	1.402 ± 0.037	14.0	7.857 ± 0.252	91.1	9.259 ± 0.255	92.4
13	0.416 ± 0.015	18.7	1.394 ± 0.039	77.3	1.810 ± 0.042	81.5	2.386 ± 0.060	23.8	5.635 ± 0.148	73.8	8.021 ± 0.160	80.0
14	0.281 ± 0.014	12.7	0.874 ± 0.050	45.1	1.155 ± 0.052	52.0	1.448 ± 0.041	14.4	4.827 ± 0.210	56.3	6.275 ± 0.214	62.6

[a] Values represent mean ± SD; content in feces samples related to dry matter; the combined feces samples of individual subjects were divided into three aliquots and worked up in triplicate

[b] Value represents the sum of campesterol and fecal metabolites (24-methylcoprostanol, 24-methylcoprostanone and 24-methylcholest-4-en-3-one)

[c] Value represents the sum of sitosterol and fecal metabolites (24-ethylcoprostanol, 24-ethylcoprostanone and 24-ethylcholest-4-en-3-one)

[d] Recovery related to consumed esters

[e] Recovery related to amount of hydrolyzed esters

It is noteworthy that this phenomenon also becomes obvious when calculating total recoveries on the basis of the substrate amounts reported in the study with ileostomy patients [20]. The sum of sterols/stanols and intact esters recovered in the ileostomy excreta amounted only to 82 % of the phytosterol esters and to 88 % of the phytostanyl esters consumed by the patients. In the present study, this discrepancy between the amounts of sterols/stanols actually recovered in the feces and the amounts expected from the remaining intact esters was in addition strongly dependent on the individuals (Tab. 13 and Tab. 14).

5.5.1.5 Total contents of fecal phytosterols/-stanols after alkaline hydrolysis

In order to confirm these at first glance conflicting data on the amounts of recovered intact phytosteryl/-stanyl esters and liberated sterols/stanols in the feces and to rule out methodological flaws of the employed quantification of intact esters, the calculated total phytosterol/-stanol contents in feces were compared to those obtained by the classical method based upon saponification. As shown in Tab. 15 for three randomly selected subjects from the Benecol®- and the γ-oryzanol-trial, the data sets were rather consistent. The correspondence between the total phytosterol/-stanol concentrations across the individuals ranging from 96.9 % to 99.8 % (Benecol®) and 91.0 % to 99.4 % (γ-oryzanol) definitely demonstrated the accuracy of quantitation of intact esters.

5.5.1.6 Recovery of individual intact phytosteryl/stanyl esters

In all three trials the spectra of esters found in the feces of the subjects corresponded qualitatively to those consumed with the enriched skimmed milk drinking yogurts. However, there were significant quantitative differences between the individual esters (Tab. 16) Mean recoveries of individual intact phytosteryl/-stanyl fatty acid esters in feces ranged from 18.5 ± 2.7 % for campestanyl linolenate to 70.1 ± 4.8 % for campestanyl palmitate in the Benecol®-trial and from 17.4 ± 3.8 % for campesteryl linoleate to 47.6 ± 5.4 % for sitosteryl palmitate in the pro.activ®-trial.

Tab. 15 Comparison of total phytosterol/-stanol contents in feces of three randomized selected subjects before hydrolysis (BH)[a] and after alkaline hydrolysis (AH)[b] (mean ± SD)

Substrate and sterols / stanols	Subject 1		Subject 2		Subject 3	
	BH	AH	BH	AH	BH	AH
	[mmol/dry feces]					
Benecol®						
Campestanol	3.029 ± 0.069	3.043 ± 0.045	3.378 ± 0.142	3.273 ± 0.048	2.739 ± 0.064	2.745 ± 0.192
Sitostanol	9.907 ± 0.233	9.787 ± 0.140	9.808 ± 0.397	9.791± 0.049	8.171 ± 0.169	7.940 ± 0.116
γ-Oryzanol						
Campesterol	1.535 ± 0.015	1.441 ± 0.036	1.007 ± 0.048	0.933 ± 0.030	1.305 ± 0.057	1.267 ± 0.027
Campestanol	0.194 ± 0.004	0.183 ± 0.005	0.143 ± 0.005	0.133 ± 0.004	0.154 ± 0.008	0.144 ± 0.008
Sitosterol	0.649 ± 0.008	0.642 ± 0.015	0.487 ± 0.021	0.455 ± 0.025	0.410 ± 0.019	0.425 ± 0.038
Cycloartanol	0.217 ± 0.002	0.197 ± 0.008	0.117 ± 0.006	0.114 ± 0.005	0.187 ± 0.012	0.170 ± 0.004
Cycloartenol	6.386 ± 0.015	6.224 ± 0.198	3.669 ± 0.212	3.806 ± 0.088	5.482 ± 0.299	5.565 ± 0.095
24-Methylenecycloartanol	7.100 ± 0.065	6.891 ± 0.196	4.041 ± 0.316	4.015 ± 0.084	6.061 ± 0.385	5.943 ± 0.083

[a] Values represent the sum of phytosterols/-stanols determined as total intact ester, in their free form and as fecal metabolites (in the case of campesterol and sitosterol); three feces aliquots of each subject were worked up in triplicate

[b] Values represent the sum of phytosterol/-stanols and their fecal metabolites (in the case of campesterol and sitosterol);onefold analysis of three feces aliquots of each subject

Only for the ferulates administered in the γ-oryzanol-trial recoveries in a more narrow range (between 73.7 ± 3.5 % and 84.4 ± 3.7 %) was observed. When comparing esters with the same acyl moieties, stanyl esters always exhibited higher recoveries than the respective steryl esters.

Tab. 16 Contents of individual phytosteryl/-stanyl esters in the substrates and in collected feces samples (n = 14)

No[a]	Phytosteryl/-stanyl ester	Substrate[b] [mmol]	Recoveries in feces[c] [mmol]	[%]
Benecol® (stanyl fatty acid ester)				
1	Campestanyl-C16:0	0.213	0.149 ± 0.010	70.1 ± 4.8
3	Campestanyl-C18:1	2.309	0.595 ± 0.080	25.8 ± 3.6
4	Campestanyl-C18:2	0.791	0.171 ± 0.028	21.6 ± 3.6
5	Campestanyl-C18:3	0.323	0.060 ± 0.009	18.5 ± 2.7
2	Sitostanyl-C16:0	0.566	0.367 ± 0.024	65.2 ± 4.2
6	Sitostanyl-C18:1	6.927	1.743 ± 0.227	25.2 ± 3.4
7	Sitostanyl-C18:2	2.297	0.573 ± 0.096	24.9 ± 4.3
8	Sitostanyl-C18:3	0.806	0.186 ± 0.029	23.1 ± 3.7
9	Sitostanyl-C20:0	0.192	0.083 ± 0.009	43.5 ± 4.7
Pro.activ® (steryl fatty acid ester)				
10	Campesteryl-C16:0	0.141	0.067 ± 0.006	47.3 ± 4.5
12	Campesteryl-C18:1	0.767	0.144 ± 0.022	18.8 ± 2.9
13	Campesteryl-C18:2	1.312	0.228 ± 0.048	17.4 ± 3.8
11	Sitosteryl-C16:0	0.643	0.306 ± 0.033	47.6 ± 5.4
14	Sitosteryl-C18:1	3.710	0.685 ± 0.109	18.5 ± 3.0
15	Sitosteryl-C18:2	5.671	1.071 ± 0.239	18.9 ± 4.4
γ-Oryzanol (steryl/stanyl ferulic acid ester)				
16	Campesteryl ferulate	1.456	1.082 ± 0.060	74.3 ± 3.4
17	Campestanyl ferulate	0.173	0.145 ± 0.007	83.8 ± 2.8
19	Sitosteryl ferulate	0.491	0.362 ± 0.021	73.7 ± 3.5
18	Cycloartanyl ferulate	0.215	0.181 ± 0.010	84.4 ± 3.7
20	Cycloartenyl ferulate	6.386	5.362 ± 0.287	84.0 ± 3.6
21	24-Methylenecycloartanyl ferulate	7.100	5.899 ± 0.329	83.1 ± 3.7

[a] Peak numbers correspond to Fig. 34A-C (II)

[b] Content in the substrates consumed within 3 days; determined according to [224]

[c] Content in feces samples (mean ± SEM, n = 14) related to dry matter and collection time of 5 days; the combined feces samples (5 days) of individual subjects were divided into three aliquots and worked up in triplicate

In the present study, the hydrolysis of dietary phytosteryl/-stanyl esters during gastrointestinal passage in healthy humans was significantly affected by their molecular structures. Fecal recoveries of individual fatty acid esters also revealed large variability within the study population indicating large inter-individual variations of the hydrolysis rates of the different phytosteryl/-stanyl esters analogous to the variability seen for the total ester hydrolysis. The data shown in Fig. 37 demonstrate that the three selected subjects did not only differ in the recoveries of total sitostanyl fatty acid esters (Fig. 37 A) and total sitosteryl fatty acid esters (Fig. 37 B) but also showed clear differences in the profiles of the recovered esters compared to the consumed substrates. This variability was less pronounced for the ferulates of desmethylsterols/-stanols (Fig. 37 C) and dimethylsterols (Fig. 37 D).

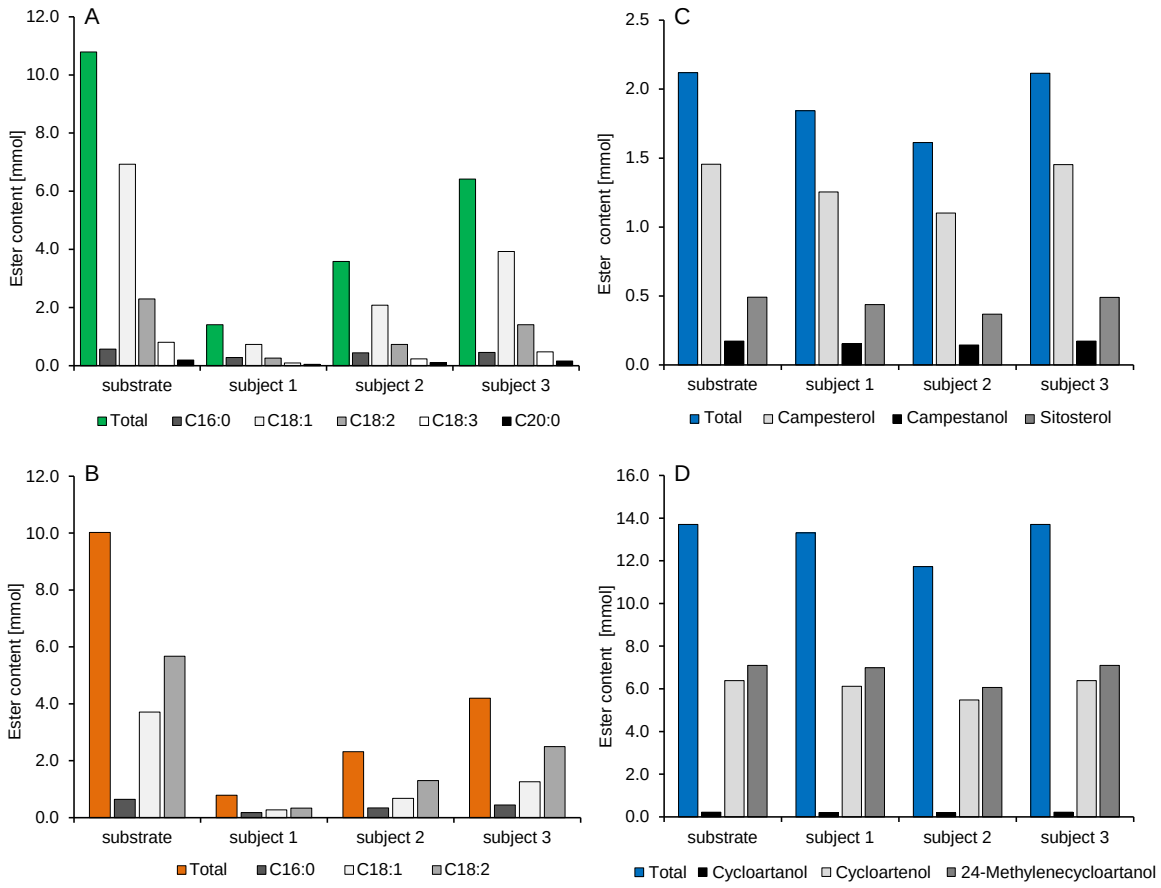

Fig. 37 Contents of sitostanyl fatty acid esters (A), sitosteryl fatty acid esters (B), desmethylsteryl ferulates (C) and dimethylsteryl ferulates (D) in feces of 3 subjects after oral administration and in the consumed substrate; (A) = Benecol®, (B) = pro.activ® and (C, D) = γ-oryzanol

Despite this inter-individual variability, the data summarized in Fig. 38 demonstrate the impact of the acyl moieties (Fig. 38 A & B) and of the steryl/stanyl moieties (Fig. 38 C & D) on the recoveries of the administered esters. The impact of the acid moiety seems to be more relevant. Ferulates were consistently recovered at higher rates than all other esters. Palmitates of both, campestanol and sitostanol, were recovered to significantly higher degrees than the respective esters of unsaturated C18 fatty acids (Fig. 38 A). The recovery rate of sitostanyl eicosanoate was significantly lower than that of sitostanyl palmitate but significantly higher than those of the respective esters of unsaturated C18 fatty acids. The recovery rates of oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acid esters were in the same range. Differences between campestanyl esters and the corresponding sitostanyl esters could not be observed (Fig. 38 A). The results obtained for steryl fatty acid esters confirmed these findings. The C16:0 esters of both phytosterols were also recovered to a significantly higher degree than the respective C18:1 and C18:2 esters, whereas the recovery rates of the campesteryl and the corresponding sitosteryl esters were in the same range (Fig. 38 B). In conclusion, for the rates of hydrolysis the following order depending on the acid moiety was determined: oleate = linoleate = linolenate > eicosanoate > palmitate > ferulate.

For rats fed oleates and stearates mainly of phytosterols also a significant impact of the acid moiety has been described; oleic acid esters were virtually absent in feces, whereas almost 80 % of the stearates were recovered in their intact form [17]. A recent *in vitro* study focussing on the substrate specificity of pancreatic cholesterol esterase further supports the findings of this study; oleates were preferred substrates for hydrolysis by pancreatic cholesterol esterase compared to the respective palmitic and stearic acid esters which were in the same order of magnitude [12].

The comparison of different palmitates, oleates and linoleates demonstrates the impact of the steryl/stanyl moieties on the recoveries (Fig. 38 C); the palmitates of campestanol and sitostanol were recovered at significantly higher rates than the corresponding esters of campesterol and sitosterol. There were similar trends for the oleates and linoleates; however, the differences in the recoveries between stanyl and steryl esters were not significant.

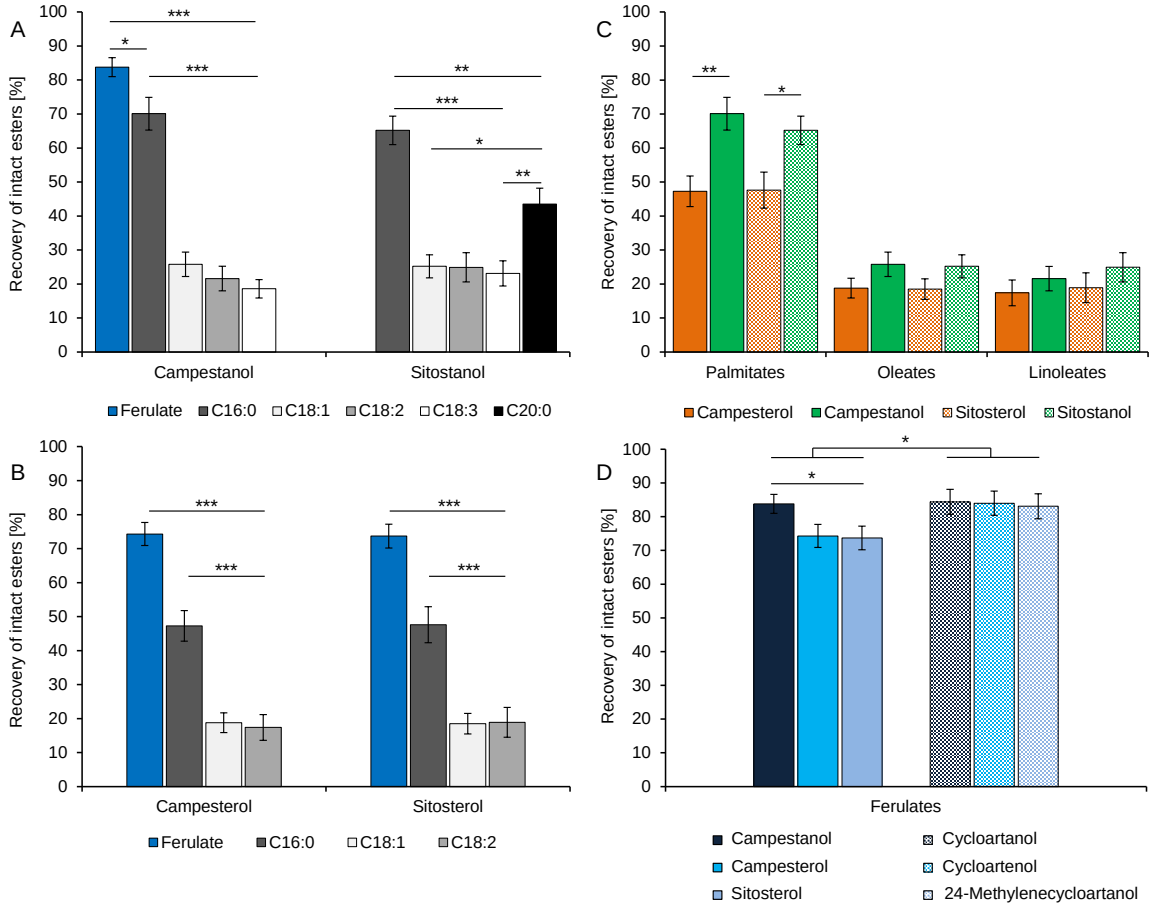

Fig. 38 Structure-dependent recoveries of intact phytosteryl/-stanyl esters in human feces after oral consumption of enriched skimmed milk drinking yogurts; (A, B) = impact of acid moiety; (C, D) = impact of phytosterol/-stanol structure; significance levels: $P \leq 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***)

ANOVA comparing the ferulic acid esters of all containing phytosterols/-stanols within the γ-oryzanol-trial revealed borderline significance ($P = 0.066$).To control this overall trend, the ferulates of desmethylsterols/-stanols (campestanol, campesterol and sitosterol) as one group and of dimethylsterols (cycloartanol, cycloartenol and 24-methylenecycloartanol) as second group were investigated using Student's unpaired *t*-test. Significant differences could be proven between the two groups ($P \leq 0.05$) and within the desmethylsteryl/-stanyl ferulic acid esters when comparing the recoveries of campesteryl and sitosteryl ferulate with campestanyl ferulate ($P \leq 0.05$) (Fig. 38 D). In summary, a significant impact of the desmethylsterol/-stanol structure was proven for the palmitic acid and ferulic acid esters in this order of rate of hydrolysis: desmethylsterol > desmethylstanol. But the consistently lower recoveries of the remaining desmethylsteryl esters

compared to the analogous desmethylstanyl esters indicated at least an additional impact of the sterol/stanol structure (Tab. 16).

Similar results were reported in hamsters fed either sitosteryl stearate or sitostanyl stearate [16]. Although the hydrolysis rates of both esters were very low, almost 5 % for sitosteryl-C18:0 and 1 % for sitostanyl-C18:0, the difference was described as being significant. In contrast, no difference has been reported for the hydrolysis of sitosteryl fatty acid esters compared to the respective sitostanyl esters in an *in vitro* study investigating the substrate specificity of pancreatic cholesterol esterase [12]. Further indications for an impact of the sterol/stanol moiety can be inferred from the results obtained by *in vitro* hydrolysis of steryl ferulates compared to sitostanyl ferulate [15]. Steryl ferulate mixtures from rye and wheat composed of 80 % stanyl (campestanol and sitostanol) and 20 % steryl (campesterol and sitosterol) ferulates were hydrolyzed by cholesterol esterases to a significantly higher degree. Employing several mammalian digestive enzyme preparations, it has been concluded that dimethylsteryl ferulates are not hydrolyzed [13] or that desmethylsteryl ferulates are preferentially hydrolyzed compared to dimethylsteryl ferulates [14, 15]. The hypothesis arising from the available *in vitro* data that dimethylsteryl ferulates are not hydrolyzed upon human digestion is confirmed by the results of the present study. In line with this, the exact mechanism underlying the cholesterol-lowering activity reported for γ-oryzanol and rice bran oil [8-11, 215, 220] remains unclear.

5.5.1.7 Influence of the collection period on the fecal recoveries of intact phytostanyl esters and released phytostanols

The lengths of the periods of feces collection during the three interventions were based on an average transit time of the diet described as 55 – 72 h [238, 264]. Nevertheless, it might be hypothesized that delayed excretion of free phytosterols/-stanols owing to their turnaround time in the intestinal and enterohepatic circulation might be responsible for the differences between experimentally determined and expected contents in the feces. However, it is important to note that this delayed excretion did not result in permanently increased plasma phytosterol/-stanol concentrations (section 5.5.2.3). In order to

prove whether intact phytosteryl/-stanyl esters and liberated phytosterols/-stanols have been excreted in feces quantitatively within a period of 5 days, a subsequent trial using Benecol® as substrate was performed with one volunteer, who did not participate in the randomized trial. In contrast to the main study (Fig. 33), the period of feces collection starting on the second day of application was extended to 7 days and daily feces samples were not pooled for analysis. Each daily feces sample was prepared separately according to Fig. 16. Data on daily feces weights and the corresponding dry matter contents are given in Tab. 17. Both the mean weight of daily feces of 139.0 ± 19.9 g and the averaged feces dry weight of 23.6 ± 4.4 % were very similar to the corresponding values obtained for the entire population within the main study.

Tab. 17 Daily weights and dry matter contents of feces excreted by one participant within a subsequent consumption study related to the investigation of the influence of the collection period on fecal recoveries of intact phytostanyl esters and liberated phytostanols

Feces	Day of collection						
	1	2	3	4	5	6	7
Weight [g]	144.9	106.2	139.1	119.3	171.3	137.9	154.4
Dry matter [%]	20.5	32.0	23.2	27.4	18.7	23.4	19.8

Data regarding the daily and total recoveries of individual phytoststanyl fatty acid esters in the feces are presented in Tab. 18. Within the corresponding limits of detection, intact phytostanyl esters could be analyzed up to day 6. At day 7 neither campestanyl esters nor sitostanyl esters were detectable in the feces. In total, 51.5 % of the campestanyl fatty acid esters and 53.7 % of the sitostanyl fatty acid esters were recovered in feces upon consumption of Benecol® skimmed milk drinking yogurts indicating hydrolysis rates of 48.5 % and 46.3 %, respectively. Compared to the main study, this is in the order of magnitude of the lowest rates of total ester hydrolysis described for subject 7 (section 5.5.1.4); the impact of the acid moeity was also confirmed. Although intact esters were detectable on day 6 of defecation, 99.4 % and 99.6 % of total recovered intact campestanyl esters and sitostanyl esters, respectively, were excreted within five days of starting feces collection. This indicates that the chosen feces collection period of five days within the main study starting with the second day of application was sufficient for the quantitation of intact phytosteryl/-stanyl esters.

Tab. 18 Data on time-dependent fecal recoveries of intact phytostanyl esters upon consumption of Benecol® drinking yogurts in one human volunteer

Day	Campestanyl esters [mmol]						Sitostanyl esters [mmol]						
	C16:0	C18:1	C18:2	C18:3	Sum	Rec[c] [%]	C16:0	C18:1	C18:2	C18:3	C20:0	Sum	Rec[c] [%]
Substrate[a]	0.213	2.309	0.791	0.323	3.637		0.566	6.927	2.297	0.806	0.192	10.788	
Feces[b]													
1	0.031	0.224	0.063	0.020	0.338	9.3	0.080	0.675	0.222	0.068	0.025	1.070	9.9
2	0.045	0.352	0.101	0.036	0.534	14.7	0.115	1.089	0.299	0.107	0.037	1.647	15.3
3	0.053	0.397	0.113	0.036	0.599	16.5	0.134	1.200	0.364	0.121	0.044	1.863	17.3
4	0.037	0.213	0.055	0.019	0.324	8.9	0.096	0.625	0.193	0.057	0.025	0.996	9.2
5	0.007	0.043	0.012	0.004	0.066	1.8	0.017	0.116	0.034	0.016	0.005	0.188	1.7
6	0.003	0.008	n.d.[e]	n.d.[e]	0.011	0.3	0.006	0.020	n.d.[e]	n.d.[e]	n.d.[e]	0.026	0.2
7	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]	-		n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]	-	
Sum [mmol]	0.176	1.237	0.344	0.115	1.872	51.5	0.448	3.725	1.112	0.369	0.136	5.790	53.7
Rec[d] [%]	82.6	53.6	43.5	35.6	51.5		79.2	53.8	48.4	45.6	70.8	53.7	

[a] Amounts consumed with the skimmed milk drinking yogurt within three days of application

[b] Mean values; content in feces samples related to dry matter; the daily feces samples were divided into three aliquots and worked up in triplicate; standard deviations are unembodied for reasons of clarity of the illustration

[c] Recovery of total phytostanyl ester excreted with daily feces related to the sum of consumed esters

[d] Recovery individual phytostanyl fatty acid esters excreted within seven days related to the respective content consumed with the substrate

[e] Below limits of detection determined according to DIN 32645 (2008-11) [229]; LODs of individual phytostanyl fatty acid esters given in Tab. 9

As presented in Tab. 19, ester-derived campestanol and sitostanol were quantified in the daily feces samples up to the seventh day. The time courses of fecal excretions of liberated phytostanols and intact phytostanyl fatty acid esters were comparable and revealed highest recoveries on day three. On average, 90 % of the amounts of campestanol and sitostanol expected from the hydrolysis rates calculated on the basis of recovered intact esters were found upon seven days of feces collection.

Tab. 19 Data on time-dependent fecal recoveries of liberated phytostanols upon consumption of Benecol® drinking yogurts in one human volunteer

Day	Phytostanol			
	Campestanol [mmol]	Rec[c] [%]	Sitostanol [mmol]	Rec[c] [%]
Hydrolysis[a]	1.765		4.998	
Feces[b]				
1	0.306 ± 0.008	17.4	0.881 ± 0.007	17.6
2	0.317 ± 0.018	18.0	0.944 + 0.022	18.9
3	0.440 ± 0.006	24.9	1.229 ± 0.013	24.6
4	0.366 ± 0.005	20.7	1.049 ± 0.011	21.0
5	0.102 ± 0.004	5.8	0.301 ± 0.006	6.0
6	0.044 ± 0.001	2.5	0.154 ± 0.002	3.1
7	0.010 ± 0.001	0.6	0.055 ± 0.003	1.1
Sum [mmol]	1.585 ± 0.022	89.8	4.613 ± 0.030	92.3
Rec[d] [%]	89.8		92.3	

[a] Expected amounts related to hydrolyzed esters

[b] Mean values ± SD; content in feces samples related to dry matter; daily feces samples were divided into three aliquots and worked up in triplicate

[c] Recovery of phytostanols excreted with daily feces related to the expected amounts

[d] Recovery of phytostanols excreted within seven days related to expected amounts

However, the averaged recovery rate after five days was only slightly lower (87 %). Although these data indicate that fecal excretion of phytostanols compared to intact esters might be prolonged, this cannot be the sole explanation for the partly significant discrepancies observed in the main study, irrespective of a large inter-individual varability (Tab. 13 and Tab. 14)

5.5.1.8 Impact of colonic microflora

The potential impact of the gut microbiota on the *in vivo* metabolization of intact phytosteryl/-stanyl esters or liberated PS upon large intestine transit is determined by two aspects: The converter status of the subject determined on the basis of fecal excretion of cholesterol and coprostanol in the respective blank samples using Eq. 8 (section 4.2.1), and the point of time of last antibiotic treatment according to the personal statements of the participants. Characteristics of the fourteen subjects regarding their converter-status and the period since last use of antibiotics are presented in Tab. 20. The extent of cholesterol transformation by the individual gut microbiota remained stable during the three periods indicated by low standard deviations of the averaged conversion rates. The converter status of the subjects was also reflected by the amounts of comparable microbial transformation products of ester derived PS quantified in

Tab. 20 Individual characteristics on converter status and last antibiotic treatment

Subject	Converter status		Antibiotics
	Conversion rate[a] [%]	Category[c]	[months since treatment]
1	89.4 ± 5.5	HC	60
2	89.7 ± 5.5	HC	> 120
3	85.4 ± 6.2	HC	72
4	84.5 ± 2.4	HC	48
5	87.3 ± 6.5	HC	no treatment[d]
6	27.5 ± 2.6	LC	8
7	2.4 ± 0.4	NC	12
8	88.6 ± 2.6	HC	12
9	77.0 ± 6.8	HC	> 120
10	no conversion[b]	NC	24
11	92.2 ± 3.6	HC	42
12	94.0 ± 0.2	HC	84
13	71.0 ± 3.3	HC	24
14	32.4 ± 3.6	LC	60

[a] Conversion rate corresponds to the percentage coprostanol of the sum of cholesterol and coprostanol, excreted in feces blanks of individual subjects prior to each intervention; values represent mean ± SD; feces blanks were divided into three aliquots and worked up in triplicate

[b] Coprostanol has not been identified in feces

[c] Categories according to [252]; HC = “high-converter” (> 40 %); LC = “low-converter” (5 to ≤ 40 %); NC = “non-converter” (< 5 %)

[d] No history of antibiotic treatment

feces. The sum of these metabolites (Fig. 9) was highest in “high-converter” and lowest in “non-converter”, especially within the pro.activ®-trial. In accordance with the literature [81] fecal metabolites of liberated phytostanols during the Benecol® exposure could not be identified. Although there is no direct correlation between the converter-status of the subjects and the point of time of the last antibiotic treatment (Tab. 20), fecal profile of intact phytosteryl esters determined for subject 5, the only one who never had used antibiotics before, upon pro.activ® consumption varied significantly from all other subjects. By way of comparison,

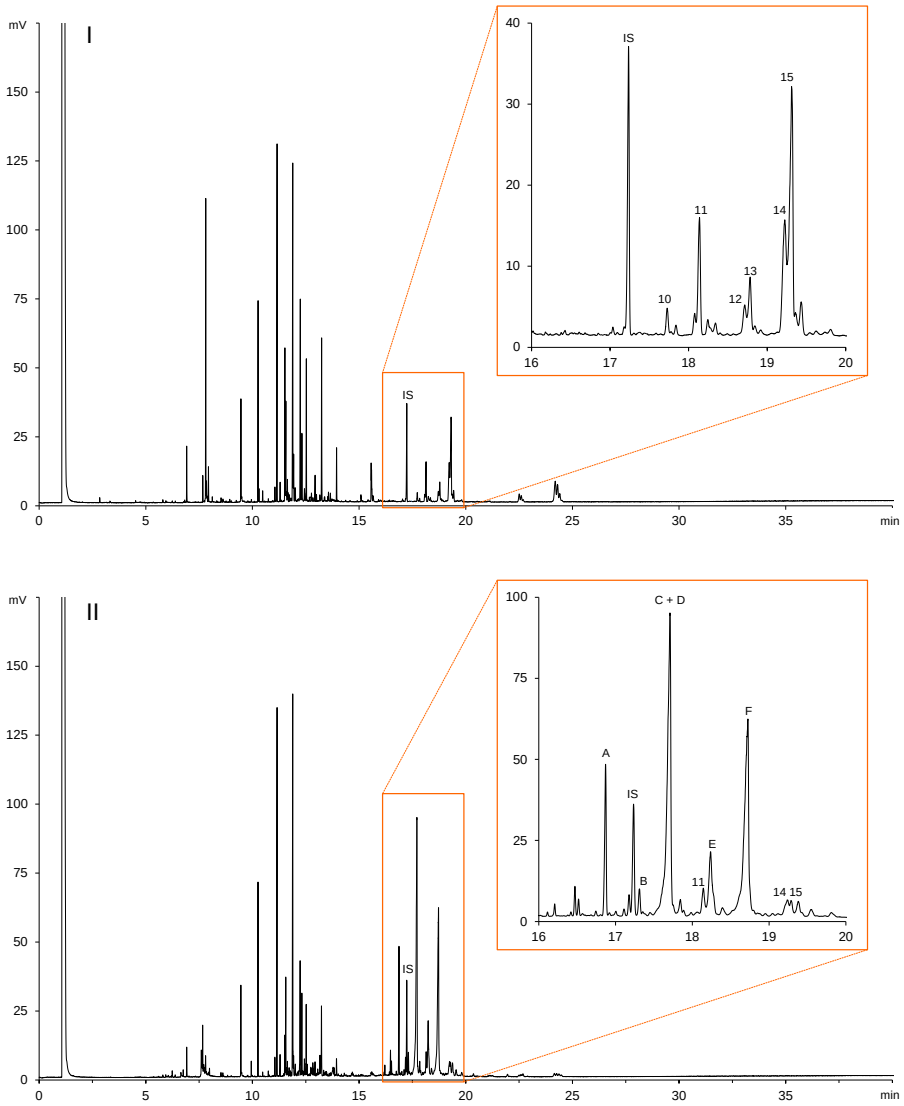

Fig. 39 GC/FID analysis of fecal recovered intact esters in two subjects both characterized as “high-converter” according to [252] upon consumption of Becel pro.activ® skimmed milk drinking yogurts; (I) subject 3; (II) subject 5; peak-numbering according to Tab. 9; A = coprostanyl palmitate; B = 24-methylcoprostanyl palmitate (tentatively identified); C = coprostanyl stearate; D = 24-ethylcoprostanyl palmitate; E = 24-methylcoprostanyl stearate (tentatively identified); F = 24-ethylcoprostanyl stearate; IS = internal standard (cholesteryl palmitate)

intact phytosteryl ester profiles determined by gas chromatographic separation of feces samples obtained from subjects 5 and 3 (both defined as “high-converter”) during the pro.activ®-trial are illustrated in Fig. 39. Whereas in the feces of subject 3 the spectra of identified intact esters corresponded qualitatively to that consumed with the drinking yogurt (Fig. 39 I), the region of intact esters in the feces of subject 5 was dominated by six peaks (A-F) probably attributed to metabolized phytosteryl fatty acid esters consumed with Becel pro.activ® (Fig. 39 II). Due to the high degree of hydrolysis of almost 96 % of consumed steryl esters determined for subject 5 (Tab. 14), the assignment of individual intact campesteryl esters was not presentable in the used enlargement. Based on the comparisons of RRT related to cholesteryl-C16:0 (IS) and MS spectra with chemically synthesized references [230], the unknown esters were identified as coprostanyl palmitate (A; RRT = 0.979), coprostanyl stearate (C; RRT = 1.024), 24-ethylcoprostanyl palmitate (D; RRT = 1.025) and 24-ethylcoprostanyl stearate (F; RRT = 1.086). As examples the MS spectra of synthesized 24-ethylcoprostanyl-C16:0 and 24-ethylcoprostanyl-C18:0 are presented in Fig. 40. Due to GC/MS-SIM analysis

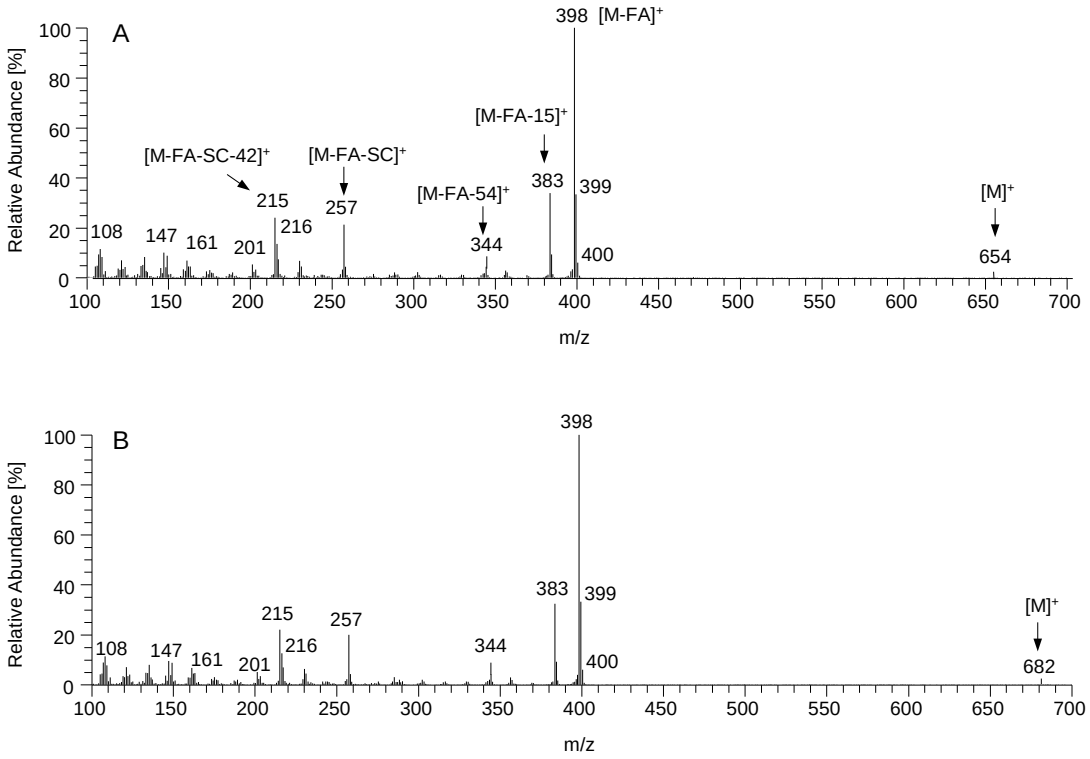

Fig. 40 EI-MS spectra of 24-ethylcoprostanyl palmitate (A) and 24-ethylcoprostanyl stearate (B) chemically synthesized according to [230]; M = molecular ion; FA = fatty acid; SC = side chain

using the fragment $[M\text{-}FA]^+$ the proportions of 24-ethylcoprostanyl-C16:0 (*m/z* 398) and coprostanyl-C18:0 (*m/z* 370) in the coeluted peak (C+D) were determined as 30 % and 70 %, respectively. The mass spectra of B (RRT = 1.005) and F (RRT = 1.058) both characterized by a base fragment of *m/z* 384 as well as molecular ions of *m/z* 640 and *m/z* 668, respectively, were in all remaining fragments in accordance to those obtained for synthesized 24-ethylcoprostanyl esters (Fig. 40). Therefore, these esters were tentatively identified as 24-methylcoprostanyl palmitate (B) and 24-methylcoprostanyl stearate (E).

The formation of these esters might be explained by two pathways: (I) The direct redecution of intact cholesteryl esters endogenously synthesized and of the consumed sitosteryl and campesteryl esters; (II) the microbial reduction of liberated free desmethylsterols (cholesterol, sitosterols and campesterol) according to Fig. 9 and subsequent re-esterification. Considering the research done by ROSENFELD in the mid 1960's [265-267], the direct reduction of intact desmethylsteryl esters appears to be extremely unlikely.

5.5.1.9 Fecal cholesterol excretion

Due to the mechanisms (section 2.1.4) described for the cholesterol-lowering properties of phytosterols/-stanols, the consumption of foods enriched with these substances or corresponding phytosteryl/-stanyl esters results in an increased fecal cholesterol excretion. In order to compare the effectivity of the phytosteryl/-stanyl esters consumed on the inhibiton of intestinal cholesterol absorption, fecal excretion of CS was monitored during the main study. Data on total fecal cholesterol contents calculated as sum of CS and related microbial transformation products are given in Tab. 21. The increase in fecal cholesterol excretion within the γ-oryzanol exposure (17.6 %) was in same order of magnitude as those determined upon consumption of Benecol® (18.4 %) and pro.activ® (19.2 %) although the averaged fecal recovery of almost 80 % of intact PSF indicate a three up to four times lower dergree of hydrolysis compared to PSA (27 % intact esters) and PSE (20 % intact esters) (Tab. 12), respectively. These data consistently suggest that an increase in fecal cholesterol excretion in reponse to consumption of phytosteryl/-stanyl esters enriched food products is not necessarily restricted to

the underlying mechanisms described for the cholesterol-lowering properties of liberated phytosterols/-stanols. Thus, taken increased fecal cholesterol excretion as an indicator of decreased plasma cholesterol levels, it might be that intact phytosteryl/-stanyl ferulates show their cholesterol-lowering effects on the basis of other previously unidentified mechanisms.

Tab. 21 Fecal cholesterol excretion in reponse to consumption of skimmed milk drinking yogurts enriched wich phytosteryl/-stanyl esters (n = 14)

Trial	Cholesterol[a]			
	Blank [mg/d]	$Feces_{pooled}$ [mg/5 d]	Feces [mg/d]	Changes [%/d]
Benecol®	422.7 ± 61.2	2501.8 ± 244.7	500.3 ± 48.9	+ 18.4
Pro.activ®	433.0 ± 67.2	2581.1 ± 238.5	516.2 ± 47.7	+ 19.2
γ-Oryzanol	400.5 ± 60.9	2354.7 ± 185.5	470.9 ± 37.1	+ 17.6

[a] Values represent mean ± SEM of the sum of cholesterol, coprostanol, coprostanone and cholest-4-en-3-one; contents in feces samples related to dry matter; the combined feces samples of individual subjects were divided into three aliquots and worked up in triplicate

5.5.2 PLASMA ANALYSIS

5.5.2.1 GC/MS calibration and validation data

Analytical validation data (Tab. 22) for the GC/MS determination of plasma phytosterol/-stanol concentrations in response to functional food consumption were calculated according to equations 1 – 5 (section 3.2.4.1.1) on the basis of triplicate linear regression analysis in coordinate ratios of peak areas (phytosterol/-stanol / IS) and amounts (phytosterols/-stanol / IS). Due to the lack of a reference substance the characteristics determined for cycloartenol were also transferred to cycloartanol.

Tab. 22 Relative retention times (RRT) compared to 5α-cholestane, coefficients of correlation (R^2), limits of detection (LOD), limits of quantitation (LOQ) and coefficient of variation (CV) of liberated phytosterols/-stanols sterols determined on the basis of the linear regression methodology according to [229] by means of GC/MS using selected ion monitoring

Phytosterol/-stanol	RRT	R^2	LOD	LOQ	CV
	[min]		[µg/dL]		[%]
Campesterol	1.162	0.9973	0.52	1.45	6.0
Campestanol	1.170	0.9967	0.47	1.32	6.8
Sitosterol	1.192	0.9972	0.53	1.48	5.5
Sitostanol	1.200	0.9979	0.49	1.34	6.1
Cycloartenol	1.258	0.9981	0.57	1.55	5.4
24-methylenecycloartanol	1.290	0.9940	0.93	2.47	6.4

5.5.2.2 Plasma lipids and lipoproteins in response to functional food consumption

Concentrations of LDL-C, HDL-C, and TG measured across all dietary treatments were in the ranges described for normolipidemic subjects (Tab. 23). As expected due to the shortness of administration, no treatment-related differences were observed for any of the parameters.

Tab. 23 Plasma lipid levels at day 0, day 3 and day 6 of each dietary period (mean ± SEM; n = 14)

Plasma lipid and study day	Benecol®	Pro.activ®	γ-Oryzanol
		[mg/dL]	
LDL cholesterol			
Day 0	93.9 ± 8.3	94.9 ± 8.0	89.9 ± 6.5
Day 3	93.9 ± 6.6	95.5 ± 7.2	94.8 ± 7.2
Day 6	92.0 ± 7.1	95.8 ± 6.9	90.3 ± 6.4
HDL cholesterol			
Day 0	57.5 ± 4.9	58.3 ± 4.4	56.6 ± 4.0
Day 3	60.7 ± 4.5	61.2 ± 4.3	59.2 ± 4.7
Day 6	59.6 ± 4.3	61.8 ± 4.0	60.3 ± 5.1
Total triacylglycerols			
Day 0	59.8 ± 13.2	51.5 ± 3.5	58.3 ± 6.2
Day 3	79.9 ± 12.2	74.2 ± 11.5	69.6 ± 5.4
Day 6	63.9 ± 6.2	72.1 ± 6.8	59.1 ± 5.2

5.5.2.3 Phytosterol/-stanol profile in response to functional food consumption

Plasma phytosterol/-stanol concentrations and their ratios to plasma TC are presented in Tab. 24. Baseline phytosterol/-stanol levels across all volunteers were comparable to published data [57, 79, 212]. Thus, mutations and polymorphisms in the ABCG5 or ABCG8 gene in the enterocytes and hepatocytes responsible for hyperabsorption and hyposecretion pf PS [70, 82-84] in any of the subjects appear to be unlikely.

Mean plasma concentrations of campestanol and sitostanol were significantly increased on day 3 ($P < 0.001$ and $P < 0.01$) compared with day 0 after consumption of Benecol® by 275 and 127 %, respectively, but were not significantly different compared with the concentrations on day 6. Without further consumption, plasma concentrations of campestanol and sitostanol on day 6 compared with day 3 decreased by 51.7 and 38.2 %. This phenomenon has also been described by VON BERGMANN *et al.* [81]. Concentrations of sitostanol in the plasma of twelve volunteers increased fourfold ($P < 0.001$) on day 8 upon one week of oral adminstration of sitostanyl oleate (0.5 g three times daily) and returned nearly to baseline levels at least after fourteen days of stopping consumption of sitostanyl oleate [81]. In the pro.activ®-trial plasma concentrations of campesterol and sitosterol on day 3 increased ($P < 0.02$ and $P < 0.005$) relative to day 0 by 43.2 and 66.2 %, respectively. On day 6, concentrations of campesterol and sitosterol decreased ($P = 0.01$ and $P < 0.005$) compared with day 3 by 24.6 and 27.5 %, but were not significantly different from day 0. Consumption of γ-oryzanol-enriched skimmed milk yogurt drinks solely resulted in a significant higher plasma level of cycloartenol on day 3 ($P < 0.01$) compared with day 0 by 23.7 %. The plasma cycloartenol concentration decreased on day 6 compared with day 3 by 8.5 % and was neither significantly different compared with day 3 nor with day 0.

Tab. 24 Plasma phytosterol/-stanol concentrations determined by GC/MS selected ion monitoring and their ratios to cholesterol during the treatment periods (mean ± SEM; n = 14)

Phytosterol/-stanol	Day 0		Day 3		Day 6	
	[µg/dL]	[x10^2 µg/mg][a]	[µg/dL]	[x10^2 µg/mg][a]	[µg/dL]	[x10^2 µg/mg][a]
Benecol®						
Total cholesterol [mg/dL]	153.3 ± 11.0		162.4 ± 9.3		158.1 ± 9.2	
Campestanol	2.5 ± 0.4	1.6 ± 0.3†	9.3 ± 1.4	6.0 ± 1.0‡	4.7 ± 0.8	2.9 ± 0.5†,‡
Sitostanol	7.2 ± 1.1	4.5 ± 0.8†	15.9 ± 2.1	10.2 ± 1.5‡	9.7 ± 1.3	6.3 ± 1.0†,‡
Pro.activ®						
Total cholesterol [mg/dL]	157.3 ± 12.5		165.7 ± 9.6		172.4 ± 11.8	
Campesterol	343.9 ± 65.6	198.9 ± 26.5†	493.9 ± 79.8	284.8 ± 30.3‡	395.4 ± 70.7	214.8 ± 22.6†
Sitosterol	215.0 ± 42.6	123.6 ± 18.5†	353.6 ± 53.3	205.4 ± 21.5‡	273.4 ± 46.7	149.0 ± 16.9†
γ-Oryzanol						
Total cholesterol [mg/dL]	149.4 ± 8.2		160.0 ± 9.7		157.5 ± 9.5	
Campesterol	303.2 ± 40.3	187.3 ± 19.9	339.3 ± 40.7	213.3 ± 23.0	303.0 ± 41.0	189.8 ± 20.6
Campestanol	1.9 ± 0.2	1.2 ± 0.2	1.9 ± 0.2	1.2 ± 0.1	1.8 ± 0.2	1.2 ± 0.1
Sitosterol	171.4 ± 26.2	105.9 ± 13.5	167.9 ± 25.1	105.6 ± 15.1	153.0 ± 24.4	95.8 ± 13.0
Cycloartanol	n.d.[b]		n.d.[b]		n.d.[b]	
Cycloartenol	5.9 ± 0.5	3.8 ± 0.3†	7.3 ± 0.6	4.7 ± 0.5‡	6.6 ± 0.4	4.3 ± 0.4†,‡
24-Methylenecycloartanol	n.q.[c]		n.q.[c]		n.q.[c]	

[a] Values represent ratio to total cholesterol calculated as mean of the individual ratios of the subjects; different superscript symbols indicate significant differences ($P < 0.05$ or less) between time points within each treatment

[b] Not detectable within limit of detection determined according to DIN 32645 (2008-11) [229]; LOD [µg/dL]: cycloartanol/cycloartenol = 0.57

[c] Not quantifiable within limit of quantification determined according to DIN 32645 (2008-11) [229]; LOQ [µg/dL]: 24-methylenecycloartanol = 2.47

5.5.3 URINE SCREENING

Urinary excretion not only plays an important role in the regulation of the water and electrolyte balance of the human body but also in the elimination of potentially harmful xenobiotics (e.g. drugs and anabolic steroids) as well as metabolites produced physiologically in the human body (e.g. steroid hormones and bile pigments). Once these lipohilic and unpolar substances have reached the liver, they usually are subjected to enzyme-catalyzed transfomations in terms of functionalization within phase I reactions (nonsynthetic reactions) followed by the formation of highly polar conjugates by phase II reactions (conjugation reactions) for readily elimination. Steroids and related metabolites usually are conjugated either to glucuronic acid (glucuronidation) or to sulfate (sulfation) prior to urinary excretion.

5.5.3.1 Method development

To ensure the quantitative extraction of potential urinary steroid conjugates and the suitability of the sample preparation (section 3.2.4.3), the recovery of cholesterol derived either from sodium cholesteryl sulfate or cholesteryl-β-D-glucuronide as representatives was determined in spiked urine samples in triplicate, respectively. Separate spiking solutions of sodium cholesteryl sulfate (solution A; 105 µg/mL) and cholesteryl-β-D-glucuronide (solution B; 98 µg/mL) corresponding to 81.1 µg and 65.6 µg cholesterol/mL, respectively, were prepared using ethanol as solvent. After adding of 1.0 mL of either solution A or B to 50 mL of steroid free uirne obtained from a non-participating volunteer sample preparation was performed as described in section 3.2.4.3. Data on the recovery rate of cholesterol derived from sulfate and glucuronide conjugates calculated according to Eq. 7 using RRF (0.9475) of CS compared to 5αC as internal standard are presented in Tab. 25. Furthermore, the LOD of cholesterol (0.05 µg/50 mL urine) defined as a signal to noise ratio of 3:1 was empirically determined by repeated analysis of urine spiked with cholesteryl-β-D-glucuronide.

Tab. 25 Recovery of cholesterol (CS) in urine samples spiked either with sodium cholesteryl sulfate or cholesteryl-β-D-glucuronide determined by GC/FID

Sample	$Area_{IS}$[a] [pA × s]	$Area_{CS}$ [pA × s]	$Content_{CS}$[c] [µg/50 mL urine]	$Recovery_{CS}$ [%]
Sodium cholesteryl sulfate			*81.1*[b]	
I	44.8	611.9	80.9	99.7
II	45.6	627.7	81.5	100.5
III	42.8	567.9	78.6	96.9
Mean			80.3	99.0
SD			1.3	1.6
Cholesteryl-β-D-glucuronide			*65.6*[b]	
I	43.3	469.1	64.2	97.8
II	48.4	531.4	65.0	99.1
III	46.3	514.2	65.8	100.2
Mean			65.0	99.1
SD			0.7	1.0

[a] Area obtained for the internal standard (IS) 5α-cholestane [6.25 µg/50 mL urine]

[b] Values related to 1.0 mL of spiking solutions of sodium cholesteryl sulfate [105 µg/mL ≡ 81.1 µg CS/mL] and cholesteryl-β-D-glucuronide [98 µg/mL ≡ 65.6 µg CS/mL] added to 50 mL urine

[c] Calculated on the basis of a relative response factor of cholesterol (0.9475) compared to 5α-cholestane

5.5.3.2 Sample analysis

In order to prove whether urinary excretion of PS and their metabolites might be accountable for the conflicting amounts of liberated phytosterols/-stanols determined in feces and expected from the calculated hydrolysis rates, urine samples of three subjects (numbers 3, 5, and 10; Tab. 13 and Tab. 14) within the Benecol®- and pro.activ®-trial revealing maximum discrepancies were chosen for analysis. In the urine investigated phytosterols/-stanols or related derivatives could not be identified considering the LOD of 0.05 µg/50 mL determined for cholesterol at any time point of sample collection (day 0, day 3, and day 6; Fig. 33). Thus, in the context of the applied methodology it seems unlikely that PS have been subject to metabolization by phase I and II reactions and finally elimination via urine in significant quantities.

5.6 CONCLUSIONS

In conclusion, this is the first human study that demonstrates the structure-dependent hydrolysis of phytosteryl/-stanyl esters upon digestion of complex ester mixtures via enriched skimmed milk drinking yogurts. Despite large inter-individual variability, the results clearly indicate that hydrolysis is significantly affected by the acid moiety (order of hydrolysis: oleate = linoleate = linolenate > eicosanoate > palmitate > ferulate) and the phytosterol/-stanol moiety (order of hydrolysis: desmethylsterols > desmethylstanols (=dimethylsterols)). However, the impact of the sterol/stanol moiety is less pronounced than the impact of the acid moiety. Considering the large variability observed in this study, more detailed investigations on the correlations between the molecular structures of phytosteryl/-stanyl fatty acid ester, their hydrolysis rates and the resulting cholesterol-lowering properties for individual subjects seem reasonable. The simultaneous quantitation of individual intact esters and liberated sterols/stanols revealed subject-dependent discrepancies between the amounts of phytosterols/-stanols found in the feces and those expected from the calculated hydrolysis rates. Due the fact that these discrepancies are obviously not explainable by either delayed fecal excretion of liberated phytosterols/-stanols or increased plasma phytosterol/-stanol levels as well as urinary elimination, this phenomenon should also be further investigated, in particular in the light of the ongoing discussions regarding effects of plant sterols on vascular function [125-128].

6 SUMMARY

The metabolic fate of individual phytosteryl/-stanyl fatty acid and ferulic acid esters upon consumption and digestion by healthy human subjects has been investigated by *in vitro* fermentation experiments and in a randomized human trial.

For the *in vitro* experiments focussing on the metabolic activity of the gut microflora, the conditions such as period of fermentation, inoculum preparation, substrate concentration and substrate addition, described for dietary fiber fermentations were adapted to the more lipohilic steryl/stanyl esters. Incubation experiments were performed using an incoulum made up of fresh feces of two volunteers defined as “high-converter” of cholesterol to ensure that the respective microflora is at least capable to metabolize sterols in their free form. The two media used were on the one hand a simple carbonate- phosphate buffer solution enriched with urea and trace elements and on the other hand thioglycollate broth, an all-purpose medium for the cultivation and isolation of fastidious anaerobic microorganisms. In order to verify the suitability of the adjusted set up *trans*-ferulic acid, as a potential product of ester hydrolysis, was employed as substrate. The impact of the large intestine bacteria on the metabolization of intact esters exhibiting different molecular structures was investigated by fermentations of cycloartenyl ferulate, cholesteryl cinnamate, cholesteryl palmitate as well as ethyl ferulate. In the two media used, *trans*-ferulic acid was almost completely degraded; however, the metabolites identified based on the comparison of relative retention times and mass fragmentation pattern of reference substances varied upon fermentation. Metabolites obtained in the nutritive buffer were identified as caffeic acid, dihydrocaffeic acid and 3-(3-hydroxyphenyl)propionic acid, whereas microbial transformation of ferulic acid in the thioglycollate broth led to 3-(3-methoxyphenyl)propionc acid, 3-(3-hydroxyphenyl)propionic acid and dihydro-cinnamic acid. For the steryl esters tested neither ester hydrolysis nor other types of metabolization could be proven in any of the media under employed conditions. Hydrolytic and metabolic acitivity of the colonic microflora was solely observed for ethyl ferulate, a non-steryl ester exhibiting the simplest molecular structure, whereby the metabolic fates differed in the two media used. Using inoculated nutritive buffer, ethyl ferulate was only partially hydrolyzed and the liberated ferulic

acid was not subject of further microbial transformations. Upon incubation with the inoculated thiglycollate broth, hydrolysis was virtually complete and intermediately liberated ferulic acid was further metabolized to 3-(3-methoxyphenyl)propionic acid and dihydrocinnamic acid, metabolites also identified using *trans*-ferulic acid as substrate.

In the performed human trial hydrolysis rates for a total of twenty-one phytosteryl/-stanyl esters were determined by simultaneous determination of individual intact esters, liberated phytosterols/-stanols and their metabolites recovered in the feces of fourteen healthy subjects. Almost 73 % of phytostanyl fatty acid esters, 80 % of phytosteryl fatty acid esters, 26 % of desmethylsteryl ferulates as well as 16 % of desmethylstanyl ferulates were hydrolyzed upon digestion of complex mixtures via enriched skimmed milk drinking yogurts. Liberated dimethylsterols were not identified in the feces indicating that the *in vivo* hydrolysis of the corresponding ferulic acid esters by digestive enzymes is extremely unlikely. Contrary to expectations, large inter-individual variabilities regarding the total hydrolysis rates of phytosteryl fatty acid esters and phytostanyl fatty acid esters ranging from 49 % – 96 %, and 41 % – 87 %, respectively, were observed. Furthermore, subject-dependent discrepancies between the amounts of phytosterols/-stanols, including microbial metabolites, found in the feces and those expected from the calculated hydrolysis rates were determined. The fate of the missing phytosterols/-stanols remained unclear due to the fact that these discrepancies were not explainable by either delayed fecal excretion of liberated phytosterols/-stanols, increased plasma phytosterol/-stanol levels or urinary elimination.

Based on the comparison of the amounts of individual intact esters recovered in feces with the respective amounts consumed, structure dependent hydrolysis rates were determined for nine phytostanyl fatty acid esters, six phytosteryl fatty acid esters and six phytosteryl/-stanyl ferulic acid esters. The hydrolysis of dietary phytosteryl/-stanyl esters by digestive enzymes during gastrointestinal passage in healthy humans is significantly affected by both the acid moiety (order of hydrolysis: oleate = linoleate = linolenate > eicosanoate > palmitate > ferulate) and the sterol/-stanol moiety (order of hydrolysis: desmethylsterols > desmethylstanols (=dimethylsterols)). However, the impact of the acid moiety was much more

pronounced than the impact of the sterol/stanol moiety. Surprisingly, the increase in fecal cholesterol excretion for fatty acid esters as well as ferulic acid esters, taken as a marker of cholesterol-lowering properties, was in the same order of magnitude independently of the degree of phytosteryl/-stanyl ester hydrolyis.

7 ZUSAMMENFASSUNG

Zur Untersuchung des metabolischen Schicksals individueller Phytosteryl/-stanylester in der Humanernährung wurden im Rahmen der vorliegenden Arbeit *in vitro* Fermentationsexperimente sowie eine randomisierte Humanstudie im cross-over Design mit oraler Applikation funktioneller Trinkjoghurts durchgeführt, die mit komplexen Estermischungen angereichert waren.

Ziel der *in vitro* Studien war es, den Einfluss der intestinalen Mikroflora des humanen Dickdarms auf den Metabolismus verschiedener Phytosterylester zu untersuchen. Zur Durchführung der Fermentationsexperimente wurde zunächst auf Grundlage einer für die Ballaststofffermentation eingesetzen Methode eine optimierte Fermentationsmethode für lipophile Substanzen durch die Adaption verschiedener Parameter wie Fermentationszeit, Inokulumkonzentration sowie Vorlage und Konzentration des Substrates entwickelt. Zur Herstellung des Inokulums wurden Fäzesproben von freiwilligen Spendern eingesetzt, deren Mikroflora eine hohe Transformationsrate für Cholesterol aufwies, wodurch diese als „High-Converter“ klassifiziert wurden. Die Fermentationen wurden in zwei verschiedenen Kulturmedien durchgeführt: (I) in einer einfachen mit Harnstoff und Spurenelementen angereicherten Carbonat-Phosphat-Pufferlösung sowie (II) in Thioglycollat-Bouillon, einem Komplexmedium speziell geeignet für die Anzucht anaerober Bakterien. Zur Verifizierung der Versuchsbedingungen wurde *trans*-Ferulasäure, die ein mögliches Hydrolyseprodukt der Phytosteryl/-stanylester darstellt, als Substrat zur Fermentation eingesetzt. Um die metabolische Aktivität der humanen Dickdarmflora im Bezug auf strukturell verschiedene Sterylester zu untersuchen, wurden Fermentationsexperimente mit Cycloartenylferulat, Cholesterylcinnamat, Cholesterylpalmitat und zusätzlich mit Ethylferulat, einem strukturell weniger komplexen Ester, durchgeführt. Ferulasäure wurde in den beiden Medien vollständig metabolisiert. Die jeweiligen Metabolite wurden durch Vergleich der relativen Retentionszeiten und Massenspektren von Referenz-substanzen identifiziert. Unter Verwendung der Carbonat-Phosphat-Pufferlösung wurde die *trans*-Ferulasäure in die Metabolite Kaffeesäure, Dihydrokaffeesäure und 3-(3-Hydroxyphenyl)-propionsäure metabolisiert. In der Thioglycollat-Bouillon erfolgte die mikrobielle Transformation zu 3-(3-Methoxyphenyl)-propionsäure,

3-(3-Hydroxyphenyl)-propionsäure und Dihydrozimtsäure. Für Cycloartenylferulat, Cholesterylcinnamat und Cholesterylpalmitat konnten indes in keinem der beiden Kulturmedien eine Esterhydrolyse oder sonstige Metabolisierungen festgestellt werden. Die hydrolytische und metabolische Aktivität der intestinalen Mikroflora konnte in den Fermentationen von Ethylferulat nachgewiesen werden. Wurde Ethylferulat in der Carbonat-Phosphat-Pufferlösung nur teilweise hydrolysiert und nur Ferualsäure als Metabolit identifizert, erfolgte in Thiglycollat-Bouillon eine vollständige Esterydroylse und analog zu den Experimenten mit *trans*-Ferulasäure als Substrat die weiterführende Metabolisierung der intermediär freigesetzten Ferulasäure zu 3-(3-Methoxyphenyl)-propionsäure und Dihydrozimtsäure.

Zur Untersuchung des Metabolismus *in vivo* wurde eine Humanstudie mit 14 männlichen Probanden durchgeführt. Basierend auf der simultanen Quantifizierung individueller intakter Ester, freigesetzter Phytosterole/-stanole sowie deren Metabolite in humanen Fäzesproben wurden die Hydrolyseraten von insgesamt 21 Phytosteryl/-stanylestern ermittelt. Nach oraler Applikation von mit komplexen Phytosteryl/-stanylestermischungen angereicherten Magermilch-Trinkjoghurts wurden 73 % der enthaltenden Stanylfettsäureester, 80 % der Sterylfettsäureester sowie 26 % bzw. 16 % der Desmethylsteryl- und stanylferulasäureester im humanen Gastrointestinaltrakt hydrolysiert. Freie Dimethylsterole oder deren Metabolite wurden nicht in den Fäzesproben identifziert, so dass die Hydrolyse von Dimethylsterylferulasäurestern durch humane Verdauungsenzyme ausgeschlossen werden kann. Entgegen den Erwartungen wurden zudem große interindividuelle Unterschiede in den Hydrolyseraten der Phytosterylettsäureester (49 % - 96 %) sowie Phytostanyl-fettsäureester (41 % - 87 %) nachgewiesen. Des Weiteren wurden in den individuellen Probanden Diskrepanzen bezüglich der im Fäzes quantifizierten Gehalte an Phytosterolen/-stanolen inklusive deren Metabolite sowie denen auf Grund der Esterhydrolyse kalkulierten Stoffmengen bestimmt. Der Verbleib dieser Phytosterole und -stanole konnte im Rahmen der durchgeführten Studie nicht abschließend geklärt werden, weil weder eine verzögerte Exkretion mit dem Fäzes oder eine Ausscheidung mit dem Urin noch erhöhte Plasmakonzentrationen von Phytosterolen/ -stanolen nachgewiesen wurden.

Durch den Vergleich der Stoffmengen individueller intakter Ester, die in den Fäzesproben quantifiziert wurden, und den entsprechenden mit den Trinkjoghurts konsumierten Stoffmengen wurden erstmalig strukturabhängige Hydrolyseraten von insgesamt neun Phytostanylfettsäureestern, sechs Phytosterylfettsäureestern und sechs Phytosteryl/-stanylferulasäureestern innerhalb des humanen Gastrointestinaltraktes bestimmt. Die Hydrolyse der Phytosteryl/-stanylester durch die Verdauungsenzyme wurde sowohl durch die Molekülstruktur der enthaltenden Säuren (Ausmaß der Hydrolyse: Oleat = Linolat = Linolenat > Eicosanat > Palmitat > Ferulat) als auch durch die Phytosterol/-stanolstruktur (Ausmaß der Hydrolyse: Desmethylsterole > Desmethylstanole (= Dimethylsterole)) signifikant beeinflusst. Dabei war der Einfluss der Säurestruktur im Vergleich zur Phytosterol/-stanolstruktur deutlich stärker ausgeprägt. Die Erhöhung der fäkalen Auscheidung von Cholesterol, allgemein beschrieben als ein Indikator für die cholesterinsenkenden Eigenschaften von Phytosteryl/-stanylestern, hingegen war für die Fettsäureester und Ferulasäurester in einer vergleichbaren Größenordnung und damit unabhängig vom Ausmaß der Esterhydrolyse.

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9 CURRICULUM VITAE

PERSONAL INFORMATION

Name:	Tim Lubinus
Date and place of birth:	March 09, 1978 in Emden · Germany
Nationality:	German
Marital status:	Married
Current address:	Abenberger Str. 1a
	D-91126 Schwabach · Germany

EDUCATION

11/2008 – 04/2012	PhD thesis: *"Fate of dietary phytosteryl/-stanyl esters upon digestion by humans: Analysis of individual intact esters in feces"* Research group of Univ.-Prof. Dr. Karl-Heinz Engel (Chair of General Food Technology, Technische Universität München) and Research fellow of the Research Training Group 1482
01/2009	2. Staatsexamen in Food Chemistry, Hamburg
11/2007 – 10/2008	Post-graduate internship, Hamburg
09/2007	Degree in Food Chemistry (1. Staatsexamen und Diplom); Diplomarbeit: *"Isolierung von oligomeren Hydroxyzimtsäuren aus Maisstroh"*, Universität Hamburg
04/2002 – 09/2007	Academic studies of Food Chemistry, Universität Hamburg
02/2001 – 03/2002	Chemical laboratory assistant, Emden
08/1998 – 01/2001	Professional training (Chemical laboratory assistant), Emden
07/1997 – 04/1998	Military service (German Air Force)
08/1990 – 06/1997	Allgemeine Hochschulreife,Gymnasium am Treckfahrtstief, Emden

PUBLICATIONS

Lubinus, T., A. Barnsteiner, T. Skurk, H. Hauner, and K.-H. Engel. **2013**. Fate of dietary phytosteryl/-stanyl esters: analysis of individual intact esters in human feces. *Eur. J. Nutr.* (52): 997-1013

Barnsteiner, A., **T. Lubinus**, A. di Gianvito, W. Schmid, and K.-H. Engel. **2011**. GC-Based Analysis of Plant Stanyl Fatty Acid Esters in Enriched Foods. *J. Agric. Food Chem.* (59): 5204-5214

CONFERRENCES

49. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung **2012** – Freising · Germany
Talk: *"Humanstudie zur Untersuchung der strukturabhängigen Metabolisierung von Phytosteryl/-stanylestern nach dem Verzehr funktioneller Lebensmittel"*

40. Deutscher Lebensmittelchemikertag **2011** – Halle a.d. Saale · Germany
Poster: *"In vivo-Metabolismus von Phytosteryl/-stanylestern: Quantitative Bestimmung individueller intakter Ester in humanen Fäzesproben"*

EuroFoodChem XVI **2011** – Gdansk · Poland
Poster: *"Fate of dietary phytosteryl/-stanyl esters upon digestion by humans: Analysis of individual intact esters and their metabolites in feces"*

AWARDS

Young Researcher Award **2011** – EuroFoodChem XVI
Competence in Food Award **2009** – Universität Hamburg
Innovations-Preis der Unilever Deutschland GmbH **2007** – Universität Hamburg