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ABTEILUNG GASTROINTESTINALE MIKROBIOLOGIE

Impact of intestinal bacteria on the anatomy and physiology of the intestinal tract in the PRM/Alf mouse model

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ABSTRACT

Introduction: Intestinal bacteria influence gut morphology by affecting epithelial cell proliferation, development of the lamina propria, villus length and crypt depth [1]. Gut microbiota-derived factors have been proposed to also play a role in the development of a 30 % longer intestine, that is characteristic of PRM/Alf mice compared to other mouse strains [2, 3]. Polyamines and SCFAs produced by gut bacteria are important growth factors, which possibly influence mucosal morphology, in particular villus length and crypt depth and play a role in gut lengthening in the PRM/Alf mouse. However, experimental evidence is lacking. **Aim:** The objective of this work was to clarify the role of bacterially-produced polyamines on crypt depth, mucosa thickness and epithelial cell proliferation. For this purpose, C3H mice associated with a simplified human microbiota (SIHUMI) were compared with mice colonized with SIHUMI complemented by the polyamine-producing *Fusobacterium varium* (SIHUMI + Fv). In addition, the microbial impact on gut lengthening in PRM/Alf mice was characterized and the contribution of SCFAs and polyamines to this phenotype was examined. **Results:** SIHUMI + Fv mice exhibited an up to 1.7-fold higher intestinal polyamine concentration compared to SIHUMI mice, which was mainly due to increased putrescine concentrations. However, no differences were observed in crypt depth, mucosa thickness and epithelial proliferation. In PRM/Alf mice, the intestine of conventional mice was 8.5 % longer compared to germfree mice. In contrast, intestinal lengths of C3H mice were similar, independent of the colonization status. The comparison of PRM/Alf and C3H mice, both associated with SIHUMI + Fv, demonstrated that PRM/Alf mice had a 35.9 % longer intestine than C3H mice. However, intestinal SCFA and polyamine concentrations of PRM/Alf mice were similar or even lower, except *N*-acetylcadaverine, which was 3.1-fold higher in PRM/Alf mice. When germfree PRM/Alf mice were associated with a complex PRM/Alf microbiota, the intestine was one quarter longer compared to PRM/Alf mice colonized with a C3H microbiota. This gut elongation correlated with levels of the polyamine *N*-acetylspermine. **Conclusion:** The intestinal microbiota is able to influence intestinal length dependent on microbial composition and on the mouse genotype. Although SCFAs do not contribute to gut elongation, an influence of the polyamines *N*-acetylcadaverine and *N*-acetylspermine is conceivable. In addition, the study clearly demonstrated that bacterial putrescine does not influence gut morphology in C3H mice.

ZUSAMMENFASSUNG

Einleitung: Die intestinale Mikrobiota beeinflusst die Morphologie des Darmes durch Beeinflussung der Epithelzellproliferation, Entwicklung der Lamina Propria, Zottenlänge und Kryptentiefe [1]. Zudem stehen bakterielle Faktoren im Verdacht, die Entwicklung eines 30 % längeren Darmes in der PRM/Alf Maus gegenüber anderen Mausstämmen zu begünstigen [2, 3]. Die von der intestinalen Mikrobiota produzierten Polyamine und kurzkettigen Fettsäuren (SCFA) stellen wichtige Wachstumsfaktoren dar, die bei der Ausbildung des Darmes sowie an der Darmverlängerung in der PRM/Alf Maus beteiligt sein könnten. **Zielstellung:** Ziel dieser Arbeit war, den Einfluss von bakteriell-produzierten Polyaminen auf die Kryptentiefe, Schleimhautdicke und Epithelzellproliferation zu untersuchen. Zu diesem Zweck wurden keimfreie C3H Mäuse mit einer vereinfachten menschlichen Mikrobiota (SIHUMI) assoziiert und mit C3H Mäusen, die mit einer SIHUMI plus dem polyaminproduzierendem *Fusobacterium varium* (SIHUMI + Fv) besiedelt worden waren, verglichen. Weiterhin sollte der mikrobielle Einfluss sowie die Rolle von SCFAs und Polyaminen bei der Ausbildung eines verlängerten Darms in der PRM/Alf Maus untersucht werden. **Ergebnisse:** Die SIHUMI + Fv Mäuse zeigten eine bis zu 1,7-fach höhere intestinale Polyaminkonzentration im Vergleich zu SIHUMI-Mäusen, welche vor allem auf eine Erhöhung von Putrescin zurückzuführen war. Trotz der höheren Polyaminkonzentrationen wurden keine Unterschiede in der Kryptentiefe, Schleimhautdicke und Epithelzellproliferation beobachtet. Die Untersuchung der Darmlänge in PRM/Alf Mäusen in Abhängigkeit vom Besiedlungsstatus ergab einen 8,5 % längeren Darm in konventionell besiedelten PRM/Alf Mäusen im Vergleich zu keimfreien PRM/Alf Mäusen. Im Gegensatz dazu wurden in C3H-Mäusen keine Unterschiede in der Darmlänge in Abhängigkeit von der Besiedlung beobachtet. Der Vergleich zwischen PRM/Alf und C3H Mäusen, die beide mit der SIHUMI + Fv Mikrobiota assoziiert wurden, zeigte einen 35,9 % längeren Darm in PRM/Alf Mäusen. Trotz des längeren Darmes waren die intestinalen SCFA- und Polyaminkonzentrationen vergleichbar bzw. geringer als in C3H Mäusen, mit einer Ausnahme: Die Konzentration von *N*-Acetylcadaverin war in PRM/Alf Mäusen 3,1-fach erhöht. Wurden keimfreie PRM/Alf Mäuse mit einer komplexen PRM/Alf Mikrobiota assoziiert, so war ihr Darm ein Viertel länger als bei PRM/Alf Mäusen, die mit einer C3H Mikrobiota besiedelt wurden. Dieser längere Darm korrelierte mit der *N*-Acetylsperminkonzentration. **Schlussfolgerung:** Die intestinale Mikrobiota ist in der Lage, die Darmlänge abhängig von der mikrobiellen Zusammensetzung und von dem Genotyp des Wirtes zu beeinflussen. Obwohl SCFAs die Darmlänge nicht beeinflussten, ist eine Beteiligung der Polyamine *N*-Acetylcadaverin und *N*-Acetylspermin denkbar. Darüber hinaus zeigte die Studie, dass Putrescin die Anatomie des Darmes in C3H Mäusen nicht beeinflusst.

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ABBREVIATIONS

16S rRNA	Prokaryotic small subunit ribosomal ribonucleic acid
2D-DIGE	Two-dimensional difference gel electrophoresis
5'MTA	5'Methylthioadenosine
A	Adenine
AccQ Fluor	6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate
Acetyl CoA	Acetyl Coenzyme A
AMQ	6-Aminoquinoline
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
Bp	Basepair
C	Cytosine
cpnDB	Chaperonin database
C_T	Cycle treshold
DNA	Desoxyribonucleic acid
dNTP	Desoxynucleotide triphosphate
DSM	Deutsche Sammlung für Mikroorganismen
dw	Dry weight
et al.	"et allis" (and others)
f	Forward primer
FD	Fluorescence detection
g	Relative centrifugal field
G	Guanine
H_2Odd	Bidistilled water
HPLC	High performance liquid chromatography
INRA	Institut National de la Recherche Agronomique
IPG	Immobilized pH gradient
LOD	Limit of detection
LOQ	Limit of quantification
MCT1	Monocarboxylate transporter 1
MOPS	Morpholinepropanesulfonic acid
n	Number
n.d.	Not detected

NanoLC-ESI/MS-MS	Nano liquid chromatography-electrospray ionization-tandem mass spectrometry
NCBI	National Center for Biotechnology Information
NCC	Nestlé Capital Corporation
NHS	*N*-hydroxysuccinimide
OD_{600}	Optical density measured at 600 nm
P	Probability value
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PRM/Alf	**PR**etty **M**ouse/ Ecole Nationale Vétérinaire d'**ALF**ort
PRM/C3H	PRM/Alf mice colonized with a C3H microbiota
PRM/PRM	PRM/Alf mice colonized with a PRM/Alf microbiota
qPCR	Quantitative real-time PCR
r	Reverse primer
RNA	Ribonucleic acid
rpm	Round per minute
RT	Room temperature
SAFE	Scientific Animal Food & Engineering
SAM	S-adenosylmethionine
SAMHC	S-adenosylmethyl homocysteamine
SCFA	Short chain fatty acid
SDS	Sodium dodecyl sulfate
SIHUMI	Simplified human microbiota
SIHUMI + Fv	Simplified human microbiota plus *Fusobacterium varium*
T	Thymine
Taq	Thermus aquaticus
v/v	Volume by volume
w/v	Weight by volume
YCFA GSC	Yeast extract-casitone-fatty acids containing glucose, soluble starch and cellobiose

An "s" behind an abbreviation is indicative of the plural.

1 INTRODUCTION

1.1 Structure and function of the gastrointestinal tract

The human gut wall is composed of four layers: tunica serosa, tunica muscularis, tela submucosa and tunica mucosa [4] (Figure 1.1 A). The tunica serosa, which consists of an epithelial layer and connective tissue, secretes a protein-rich fluid to reduce friction of muscle movements [5]. The tunica muscularis is composed of a circular and longitudinal muscle layer as well as the myenteric plexus (or Auerbach's plexus) to regulate peristaltic gut movements [4, 6]. Main components of the tela submucosa are collagen fibers, submucosal plexus (or Meissner Plexus) and blood vessels. The tela submucosa tolerates movements of tunica mucosa and tunica muscularis, regulates motility of the inner gut wall and ensures nutrient supply for the gut wall. The layer adjoining the intestinal lumen is the tunica mucosa consisting of lamina epithelialis, lamina propria and lamina muscularis mucosae. The lamina muscularis mucosae is a small muscle layer responsible for gut motility and gut distension. The lamina propria regulates the defense against pathogens, while the lamina epithelialis functions for digestion and absorption of nutrients [7].

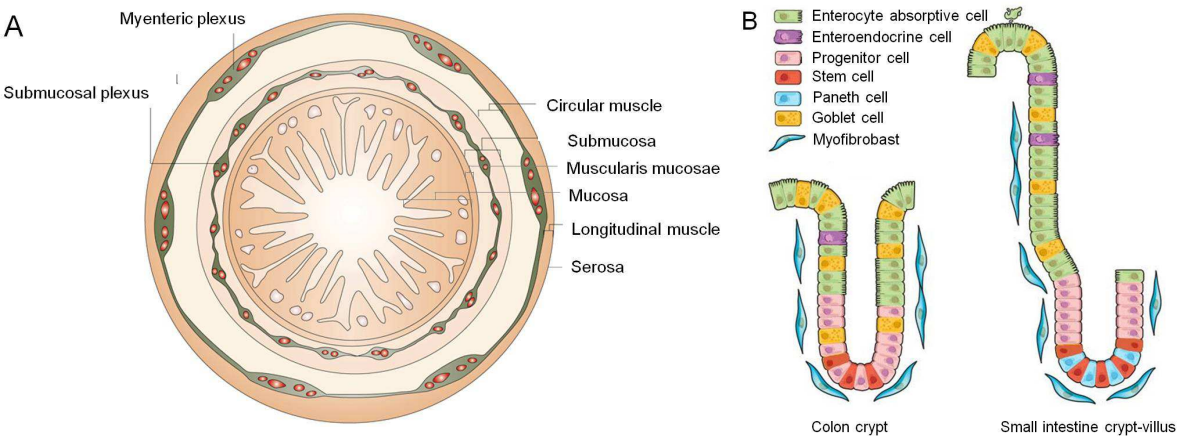

Figure 1.1 A: Schematic representation of a transverse section through the small intestine. The small intestinal mucosa is characterized by the villus-crypt unit. Adapted from [8]. **B: Schematic representation of the organization of colon crypt and small intestinal crypt-villus unit.** Adapted from [9]

The small intestinal epithelium is composed of enterocytes, enteroendocrine cells, Paneth cells and goblet cells, whereas the colonic epithelium lacks Paneth cells (Figure 1.1 B) [7, 10]. Enterocytes are the most abundant intestinal epithelial cells with a proportion of up to 80 %. The cells are columnar with apical microvilli on top and lateral junctions to their neighboring cells. They have hydrolytic and absorptive functions and are responsible for nutrient degradation. The turnover of enterocytes is around three days [6, 7]. Goblet cells are characterized by specific mucous granules located in the cytoplasm. They synthesize and release mucin, which creates a barrier to prevent the direct contact between large particles of the intestinal content and the epithelial cell layer [7, 11]. Their turnover is around three days [7]. Enteroendocrine cells represent a small percentage of the intestinal epithelium and secrete several peptides including cholecystokinin, secretin, somatostatin, gastrin and serotonin. Paneth cells are columnar with apical cytoplasmic granules. Due to their secretion of lysozymes, lactoferrin, peptidases and defensins, they contribute to the antimicrobial defense of the intestine. Around ten Paneth cells are present per crypt located at the crypt bottom. Their turnover period is around 20 days [4, 7].

1.2 Mechanism of mucosal growth

Intestinal growth during fetal and postnatal life involves two independent processes (Figure 1.2) [12]. First, the number of crypts increases via binary crypt fission (duplication). This process occurs mainly in early fetal life and is associated with cylindrical growth of intestinal length and diameter [13, 14]. Small indentations are formed at the crypt base and divide the crypt longitudinally into two daughter crypts (Figure 1.2 A) [12-14]. It is believed that this process is triggered by a 2-fold increase in the number of intestinal stem cells, but this has not yet been confirmed experimentally [15, 16]. Second, crypt hyperplasia as a result of increased cell proliferation promotes luminal mucosal growth mainly during and after weaning (Figure 1.2 B) [12]. Although the maximum performance of both processes is temporally shifted, there is an overlap to ensure the continuous increase of intestinal surface [17, 18].

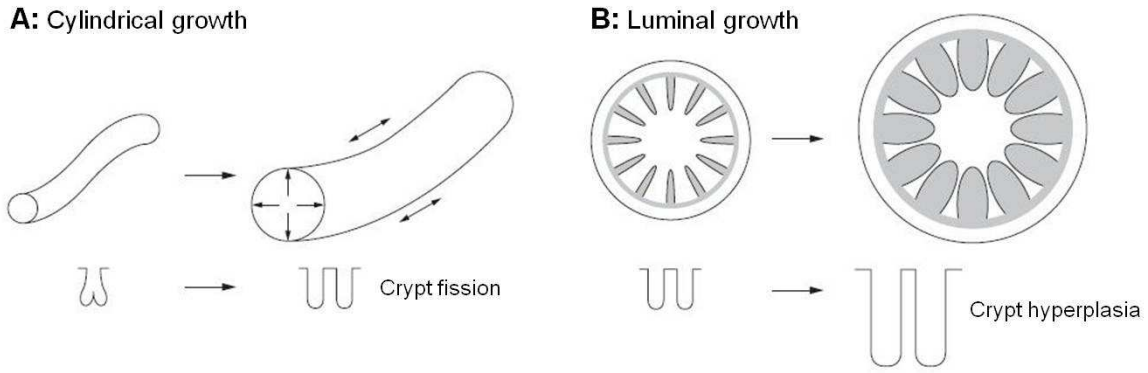

Figure 1.2: Postnatal growth of the small intestine. Cylindrical growth (A) is associated with crypt fission, whereas luminal growth (B) accompanies crypt hyperplasia. Adapted from [12]

Intestinal growth is a complex process, whose mechanisms have not yet been fully identified. It is suggested that not only genetic pathways such as the Wnt/β-catenin and the Notch pathway stimulate intestinal growth by promoting intestinal crypt fission [18, 19], but also environmental factors such as nutritional components (e.g. fiber, fatty acids, triglycerides, glutamine, polyamines, lectins) [20]. Further factors associated with intestinal growth include peptide growth factors (e.g. epidermal growth factor, transforming growth factor-α and -β, keratinocyte growth factor, growth hormone, erythropoietin, hepatocyte growth factor, glucagon-like peptide-2, insulin, insulin-like growth factor, neurotensin and gastrin), pancreatic secretions and cytokines (e.g. interleukin (IL) -11, IL-3, IL-15) [12, 20-22]. Moreover, there is increasing evidence that crypt fission and cell proliferation are controlled independently. For instance, epidermal growth factor, growth hormone, keratinocyte growth factor and polyamines are factors that reduce crypt fission, but promote crypt hyperplasia [12, 23-25].

Although there are many suggestions about factors stimulating intestinal growth, the regulatory mechanisms are not yet completely clarified. Moreover, it is still unknown which processes are genetically encoded and which role environmental factors play. For studies investigating intestinal growth, the PRM/Alf mouse model offers a unique opportunity.

1.3 PRM/Alf mouse: A model for intestinal lengthening

The PRM/Alf mouse is an inbred strain showing an one third longer intestine compared to other mouse strains including C3H/He, C57BL/6J and DBA/2J mice [2]. Both small and large intestine are proportionally elongated. The increased intestinal length does not impair digestive functions since the gut transit time is reduced [26]. The faster transport of

digesta results from an increased number of interstitial cells of Cajal, which are the pacemarkers for slow bowel contractions, increased contractility and slow-wave frequency.

Gut elongation in the PRM/Alf mouse arises from an interaction of genetic as well as environmental factors contributing approximately 60 % and 40 %, respectively, to this process [2, 3]. Since intestinal lengthening occurs before weaning, growth-promoting and genotype-specific factors in the milk as well as factors derived from the intestinal microbiota have been considered important [3]. However, the intestinotrophic factors causing gut elongation in the PRM/Alf mouse have not yet been identified. This identification would be important to unravel the mechanisms underlying intestinal lengthening and might help to better understand the factors controlling mucosal growth. Such knowledge could help to develop noninvasive, postoperative treatments after small and large bowel resection. In this project, the identification of intestinotrophic factors is focused on microbial factors, since they have been considered important.

1.4 Role of intestinal bacteria on intestinal morphology and physiology

The intestinal microbiota is a complex and dynamic ecosystem inhabiting the gastrointestinal tract. Their composition changes fundamentally at four life stages: immediately after birth, from birth to weaning, between weaning and receiving solid food as well as during old stage [27]. Immediately after birth, the gut is colonized with facultative anaerobes, which create anaerobic conditions for obligate anaerobes [27]. The change from a milk-based diet to a solid diet rich in complex carbohydrates results in an increasing bacterial diversity reaching more than 400 different species in adults [28, 29]. In elderly, there is a decline in microbial diversity, which is coincident with a shift in dominant bacterial species: Whereas Bacteroidetes become more abundant, Firmicutes are less abundant compared to adult controls [30, 31].

The changing microbial ecology during weaning is accompanied by functional and morphological gut maturation [29, 32] including immune function, intestinal metabolic activity and intestinal angiogenesis [33]. Whether those intestinal developmental changes are genetically encoded or whether some of them are triggered by intestinal bacteria is still a key question, but there is increasing evidence that bacteria are able to influence postnatal gut development [33]. That gut bacteria affect intestinal development may be deduced from numerous phenotypic differences between germfree and conventional animals [1, 28, 34, 35]. Morphological and physiological characteristics of germfree

animals are listed in Table 1.1 and include decreased intestinal surface, reduced crypt depth, underdeveloped lamina propria, decreased epithelial cell turnover, drastically enlarged cecum as a result of mucus accumulation and increased intestinal motility due to an increased propulsion rate [1, 28]. In addition, the intestinal microbiota modulates host immune functions, influences host metabolism through the degradation of dietary fibers and provides colonization resistance against pathogens [1, 28, 34].

Although the microbial factors that trigger these effects are poorly defined, one bacterium has been demonstrated to stimulate some intestinal developmental changes during weaning: *Bacteroides thetaiotaomicron*, inoculated into germfree mice, induces fucosylation of intestinal epithelial cells, stimulates villus capillary network development and increases the expression of the microbicidal protein Angiogenin 4 to levels similar to that of conventional mice [36-38]. From these experiments it is assumed that the intestine is either genetically pre-programmed to receive bacterial signals or is poised for interactions with its microbiota, which are essential for normal gut development [33]. However, bacterial signals or factors stimulating intestinal maturation have not yet been identified. Moreover, it is so far unknown how gut morphological and physiological changes are brought about and which bacterial factors are responsible for these changes.

Table 1.1: Phenotypic characterization of germfree animals focused on intestinal morphology and physiology[1]. Adapted from [1]

Observation in germfree animals	References
Intestinal morphology	
Total mass of the intestine ↓	[39-43]
Cecum size ↑	[28, 44, 45]
Total surface of the small intestine ↓ (30 %)	[43, 46]
Mucosa thickness ↓	[35, 44, 47]
Length of ileal villi ↓, length of duodenal villi ↑	[48-50]
Crypt depth ↓	[44, 47-49, 51]
Thickness and cellularity of lamina propria ↓	[35, 44, 48, 49]
Thickness of epithelial layer in cecum ↑	[44, 52]
Number of small intestinal goblet cells ↑, cecal goblet cells ↓	[1, 35, 53, 54]
Size of Paneth cells ↓, number of Paneth cells ↑	[35, 55]
Shape of epithelial cells: cecum = longer and slender; small intestine = uniform	[44, 56]
Microvilli length ↑	[44, 48]
Crypt cell proliferation ↓	[48, 51, 56]
Epithelial cell turnover ↓	[48, 50, 51]
Cellular renewal rate in lamina propria and Peyer's patch ↓	[35, 48]
Cecal mucus quantity ↑,	[53]
Mucus composition:	[53]
ratio of neutral to acidic mucin ↑, ratio of sulfomucin to sialomucin ↑	
Villus capillary network ↓	[33, 38]
Intestinal physiology	
Intestinal transit time ↓	[57, 58]
Intestinal motility ↓ due to propulsion rate ↓	[58, 59]
Spontaneous muscle contraction in cecum ↓	[60]
Luminal pH ↑	[58]
Brush border enzyme concentration ↑	[58, 61, 62]
(e.g. lactase, maltase, sucrose, alkaline phosphatase)	
Activity of Mg^{2+}-dependent and (Na^+ + K^+)-stimulated ATPase ↑	[63]
Requirement of vitamin K and vitamin B ↑	[2, 58, 64-66]
Oxygen tension and redox potential ↑	[35, 67]
Absorption of minerals (Ca, Mg,) ↑ and amino acids ↑	[58, 68]
Absorption of dietary fat ↑, cholesterol ↑	[69, 70]
Reabsorption of bile acids ↑	[71]
Excretion of bilirubin ↑, mucin ↑, urea ↑, nitrogen ↑ and ammonia ↓	[69, 72-74]

[1] Microbial effects on host immune system, energy metabolism, endocrine system, host susceptibility to infections and effects on liver, lungs, heart, skin, kidney and vessels were not included.

↑ increased, ↓ reduced

1.5 Growth factors produced by intestinal bacteria

Bacterial factors that potentially stimulate intestinal growth include short chain fatty acids (SCFAs) and polyamines, since they possess trophic functions [75, 76]. SCFAs are organic fatty acids with 2 to 6 carbon atoms. They arise from bacterial breakdown of polysaccharides, oligosaccharides, proteins, peptides and glycoproteins [77]. The major SCFAs are acetate, propionate and butyrate, with typical ratios in human feces of around 3:1:1 [78]. The total concentration of SCFAs detected in feces is less than 5 % of the total amount produced by intestinal bacteria. This is due to a very effective colonic absorption of SCFAs via passive diffusion and the carrier transporter protein MCT1 [77, 79, 80]. SCFAs increase the frequency of spontaneous contractions of smooth muscles [81], reduce the thickness of the mucus layer [82] and regulate cell proliferation, cell differentiation as well as gene expression [77]. The trophic effects of SCFAs have been reported repeatedly [76, 83-86]. In rodents, direct infusions of SCFAs into cecum or colon caused trophic effects on villus height and crypt depth [83, 84], increased cell proliferation [84, 87] and stimulated mucosal growth by increasing mucosal mass, DNA, RNA and protein content [83, 85]. Moreover, supplementation of parenteral food with SCFAs prevents mucosal atrophy, which occurs without the supplementation [88, 89]. SCFAs also improve structural markers of adaptation to small bowel resection in adult rats [90-92]. Since similar trophic effects were observed when total parenteral nutrition was either supplemented with a mixture of SCFAs or butyrate alone, butyrate appears to be the primary trophic factor among the SCFAs [85, 93]. Addition of butyrate to total parenteral nutrition stimulated mucosal growth and increased villus height, crypt depth and cell proliferation [85, 93].

There are two main mechanisms discussed in the literature how SCFAs can stimulate cell proliferation in non-malignant cells. First, SCFAs, in particular butyrate, are the main energy source for colonocytes and may stimulate cell proliferation directly as an energy-rich substance [94]. Approximately 95 % of the butyrate produced by colonic bacteria is transported across the epithelium [95] and used for ATP formation via acetyl-CoA [96, 97]. Second, SCFAs are able to stimulate the release of gastrointestinal peptides such as glucagon-like peptide-2 [98-100]. This peptide is secreted by enteroendocrine L cells [91, 101] and it acts as an intestinotrophic factor [102-106] leading to increases in intestinal weight, colonic length, villus height and crypt depth [107, 108]. Glucagon-like peptide-2 can stimulate intestinal mucosal growth due to an induction of cell proliferation and to an inhibition of enterocyte apoptosis [98, 109, 110]. Moreover, it reduces experimental intestinal injury in rodents with ischemic intestinal injury or colitis [107, 111] and also

stimulates intestinal adaptation after small bowel resection in the rat [108]. In addition to glucagon-like peptide-2, SCFAs can also stimulate the secretion of gastrin [112], however, its trophic effect on small and large intestinal mucosa is very controversially discussed [113].

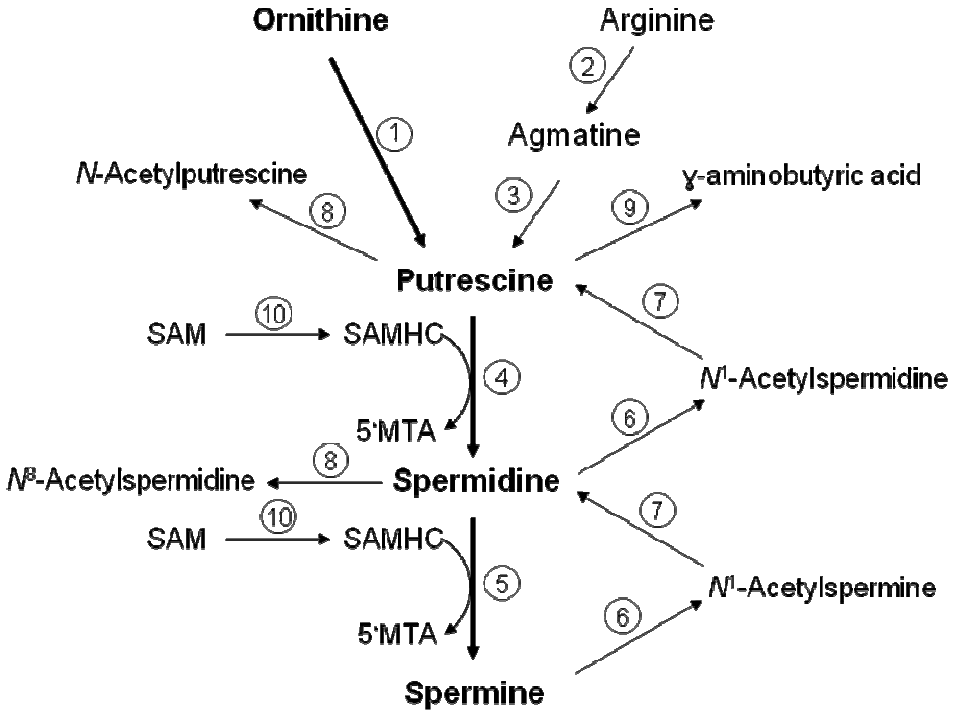

Figure 1.3: Synthesis and interconversion of polyamines. Adapted from [114]. 1. Ornithine-decarboxylase, 2. Arginine decarboxylase, 3. Agmatine decarboxylase, 4. Spermidine synthase, 5. Spermine synthase, 6. Acetyl-CoA:spermidine/spermine N^1-acetyltransferase, 7. Polyamine oxidase, 8. N^8-acetyltransferase, 9. Diamine oxidase, 10. SAM-decarboxylase, SAM: S-adenosylmethionine; SAMHC: S-adenosylmethyl homocysteamine; 5'MTA: 5-methylthioadenosine

Another trophic factor produced by intestinal microbiota are the polyamines with putrescine, spermidine and spermine as the major representatives. Cadaverine is structurally related to putrescine, but is not included in the general term of polyamines [75, 114, 115]. Since it has functions similar to that of putrescine (e.g. binding to macromolecules) [116, 117], cadaverine is mentioned at this point.

Polyamines are polycationic compounds composed of a hydrocarbon matrix with multiple amino groups [117]. They are ubiquitously distributed and can be synthesized by both prokaryotes and eukaryotes [118]. The main precursor of polyamines is putrescine, which is synthesized either from ornithine by ornithine decarboxylase (used by eukaryotes and prokaryotes) or from arginine by arginine decarboxylase and agmatine ureohydrolase

(used by some prokaryotes) (Figure 1.3) [75, 119]. Putrescine is converted into spermidine by spermidine synthase, which transfers one propylamine group derived from the decarboxylated S-adenosylmethionine (SAM). The conversion into spermine from spermidine occurs similarly by the enzyme spermine synthase. Polyamines can also be interconverted, which involves two reactions: acetylation via acetyl-CoA: spermidine/spermine N^1-acetyltransferase and cleavage via polyamine oxidase [75]. Sources of intestinal polyamines are either endogenous (intracellular de novo synthesis, interconversion pathways) or exogenous (diet, intestinal bacteria). Exogenous polyamines are rapidly and completely absorbed in duodenum via endocytosis and carrier-mediated active transport, but also via paracellular diffusion [115, 120].

The physiological importance of polyamines is based on their strong interaction with polyanionic macromolecules (DNA, RNA, proteins) and membrane structures (phospholipids) [75]. Due to these interactions, they are involved in essential cellular processes including replication, transcription and protein synthesis, but also affect cell growth, cell proliferation and neonatal gut maturation [75]. Since ornithine decarboxylase activity and polyamine levels are very high in hyperplastic diseases such as cancer, they are used as biomarkers [121]. Under physiological conditions, polyamine synthesis is increased during stages with maximum development, for instance in fetus, during first days of life or in mammary gland during pregnancy and lactation [122-124]. Oral administration of polyamines to rats results in morphologic and enzymatic maturation of the intestine. This maturation includes the induction of cell proliferation, an increase of DNA, RNA and protein content and the formation of intestinal brush border enzymes such as lactase, sucrase, maltase and aminopeptidase [125-129]. In agreement with these functions of polyamines, suckling rats fed polyamine-deficient diets showed mucosal hypoplasia of the small intestine and colon [130]. The causes for the described polyamine-mediated effects on cell proliferation might rely on their ability to stabilize DNA by binding at the minor grove or to regulate transcriptional factors and protein synthesis [75, 131]. Moreover, effects of polyamines on membrane functions might be explained by covalent modifications of membrane proteins, possibly via a transglutaminase-mediated incorporation of polyamines or a cross linking of membrane proteins [75, 132].

Although growth-promoting effects of SCFAs and polyamines have been well described, it is so far unknown, whether they are responsible for the morphological and physiological differences observed between germfree and conventional animals. Demonstrating such participation would clarify their impact on postnatal gut development and would provide new insights into host-microbe interactions.

1.6 Objectives

Intestinal growth is a complex process influenced by many genetic as well as environmental factors. There is increasing evidence that the intestinal microbiota is also involved in this process by affecting epithelial proliferation, development of the lamina propria, villus length and crypt depth. Although many effects of the microbiota on gut morphology and physiology have been described, it is not known how these effects are brought about and which microbial factors are involved. In addition, the bacterial influence on gut length has rarely been investigated. For such investigations, the PRM/Alf mouse offers a unique opportunity since these mice exhibit a 30 % longer intestine than other mouse strains. It has been proposed that genetic, nutritional and microbiota-derived factors contribute to the development of this phenotype, but experimental evidence lacks.

The objective of this study was therefore to characterize the effect of the microbiota on gut length and to identify bacterial factors involved in intestinal length and mucosal growth. Specifically, it was hypothesized that SCFAs and polyamines produced by the intestinal microbiota contribute to gut lengthening and intestinal growth, since they exert trophic functions. To test this hypothesis, two different approaches were used in this study.

First, to clarify the role of bacterial polyamines on mucosal parameters, germfree C3H mice were colonized with two different microbial communities producing low and high polyamine concentrations. The following question was investigated:

- Does an increased intestinal polyamine concentration produced by intestinal bacteria affect mucosal growth including crypt depth and thickness of different gut layers as well as intestinal physiology including proliferation and apoptosis?

Second, the PRM/Alf mouse as model for intestinal lengthening was colonized with different microbiotas to answer the following questions:

- To which extent does a complex intestinal microbiota contribute to gut lengthening in the PRM/Alf mouse?
- Does the colonization of germfree PRM/Alf mice with a defined community of eight bacterial species induce gut lengthening?
- Which bacterial molecules are involved in gut lengthening? Do SCFAs and/or polyamines play a role?

In parallel to the animal experiments, the polyamine formation of selected bacterial species was analyzed *in vitro*, to draw possible conclusions about polyamine concentrations measured *in vivo*.

2 MATERIALS AND METHODS

2.1 *In vitro* fermentation experiments

To characterize the polyamine formation of bacterial strains used in this study (Table 2.1), *in vitro* fermentation experiments were carried out. Bacteria were grown anaerobically in MOPS (morpholinepropanesulfonic acid) - buffered Ringer solution (100 mM MOPS, 8.6 g/l NaCl, 0.3 g/l KCl, 0.33 g/l $CaCl_2$ x 2 H_2O, 0.25 g/l L-cysteine x HCl, 1 mg/l resazurin, pH 7.0) supplemented with 24 g/l of pestled standard chow R03-04 (SAFE, France) and incubated at 37 °C for 48 h under continuous shaking at 120 rpm. For growth determination, 1 ml of bacterial cultures were taken at time point 0 h and 48 h, centrifuged at 300 x g, 3 min at 4 °C and optical densities of resulting supernatants were measured at 600 nm (OD_{600}) using SmartSpecPlus spectrophotometer (BioRad, Munich, Germany). For polyamine analysis, 1 ml aliquots of bacterial cultures were taken at the same time points mentioned above, centrifuged at 10 000 x g for 5 min at 4 °C and supernatants were stored at -80 °C. Polyamine concentrations present in the medium at time point zero were subtracted from the total polyamine concentration in order to calculate the amount of polyamines that was formed by bacteria. Purity of cultures was controlled at 48 h by Gram staining and plating aerobically and anaerobically on Columbia agar with 5 % sheep blood (BioMérieux, Nürtingen, Germany).

2.2 Animal experiments

2.2.1 Impact of an increased bacterial polyamine exposure on mucosal parameters in C3H mice

The animal study was formally approved by the Animal Welfare Committee of the state of Brandenburg (No. of approval: 32-44456 +1). Germfree C3H/HeOuJ mice were obtained from the gnotobiotic animal facility of the German Institute of Human Nutrition Potsdam-Rehbruecke, Germany. To generate breeding stocks, germfree mice (n♂ = 2, n♀ = 4 per group) were colonized intragastrically either with a simplified human microbiota (SIHUMI) [133] or with SIHUMI supplemented with the polyamine-producing *Fusobacterium varium* ATCC 8501 (SIHUMI + Fv) [134] on two consecutive days by fecal transplantation from corresponding donor mice. The SIHUMI consists of eight bacterial strains, which are listed in Table 2.1. The colonization status of the mice was confirmed by fluorescence *in situ* hybridisation (FISH) and plating on Rogosa agar (Oxoid Limited, Hampshire, UK)

according to Becker *et al.* [133] as well as plating of *F. varium* on brilliant green agar according to Noack *et al.* [134] with minor modifications: after cooking, the brilliant green agar was cooled to 55 °C and the antibiotics josamycin CRS (3 mg/l), vancomycin hydrochloride (4 mg/l) and norfloxacin (1 mg/l) were added as filter-sterilized solutions.

Table 2.1: Bacterial strains belonging to the simplified human microbiota (SIHUMI)

Bacterial species	Strain number[1]
Anaerostipes caccae	DSM 14667
Bacteroides thetaiotaomicron	DSM 2079
Bifidobacterium longum	NCC 2705
Blautia producta	DSM 2950
Clostridium butyricum	DSM 10702
Clostridium ramosum	DSM 1402
Escherichia coli	K-12 MG 1655
Lactobacillus plantarum	DSM 20174

[1] DSM, Deutsche Sammlung von Mikroorgansimen; NCC, Nestlé Capital Corporation

After successful association, mice were bred and the female offspring of SIHUMI (n = 9) and SIHUMI + Fv mice (n = 12) were used for the animal experiment. They were housed in Trexler-type isolators (Metall & Plastik, Radolfzell, Germany) at constant room temperature (RT, 22 °C ± 2 °C), air humidity (55 %± 5.5 %), and a 12 h light-dark cycle. The isolators were equipped with polycarbonate cages. All materials were sterilized by autoclaving or irradiation at 50 kGy and introduced via an airlock after disinfection with 3 % peracetic acid or via an access tank with 10 % chloramine after disinfection with 1 % peracetic acid. Mice had free access to sterile water (autoclaved, acidified, pH = 4) and irradiated standard chow R03-40 (45 kGy, SAFE, France). Microbial status was regularly confirmed by plating feces aerobically and anaerobically on Columbia agar with 5 % sheep blood (BioMérieux, Nürtingen, Germany), Mannitol Salt Agar (Oxoid Limited, Hampshire, UK) and on KF Streptococcus Agar (Oxoid Limited, Hampshire, UK) as well as Gram staining. Germfree (n = 12, female) and conventional (n = 12, female) C3H mice were used as control groups.

After determination of body weights, mice were killed eight weeks after birth by cervical dislocation. The intestine was removed from pylorus to rectum and intestinal length and weights were measured. Cecal and colonic contents were collected and two aliquots were

stored at -20 °C for later quantification of SCFA concentrations and bacterial cell numbers. The remaining contents were diluted 1:5 (w/v) with reduced phosphate-buffered saline (reduced PBS in g/l: NaCl 8.5, KH_2PO_4 0.3, Na_2HPO_4 0.6, cysteine x HCl x H_2O 0.25, Bacto peptone 0.1, resazurine 0.001, pH 7.0), homogenized and centrifuged (300 x g, 5 min, 4 °C). The pellet was lyophilized (Gamma 1A, Christ, Osterode am Harz, Germany) to determine dry weight. The supernatant was centrifuged at 10 000 x g for 5 min at 4 °C and the resulting supernatant was stored at -80 °C to determine polyamine concentrations.

2.2.2 Influence of different conventional microbiotas on gut length in PRM/Alf mice

The animal experiment was performed by Dr. Bellier and colleagues at the Institut National de la Recherche Agronomique (INRA) in Jouy-en-Josas, France. Germfree PRM/Alf mice (n = 16 per group) were orally inoculated with a 100-fold diluted suspension of feces freshly collected either from conventional PRM/Alf (PRM/PRM) or C3H/He mice (PRM/C3H). Mice were housed as described above.

The offspring of PRM/PRM (n = 27) and PRM/C3H mice (n = 27) were killed at eight weeks of age by cervical dislocation and intestinal lengths were measured as described above. Cecal contents were sampled and prepared as described above.

2.2.3 Impact of a simplified human microbiota on gut length in PRM/Alf mice

This animal experiment was carried out in close cooperation with Dr. Bellier and colleagues from INRA in France. For generating breeding colonies, germfree PRM/Alf mice ($n♂$ = 4, $n♀$ = 4; obtained from the gnotobiotic animal facility of the INRA and germfree C3H/HeOuJ-mice ($n♂$ = 2, $n♀$ = 4; obtained from Charles River, France) were colonized intragastrically with SIHUMI + Fv on two consecutive days by fecal transplantation from corresponding donor mice. The association was confirmed as described above. After successful association, animals were bred and littermates ($n_{PRM/Alf}$ = 25; n_{C3H} = 26) were used for the animal study. Female pups of germfree ($n_{PRM/Alf}$ = 14; n_{C3H} = 12) and conventional mice ($n_{PRM/Alf}$ = 14; n_{C3H} = 12) were used as reference groups. Microbial status was regularly confirmed either by Temporal Temperature Gradient Gel Electrophoresis according to Seksik *et al.* [135] or by plating

feces aerobically and anaerobically on Columbia agar with 5 % sheep blood (BioMérieux, Germany).

All mice were housed as described above and killed eight weeks after birth by cervical dislocation. Body weights as well as small intestinal and colonic lengths were determined in all animal groups. Cecal contents of SIHUMI + Fv -colonized PRM/Alf and C3H mice were sampled and two aliquots each were stored at -20 °C for later bacterial cell number and SCFA analysis. The remaining cecal content was diluted 1:5 (w/v) with reduced PBS containing 1 x protease inhibitor mix (Roche, Grenzach-Wyhlen, Germany), vortexed for 6 min and centrifuged at 300 x g for 5 min at 4 °C. The pellet was lyophilized and the supernatant was centrifuged at 10 000 x g for 5 min at 4 °C. The resulting supernatant was stored at -80 °C for later polyamine analysis, whereas the bacterial pellet was resuspended in 500 µl washing buffer (10 mM Tris, pH 8.0, 5 mM magnesium acetate, 30 µg/ml chloramphenicol, 1 x protease inhibitor mix). This bacterial mixture was carefully layered onto 0.5 ml Nycodenz solution (60 % [w/v], Axis-shield PoC, Oslo, Norway) followed by ultracentrifugation at 186 000 x g for 15 min at 4 °C to remove particles originating from the diet. Bacterial cells were collected from the interphase and washed four times with 1 ml washing buffer (centrifugation at 10 000 x g, 3 min, 4 °C). Isolated bacterial cells were stored at -80 °C for later two-dimensional difference gel electrophoresis (2D-DIGE).

2.3 Histological tissue analysis

For histological tissue evaluation, 0.8 cm of cecal tissue was sampled from the cecal center and 0.8 cm of colonic tissue was taken 1.5 cm above the rectum. Intestinal tissues were fixed for 24 h in 4 % neutral-buffered formalin and embedded in paraffin. Serial tissue sections (thickness 2 µm; sections of 50 µm between the collected sections were discarded) were stained with Masson's trichrom to determine crypt depth and thickness of mucosa, submucosa, epithelium and muscularis externa. Mucosa thickness included the top of the crypt and the lamina muscularis mucosa. Muscularis externa thickness implied the lamina serosa. The histological parameters were measured in a blinded fashion at 50 well oriented crypts.

To count mitotic and apoptotic enterocytes, intestinal tissue sections (thickness 2 µm; separated by 10 µm sections that were discarded) were stained with haematoxylin. Mitotic cells measured as a marker of cell proliferation and defined by chromatin condensation, chromosome formation and characteristic phases of mitosis (pro-, meta-, ana-, telophase)

(Figure 2.1). Apoptotic cells were identified based on morphological criteria including cell shrinkage, nuclear condensation and perinuclear clearing [136] (Figure 2.1). Mitotic and apoptotic cells were counted in 300 longitudinally sectioned crypts per animal and reported numbers were expressed per 100 crypts.

Histological analyses were carried out with an Eclipse E600 microscope (Nikon, Germany) equipped with Lucia software version 4.51. Samples were counted in a blinded fashion.

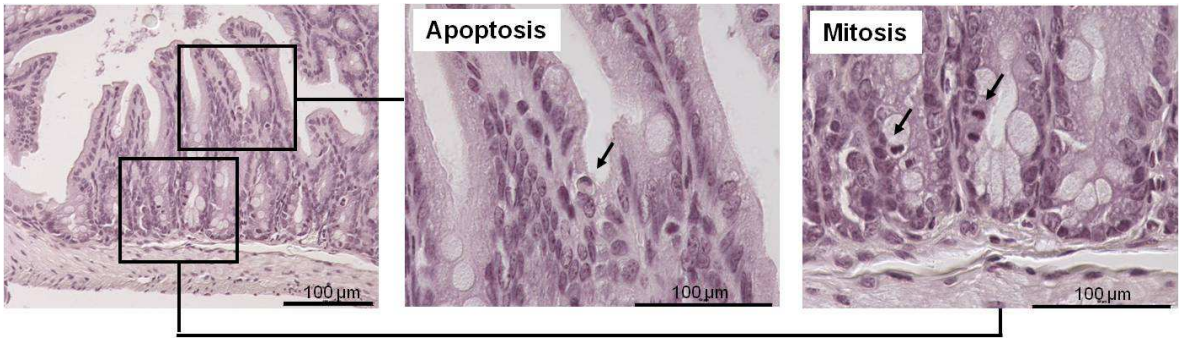

Figure 2.1: Representative picture of colonic tissue stained with haematoxylin. Apoptotic and mitotic cells are marked with arrows. Mitotic cells are arranged in telophase.

2.4 High performance liquid chromatography for polyamine determination

To determine concentrations of free polyamines in intestinal contents, a high performance liquid chromatography (HPLC) method coupled with fluorescence detection (FD) was developed, which included a pre-column derivatization using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ-Fluor) [137, 138]. AccQ-Fluor reacts with terminal amino groups of polyamines forming the derivatized polyamine and *N*-hydroxysuccinimide as by-product (Figure 2.2). Excess AccQ derivatization reagent reacts with water to 6-aminoqinoline, which is fluorescent. AccQ-Fluor was purchased from Waters (Eschborn, Germany) as AccQ-Fluor™ Reagent Kit. Polyamines used for the preparation of standards were purchased from Sigma-Aldrich (Taufkirchen, Germany): *N*-acetylcadaverine hydrochloride, *N*-acetylputrescine hydrochloride, N^8-acetylspermidine dihydrochloride, N^1-acetylspermine trihydrochloride, cadaverine dihydrochloride, putrescine dihydrochloride, spermidine, and spermine.

A polyamine standard mixture containing all eight polyamines (1 M, each) was used for method development. Each polyamine was identified based on its retention time. Standard curves generated by injecting varying amounts of polyamines were used to determine linearity and detection limit of the method. Precision and reproducibility were

monitored by triplicate derivatization of a polyamine standard mixture in both equal and different concentrations. Every tenth sample was spiked with an aliquot of the polyamine standard mixture to determine the recovery after extraction and derivatization.

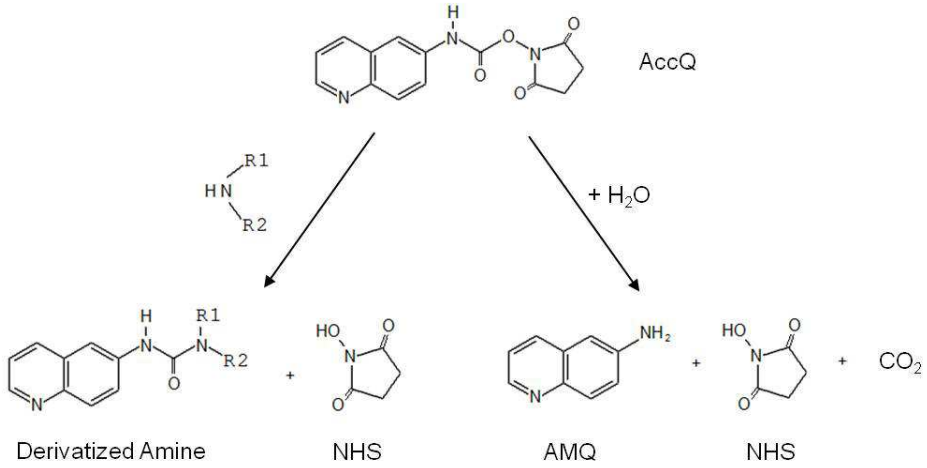

Figure 2.2: Reaction of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ) with the terminal amino group of a polyamine. NHS: *N*-hydroxysuccinimide, AMQ: 6-aminoquinoline. Adapted from [137].

Derivatization was performed in triplicate. Therefore, 10 µl of thawed intestinal supernatant or polyamine standard mixture was mixed with 10 µl AccQ-Fluor reagent and 30 µl borate buffer provided with the AccQ-Fluor™ Reagent Kit. Reagent mixture was immediately mixed, incubated at 55 °C for 20 min and centrifuged at 20 000 x g for 5 min at RT. Forty microliters of the supernatant was injected into a Summit HPLC system (Dionex, Idstein, Germany) equipped with a pump (P 680A LPG), an autosampler (ASI-100T), a column oven (TCC-100), a fluorescence detector 821-FP (Jasco, Groß-Umstadt, Germany) and the Chromeleon software 6.40 (Dionex, Idstein, Germany). The separation was performed with a 5-µm silica particle C_8 Microsorb column (150 x 4.6 mm ID) with a pore size of 10 nm (Varian, Lake Forest, USA). The mobile phase was composed of solvent A (140 mM sodium acetate, 17 mM triethylamine, pH 7.0), solvent B (HPLC-grade acetonitrile) and solvent C (bidestilled water). The mobile phase was delivered at 1.0 ml/min with the gradient conditions given in Table 2.2. The fluorescently labelled polyamines were detected using an excitation wavelength of 250 nm and an emission wavelength of 395 nm. All analyses were done at RT. Calibration curves were used for quantification. Calculated polyamine concentrations were corrected by recovery rates.

Table 2.2: Elution gradient of the HPLC/FD method to quantify intestinal polyamine concentrations

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	100	0	0
20	77	23	0
30	70	30	0
35	60	40	0
40	0	60	40
43	100	0	0
45	100	0	0

For quantification of free polyamines in standard chow, 50 mg finely pestled diet was diluted 1:10 in reduced PBS, vortexed for 5 min and centrifuged at 10 000 x g for 5 min at RT. The supernatant was derivatized and analyzed in triplicate using the HPLC/FD method described above. Polyamine concentrations were corrected by recovery rates determined by spiking dietary supernatants with the polyamine standard mixture as described above.

2.5 Analysis of short chain fatty acids by gas chromatography

For SCFA analysis, approximately 40 mg of frozen intestinal content was diluted 5-fold with reduced PBS (w/v) and centrifuged at 22 000 x g for 5 min at RT. Supernatant (100 µl) was mixed with 11.8 µl iso-butyric acid (12 mM, internal standard), 140 µl $HClO_4$ (0.36 M), 135 µl NaOH (1 M), frozen at -20 °C and lyophilized. The dry residue was re-dissolved in a mixture of 50 µl formic acid (5 M) and 200 µl acetone, centrifuged at 22 000 x g for 5 min at RT and 1 µl of the supernatant was injected into a gas chromatograph (HP 5890 series II, Agilent Technologies, Böblingen, Germany) equipped with an HP-FFAP capillary column (30 m x 0.53 mm i.d., 1 µm film thickness) and a flame ionisation detector. Helium was used as carrier gas at a flow rate of 1 ml/min. The initial column temperature of 75 °C was held for 1 min and subsequently increased at a rate of 10 °C/min to 140 °C and maintained at 140 °C for 5 min, following by an increase at a rate of 70 °C/min to a final temperature of 180 °C. After 2 min at 180 °C the temperature was decreased to 75 °C at a rate of 70 °C/min for 3 min. The temperatures of the injector and of the detector were 200 °C. The split ratio was 1:10. All samples were analyzed in duplicate.

2.6 Determination of lactate concentrations

Intestinal lactate concentrations were determined by an enzymatic assay according to manufacturer's instructions (Boehringer Mannheim/R-Biopharm, Darmstadt, Germany).

2.7 Quantification of intestinal bacteria with qPCR

For quantification of bacteria belonging to the SIHUMI and SIHUMI + Fv consortium, a quantitative real-time PCR (qPCR) was established. The *groEL* gene was selected as the target gene, because it is present as one copy per bacterial genome [139, 140].

2.7.1 Standard generation

2.7.1.1 Bacterial cultivation and purity control

Bacterial strains of the SIHUMI consortium were cultivated in medium 496 YCFA GSC (National Collection of Industrial, Food and Marine Bacteria, Aberdeen, Scotland, UK) prepared as described in Appendix A1. Each bacterial strain was grown anaerobically for 24 h at 37 °C using the Hungate technique [141]. Purity was controlled by Gram staining followed by microscopy [142] and 16S rRNA gene sequencing.

For 16S rRNA gene sequencing, 1 ml freshly grown bacterial culture was centrifuged at 20 000 x g for 5 min at 4 °C and the pellet was stored at -20 °C. Bacterial DNA was extracted using the RTP Bacteria DNA Mini Kit (Invitek, Berlin, Germany) according to the manufacturer's instructions with one minor modification: DNA was eluted with 100 µl Elution Buffer after 15 min of incubation at RT. Bacterial 16S rRNA genes were amplified by PCR with the primers 27-f (5'- AGA GTT TGA TCC TGG CTC AG -3') and 1492-r (5'- TAC CTT GTT ACG ACT T -3') [143]. PCR was performed in a 50 µl reaction mixture containing 1 x Dream Taq™ Buffer (Thermo Scientific, Walldorf, Germany), 0.1 mM dNTP (Invitek, Berlin, Germany), 0.1 µM of each Primer, 2.5 Units Dream Taq™ DNA Polymerase (Thermo Scientific, Walldorf, Germany) and 1 µl template DNA. PCR conditions were as follows: denaturation for 4 min at 94 °C; amplification with 25 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C; and extension for 10 min at 72 °C. PCR products were purified using the innuPREP PCRpure Kit (Analytik Jena, Jena, Germany) according to the manufacturer's instructions. DNA concentrations were measured by NanoDrop (PeqLab, Erlangen, Germany) and PCR product sizes were determined in 1 % agarose gels (Serva, Heidelberg, Germany) relative to a 100 bp DNA ladder (Invitrogen,

Karlsruhe, Germany). Finally, amplicons were sequenced by Eurofins MWG Operon (Ebersberg, Germany) using the above mentioned primers. The 16S rRNA gene sequences were analyzed with ContigExpress software (Vector NTI Suite 9, Invitrogen, Berlin, Germany) and compared with the database of National Center for Biotechnology Information (NCBI) by BLAST search [144].

2.7.1.2 Spiking of fecal samples

Bacterial cell numbers of freshly grown 24 h cultures were determined with a Thoma cell counting chamber and $1*10^9$ cells of each strain were added to each 50 mg feces obtained from germfree mice. These spiked fecal samples served as standards for quantification of targeted bacterial cells. To generate standards for total bacterial cells, feces was spiked with total $9*10^9$ cells, i.e. $1*10^9$ cells per strain.

2.7.2 Quantitative real-time PCR

Bacterial DNA was extracted from spiked fecal samples and intestinal contents, respectively, using the PSP® Spin Stool DNA Kit (Stratec Molecular GmbH, Berlin, Germany) according to the manufacturer's instructions with one minor modification: DNA was eluted after 15 min incubation instead of 3 min at RT with 100 µl preheated (70 °C) PCR water (Fisher Scientific GmbH, Schwerte, Germany). DNA was stored at -20 °C until use. Bacterial DNA extracted from spiked fecal samples was subjected to 10-fold serial dilutions with PCR water, to generate a standard calibration curve.

Primers were designed using Primer-Blast provided by NCBI (www.ncbi.nlm.nih.gov/tools/primer-blast). They target the *groEL* gene, whose sequence was obtained from the chaperonin database cpnDB (http://www.cpndb.ca/cpnDB/home.php). All primers used in this study (Table 2.3) were commercially synthesized by Eurofins MWG Operon (Ebersberg, Germany). Primer specificity was controlled within the SIHUMI + Fv consortium.

Since the *groEL* gene sequence of *F. varium* was not published, the gene was amplified and sequenced in triplicate. DNA of *F. varium* was isolated from over-night cultures grown in 496 YCFA GSC medium using the RTP Bacteria DNA Mini Kit (Invitek, Berlin, Germany). The target gene was amplified by PCR as described in section 2.7.1.1 using the primer pair universal-f and universal-r (Table 2.3) and the following PCR program: 94 °C for 5 min; 30 cycles 94 °C for 30 s, 55 °C fo30 s, 72 °C for 1 min; and finally 72 °C

for 10 min. PCR products were purified and sequenced as described above. Sequence identities within triplicates were controlled by multiple alignment with the program ClustalW2 version 2.1 [145] provided by the European Bioinformatics Institute (http://www.ebi.ac.uk). Based on the resulting sequence, primer pair for the amplification of the targeted DNA in *F. varium* was designed as described above.

For qPCR, a 7500 Fast Real-Time PCR System equipped with 7500 software v2.0.5 (Life Technologies, Darmstadt, Germany) was used. Amplification was performed in triplicate. Each reaction mixture of 20 µl contained the following concentrations: 1 x Hot Start Buffer complete (Analytik Jena AG, Jena, Germany), 5.5 mM $MgCl_2$, 0.25 mM dNTPs, 1.25 Units innuTag HOT-A DNA Polymerase (Analytik Jena AG, Jena, Germany), 0.1 x Sybr® Green I nucleic acid gel stain (Sigma-Aldrich, Taufkirchen, Germany), 50 nM Rox (Affymetrix, Santa Clara, USA) as passive reference dye, 0.2 µM of each forward and reverse primer (Table 2.3) and 1 µl template DNA. The PCR programs used are listed in Table 2.3. To reduce the amplification time to less than 40 min, the fast amplification program of the 7500 software v2.0.5 was chosen for almost all bacterial strains, except for *Bifidobacterium longum* and total bacteria. Post-amplification melt-curve analysis was performed as described elsewhere [140]. Bacterial cell numbers were calculated by a standard curve generated by plotting C_T-values of the 10-fold serial dilution of the standard against their known concentrations.

Table 2.3: Species specific primer sequences among the SIHUMI microbiota based on the *groEL* gene and the PCR conditions used.

Target species/ strain number	Primer	Sequence (5' to 3')[1]	Amplicon size (bp)	Annealing temp (°C)	PCR conditions	Reference
A. caccae DSM 14667	A. cac f A. cac r	AAC CTT GCG GCA GGT GCA AAT CCG AGC ATC CGC AAC TAA CTC CCC GAC	177	75	1 x 95°C 2 min 32 x (94°C 3s, 75°C 30s) (fast mode)	this study
B. thetaiotaomicron DSM 2079	B. theta f B. theta r	TGT GAC TGC CGG TGC AAG CC ACT TTG CGC ATA GCG TCA GCA	192	71	1 x 95°C 2 min 32 x (94°C 3s, 71°C 30s) (fast mode)	this study
B. longum NCC 2705	B. long f B. long r	CGG CGT YGT GAC CGT TGA AGA C TGY TTC GCC RTC GAC GTC CTC A	259	70	1 x 95°C 2 min 32 x (94°C 15s, 70°C 15s, 72°C 15s, 83°C 15s) (standard mode)	[140]
B. producta DSM 2950	B. prod f B. prod r	AAC CTG GCA GCA GGC GCT AAC TCA TCG CCT GCG GAG ATA GCT G	149	71	1 x 95°C 2 min, 32 x (94°C 3s, 71°C 30s) (fast mode)	this study
C. butyricum DSM 10702	C. buty f C. buty r	AGT AGC TGT TGA AAA GGC AGT TGA AGA TCA GCA GCA GAA ATA GCA GCA ACT C	99	71	1 x 95°C 2 min, 32 x (94°C 3s, 71°C 30s) (fast mode)	this study
C. ramosum DSM 1402	C. ram f C. ram r	TGC GAG CAA AGA GGT AGC AAA AAC T GCC GAA ATC GTT GCA ACA CTA GCA	90	71	1 x 95°C 2 min, 32 x (94°C 3s, 71°C 30s) (fast mode)	this study
E. coli K-12 MG 1655	E. coli f E. coli r	GGC TAT CAT CAC TGA AGG TCT G TTC TTC AAC TGC AGC GGT AAC	100	67	1 x 95°C 2 min, 32 x (94°C 3s, 67°C 30s) (fast mode)	[146], modified
F. varium ATCC 8501	F. var f F. var r	TGG TAA GTG CAG GAG CAA ATC CTA TGT CCT GCT GAT ACT GAT GCA ACT TGA GC	142	71	1 x 95°C 2 min, 32 x (94°C 3s, 71°C 30s) (fast mode)	this study
L. plantarum DSM 20174	L. plant f L. plant r	GCC GTT GTT CGT GTC GGT GC TTC TTC AAC GGC GGC CCG AG	99	67	1 x 95°C 2 min, 32 x (94°C 3s, 67°C 30s) (fast mode)	this study
Total bacteria	Universal f Universal r	GGN GAY GGN ACN ACN ACN GCN AC TCN CCR AAN CCN GGN GCY TTN ACN GC	ca. 500	59	1 x 95°C 2 min, 40 x (94°C 15s, 59°C 15s, 72°C 50s) (standard mode)	[147], modified

[1] IUPAC Ambiguity Codes: R (A, G); N (A, C, G, T); Y (C, T)
SIHUMI, simplified human microbiota; f, forward primer; r, reverse primer; bp, basepairs; DSM, Deutsche Sammlung von Mikroorganismen; NCC, Nestlé Capital Corporation; ATCC, American Type Culture Collection

2.8 Analysis of differently expressed proteins using two-dimensional difference gel electrophoresis (2D-DIGE)

2.8.1 Isolation and labeling of bacterial proteins

Cecal samples from ten animals per group (♀ = 5; ♂ = 5) were prepared for two-dimensional difference gel electrophoresis (2D-DIGE). All steps were performed on ice. Frozen bacterial pellets were resuspended in 800 µl lysis buffer (8 M urea, 30 mM Tris, 4 % (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 1 x protease inhibitor mix, pH 8.5) and incubated on ice for 5 min. After pipetting sample mixtures into 1.5 ml micro tubes (Sarstedt, Nümbrecht, Germany) filled with zirconia-silica beads (0.1 mm, Roth, Karlsruhe, Germany) cell disruption was performed three time at speed 6.0 for 20 s using an FP120 FastPrep cell disrupter (Thermo Scientific, Waltham, MA, USA). Between the cycles, samples were incubated for 5 min on ice. The bottom of the micro tubes was perforated with a sterile needle, placed in a 2 ml Eppendorf tube and centrifuged at 2 000 x g for 2 min. To remove unbrocken cells, samples were centrifuged at 20 000 x g for 20 min at 4 °C. The resulting supernatants were incubated with 125 U Benzonase (Novagen, Merck KGaA, Darmstadt, Germany) at 37 °C for 10 min and purified with the 2D Clean-Up Kit (GE Healthcare, Munich, Germany) according to manufacturer's instructions.

Purified protein solutions were adjusted to pH 8.5 using 50 mM NaOH and protein concentrations were determined with the Bradford assay [148] (Bio-Rad, Madrid, Spain) using bovine serum albumin as a reference.

Bacterial proteins (50 µg per sample) were labeled either with CyDye DIGE Fluor Cy3 or CyDye DIGE Fluor Cy5 (GE Healthcare, Munich, Germany) according to the manufacturer's instructions. Internal standard was prepared by mixing 25 µg protein of each sample followed by labeling with CyDye DIGE Fluor Cy2.

2.8.2 First dimension: Isoelectric focusing

For isoelectric focusing, a sample mixture was prepared containing 50 µg protein of internal standard (Cy2-labeled), 50 µg protein of sample A (Cy3-labeled), 50 µg protein of sample B (Cy5-labeled), 4.5 µl IPG-buffer (pH 4-7, GE Healthcare, Munich, Germany), 45 µl dithiothreitol (DTT, 20 % w/v, solved in rehydration buffer [7 M urea, 2 M thiourea, 4 % (w/v) CHAPS) and 1 µl bromophenol blue. The sample mixture was refilled with

rehydration buffer to a total volume of 450 µl and loaded onto an immobilized pH gradient (IPG) strip (pH range 4 to 7, 24 cm). Isoelectric focusing was performed using an Ettan IPGphor 3 device (GE Healthcare, Munich, Germany) with the following protocol: 30 V for 10 h; 500 V for 1 h; 1 000 V for 1 h and 10 000 V for 3 h followed by a linear gradient starting from 10 000 V to 42 500 V. Focused IPG strips were stored at -80 °C.

2.8.3 Second dimension: SDS-PAGE

For equilibration, frozen IPG strips were incubated with 10 ml equilibration buffer 1 (75 mM Tris-HCl, pH 8.8; 6 M urea; 3.3 M glycerol; 70 mM SDS; 0.002 % (w/v) bromophenol blue) containing 1 % (w/v) DTT for 15 min at RT under gentle shaking. After decanting the equilibration buffer 1, strips were incubated for 15 min at RT under gentle shaking with equilibration buffer 2 containing 2.5 % (w/v) iodoacetamide. Strips were placed onto 12.5 % SDS gels (size: 20 x 26 cm, 1 mm thick) and the electrophoresis was performed using an Ettan-Dalt II apparatus with the following program: 1 W per gel for 45 min, followed by 17 W per gel for 3.5 h at 20 °C.

2.8.4 Detection of differently expressed proteins

SDS-PAGE gels were scanned with the Typhoon Trio Laser Scanner (GE Healthcare, Munich, Germany) at a resolution of 100 µm using appropriate filters for excitation/emission wavelength of Cy2 (488 nm/520 nm), Cy3 (532 nm/580 nm) and Cy5 (633 nm/670 nm) dyes. Protein spots were compared between SIHUMI + Fv –colonized PRM/Alf and C3H mice using DeCyder software version 6.5 (GE Healthcare, Munich, Germany). Male and female animals were analyzed separately. Differently expressed proteins were defined by ≥ 2-fold change and a statistical difference level of $P \leq 0.05$.

2.8.5 Identification of differently expressed proteins

Preparative gels containing 500 µg of bacterial protein were prepared as described above. After staining over night using ruthenium II tris (bathophenanthroline disulfonate) [149] proteins of interests were cut out of the SDS-PAGE gel automatically by an Ettan-Dalt Spot Picker (GE Healthcare, Uppsala, Sweden) and subjected to tryptic digestion. Gel plugs were washed twice with 100 µl 50 mM ammonium hydrogen carbonate containing 50 % methanol and incubated for 30 min at RT. After addition of 100 µl acetonitrile and

incubation for 10 min, gel plugs were vacuum dried using an RCT 90 SpeedVac (Jouan Robotics, St Herblain, France). Tryptic digestion was performed over night at 37 °C in 50 µl trypsin solution (1 ng/µl trypsin [Promega, Heidelberg, Germany] dissolved in 20 mM ammonium hydrogen carbonate). Peptides were extracted from the gel plugs with 60 µl 0.1 % trifluoroacetic acid – 50 % acetonitrile for 20 min and dried by vacuum centrifugation. Proteins were identified by nano liquid chromatography-electrospray ionization-tandem mass spectrometry, electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS-MS) analysis as described elsewhere [150-152].

2.9 Statistics

Data distribution was tested with the Kolmogorov-Smirnov test and the D'Agostino and Pearson omnibus normality test using GraphPad Prism version 5.0 (San Diego, CA, USA). Parametric and non-parametric distributed data were analyzed for significance of differences between two groups using Student's *t* test and Mann Whitney test, respectively. When more than two groups were compared, one-way analysis of variance (Bonferroni) and Kruskal wallis (Dunn's Multiple Comparison Test) were used. SPSS 16.0 (SPSS® Inc., Chicago, USA) was used for description of correlations. A probability value of $P \leq 0.05$ was considered statistically significant (*), of $P \leq 0.01$ highly significant (**) and of $P \leq 0.001$ very highly significant (***). For parametric distribution, data are given as means ± standard deviation, whereas data being non-parametric distributed are expressed as medians (minimum – maximum).

3 RESULTS

3.1 Method development for polyamine determination

3.1.1 High performance liquid chromatography with fluorescence detection

To quantify free polyamines in intestinal contents and culture media, an HPLC/FD method was developed and optimized, which included a pre-column derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ.Fluor). This method afforded a complete separation of all polyamines used in this study (Figure 3.1).

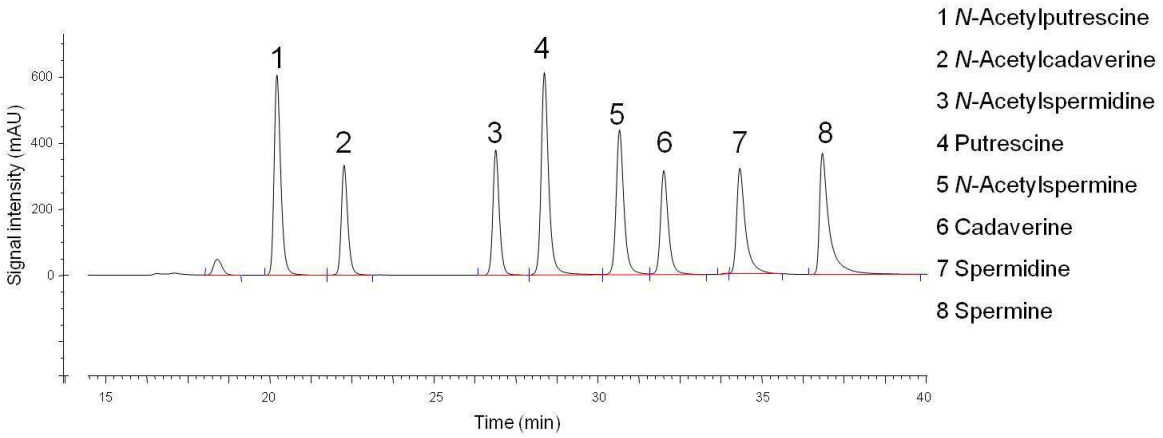

Figure 3.1: Representative HPLC/FD chromatogram showing the elution profile of a polyamine standard mixture. The polyamine mixture contained eight polyamines at a concentration of 25 µM each. Derivatization was performed with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ.Fluor). Fluorescence detection: excitation λ = 250 nm; emission λ = 395 nm

The limits of quantification (LOQ) of polyamines define the linear range, which was between 50 nM and 100 µM for most of the polyamines (Table 3.1). The method showed a high reproducibility because the coefficient of variation of repeated measurements (n = 3), which reflects the cumulative effects of derivatization and HPLC/FD detection, ranged between 10.5 % and 23.0 %, depending on the polyamine (Table 3.1). The limits of detection (LOD) of the polyamines were defined as a concentration producing a peak height three times the baseline noise. As the LOD ranged between 5 nM and 100 nM, the method is very sensitive.

Table 3.1: Characterization of the polyamine determination by HPLC/FD after derivatization

	LOD (µM)	Lower LOQ (µM)	Upper LOQ (µM)	Coefficient of variation (%)
N-Acetylputrescine	0.005	0.05	100	17.1
N-Acetylcadaverine	0.05	0.05	100	17.0
N-Acetylspermidine	0.025	0.05	100	10.6
Putrescine	0.025	0.05	100	23.0
N-Acetylspermine	0.01	0.10	100	13.1
Cadaverine	0.025	0.05	100	18.1
Spermidine	0.1	0.10	100	11.5
Spermine	0.1	1.00	50	10.5

LOD: limit of detection; LOQ: limit of quantification

3.1.2 Recovery of soluble polyamines in culture medium and intestinal contents

To determine the recovery of polyamines *in vivo* and *in vitro*, cecal and colonic contents of mice fed standard chow R03-04 (SAFE, France) as well as MOPS-buffered Ringer solution supplemented with 24 g per liter standard chow were spiked with a polyamine standard mixture.

Recoveries of polyamines from spiked *in vitro* and *in vivo* samples varied depending on both the polyamine and the sample background. The recoveries ranged between 67 % and 89 % *in vitro*, depending on the polyamine (Table 3.2), and from 65 % to 79 % *in vivo*, with the exception of *N*-acetylputrescine (53 %) and *N*-acetylcadaverine (32 %).

Table 3.2: Recoveries (%) of polyamines determined *in vitro* and *in vivo*

	In vitro[1]	*In vivo*[2]
N-Acetylputrescine	66.5	53.3
N-Acetylcadaverine	79.8	31.5
N-Acetylspermidine	70.6	65.2
Putrescine	88.5	75.5
N-Acetylspermine	86.1	73.0
Cadaverine	70.1	63.0
Spermidine	70.7	79.0
Spermine	69.9	68.8

[1] Recovery from *in vitro* samples was calculated in MOPS-buffered Ringer solution supplemented with 24 g standard chow A03 per liter (SAFE, France)
[2] Recovery from *in vivo* samples was calculated in cecal and colonic content.

3.1.3 Polyamine concentrations in standard chow

Polyamine concentrations were quantified in the standard chow R03-04 (SAFE, France). The main dietary polyamine was spermine (851.5 ± 49.4 nmol/g diet), followed by putrescine (409.0 ± 6.2 nmol/g), *N*-acetylputrescine (374.3 ± 83.7 nmol/g), spermidine (321.4 ± 24.1 nmol/g), and *N*-acetylspermine (284.4 ± 7.9 nmol/g). Cadaverine and acetylated polyamines, including *N*-acetylcadaverine and *N*-acetylspermidine, were not detected.

3.2 Polyamine formation by intestinal bacteria

Fermentation studies were carried out with the bacteria used in the animal experiments to test their ability to form polyamines. Under the conditions used, *F. varium* formed exclusively putrescine at a concentration of 23.7 µM ± 0.4 µM (Table 3.3). *E. coli* was also able to synthesize putrescine at low concentrations (6.2 µM ± 1.1 µM), but formed mainly *N*-acetylspermine, which reached a concentration of 78.5 µM ± 1.1 µM. *A. caccae* and *C. butyricum* were the only bacteria utilizing polyamines *in vitro* including putrescine and *N*-acetylspermine, as the concentration of these polyamines decreased during incubation. *C. butyricum* also formed spermidine and *N*-acetylspermidine. All other bacteria tested did not synthesize or utilize any polyamines in large amounts.

Table 3.3: Polyamine concentrations (µM) formed by SIHUMI bacteria cultivated for 48 h in MOPS-buffered Ringer solution supplemented with 24 g of standard chow A03 (SAFE, France) per liter[1].

	Concentration (µM) [2, 3]							
	N-Acetyl-putrescine	*N*-Acetyl-cadaverine	*N*-Acetyl-spermidine	Putrescine	*N*-Acetyl-spermine	Cadaverine	Spermidine	Spermine
A. caccae	0.7 ± 0.2	<0.05	1.6 ± 0.2	-9.0 ± 1.3	-7.0 ± 1.4	<0.05	<0.1	<1.0
B. thetaiotaomicron	<0.05	<0.05	<0.05	<0.05	<0.1	<0.05	<0.1	<1.0
B. producta	<0.05	0.2 ± 0.2	0.1 ± 0.1	1.2 ± 0.5	0.3 ± 0.4	<0.05	<0.1	<1.0
B. longum	<0.05	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	<0.1	<0.05	1.8 ± 0.7	<1.0
C. butyricum	0.4 ± 0.3	0.1 ± 0.1	7.9 ± 0.5	-10.3 ± 0.9	-8.7 ± 0.5	<0.05	7.8 ± 1.8	<1.0
C. ramosum	<0.05	<0.05	0.2 ± 0.02	<0.05	<0.1	<0.05	0.3 ± 0.2	<1.0
E. coli	1.3 ± 1.2	2.3 ± 0.3	0.4 ± 0.1	6.2 ± 1.1	78.5 ± 1.1	<0.05	0.3 ± 0.1	<1.0
F. varium	0.1 ± 0.1	<0.05	<0.05	23.7 ± 0.4	<0.1	<0.05	<0.1	<1.0
L. plantarum	<0.05	<0.05	<0.05	0.4 ± 0.4	<0.1	<0.05	0.1 ± 0.1	<1.0

[1] Data are given as mean ± standard deviation.
[2] Negative values indicate an utilization of polyamines because polyamine concentrations present in medium at time 0 h were subtracted from total polyamine concentration formed after 48 h.
[3] In dependence of the polyamine, limits of quantification are <0.05 µM, <0.1 µM or <1.0 µM.

3.3 Analysis of bacterially produced polyamines and their influence on gut morphology in differently colonized C3H mice

The microbiota affects various gut morphological parameters including epithelial proliferation, villus length and crypt depth, but their mechanisms and triggering microbial factors have not yet been identified. Since polyamines are known trophic factors produced by intestinal bacteria, it was hypothesized that they contribute to some of these morphological changes. To test this hypothesis, germfree C3H mice were colonized with two different microbial communities producing low and high polyamine concentrations. One group was colonized with the simplified human microbiota (SIHUMI). The other group of mice was colonized with SIHUMI supplemented with *F. varium* (SIHUMI + Fv), because the latter species is known to produce high amounts of polyamines (7, 8). The eight week old offspring of the colonized mice was used for the experiment to ensure a bacterial polyamine exposure from birth on. Germfree and conventional mice served as a negative and a positive control, respectively, because they considerably differ in gut morphology from each other (4).

3.3.1 Body weight

The body weight of eight week old mice did not differ between germfree, SIHUMI and SIHUMI + Fv mice showing final weights of 20.6 g (19.4 g-22.6 g), 20.7 g (20.2 g-25.5 g) and 20.8 g (18.5 g-22.5 g), respectively. Conventional mice had a body weight of 18.9 g (17.6 g-19.8 g) and weighed less ($P \leq 0.05$) than mice from the other groups.

3.3.2 Intestinal bacterial cell numbers

Bacterial cell numbers were quantified in cecum and colon of SIHUMI and SIHUMI + Fv -colonized mice. Moreover, total bacterial cell numbers of the gnotobiotic groups were compared with those of conventional mice. Conventional mice on standard chow R03-40 (SAFE, France) harbored 11.36 ± 0.51 $\log_{10}$ cells/g cecal content (dw) and 11.28 ± 0.34 $\log_{10}$ cells/g colonic content (dw). Total bacterial cell numbers in mice colonized with SIHUMI or SIHUMI + Fv consortium were in the same range (Table 3.4). *B. thetaiotaomicron* was the dominant bacterial species in SIHUMI as well as in SIHUMI + Fv mice. Its proportion of total bacteria was 88.0 % in cecum and 83.0 % in colon of SIHUMI mice, but only 72.7 % and 72.5 %, respectively, in SIHUMI + Fv mice

($P \leq 0.01$). Between these two mouse groups, there were only minor changes in bacterial cell numbers: numbers of *A. caccae* were higher ($P \leq 0.001$) and those of *E. coli* were lower ($P \leq 0.001$) in cecum of SIHUMI + Fv mice than in that of SIHUMI mice. *L. plantarum* was only detected in cecum and feces of SIHUMI mice. All other bacteria were similar in both colonization groups.

Table 3.4: Bacterial cell numbers in cecal and colonic contents of mice colonized with SIHUMI or SIHUMI + Fv[1]. Data are expressed as LOG numbers per g gut content (dry weight).

	Cecum		Colon	
Organism	SIHUMI	SIHUMI + Fv	SIHUMI	SIHUMI + Fv
A. caccae	9.56 ± 0.26	10.00 ± 0.24***	9.29 ± 0.33	9.66 ± 0.42
B. thetaiotaomicron	11.47 ± 0.11	11.30 ± 0.19*	11.00 ± 0.16	11.00 ± 0.18
B. longum	9.94 ± 0.23	10.05 ± 0.12	9.39 ± 0.32	9.60 ± 0.13
B. producta	10.10 ± 0.19	10.10 ± 0.17	9.90 ± 0.20	9.74 ± 0.20
C. butyricum	9.28 ± 0.15	9.33 ± 0.14	8.83 ± 0.19	8.76 ± 0.18
C. ramosum	9.66 ± 0.28	9.58 ± 0.13	9.54 ± 0.13	9.44 ± 0.16
E. coli	9.76 ± 0.16	9.14 ± 0.18***	9.46 ± 0.19	8.87 ± 0.29*
F. varium	-	10.57 ± 0.09	-	10.25 ± 0.19
L. plantarum[2]	5.73 ± 1.40	n.d.	5.41 ± 1.04	n.d.
total	11.68 ± 0.13	11.55 ± 0.16	11.27 ± 0.26	11.26 ± 0.16

[1] Values are given as mean ± standard deviation. SIHUMI $n_{cecum} = 9$, $n_{colon} = 7$; SIHUMI + Fv $n_{cecum} = 12$, $n_{colon} = 10$; Significant differences were analyzed using Student's *t*-test (unpaired): * $P \leq 0.05$; *** $P \leq 0.001$

[2] Cell numbers of *L. plantarum* were determined by plating on Rogosa agar. Due to limited colonic material *L. plantarum* was determined in cecum and feces. $n_{cecum} = 8$, $n_{feces} = 8$.

n.d., not detected; SIHUMI, simplified human microbiota; Fv, *Fusobacterium varium*

3.3.3 Intestinal polyamine concentrations

To investigate how intestinal polyamine concentrations in mice with different colonization status differ, polyamine concentrations were determined in cecal and colonic contents of germfree, conventional, SIHUMI and SIHUMI + Fv mice using HPLC/FD. Total polyamine concentrations in cecal and colonic contents of conventional, SIHUMI and SIHUMI + Fv mice were up to 7.6-fold, 2.7-fold and 4.5-fold, respectively, higher compared to germfree mice indicating that the microbiota had a strong influence on the intestinal polyamine pool. Spermidine was the major polyamine in germfree and conventional mice, while *N*-acetylspermine, putrescine and spermidine were mainly formed in the mice with the two SIHUMI consortia (Table 3.5).

Table 3.5: Polyamine concentrations (µM) in cecal and colonic contents (wet weight) in germfree mice, conventional mice and mice colonized with SIHUMI and SIHUMI + Fv

	Cecum				Colon			
Polyamines	Germfree	Conventional	SIHUMI	SIHUMI + Fv	Germfree	Conventional	SIHUMI	SIHUMI + Fv
N-Acetylcadaverine	n.d.^{a}	0^{ab} (0-21.4)	0^{ab} (0-2.4)	1.6^{b} (0-7.5)	n.d.^{a}	0^{ab} (0-9.7)	n.d.^{a}	3.1^{b} (0-8.3)
N-Acetylputrescine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
N-Acetylspermidine	3.5^{a} (0.0-5.9)	15.9^{b} (8.6-25.9)	14.2^{b} (10.9-17.9)	16.1^{b} (8.2-19.8)	2.6^{a} (0.0-4.7)	12.0^{b} (3.1-28.4)	10.1^{ab} (0.0-14.5)	13.3^{b} (6.6-18.5)
N-Acetylspermine	1.2^{a} (0.8-2.0)	4.2^{ac} (1.6-11.2)	28.9^{bc} (16.5-35.0)	34.0^{b} (17.0-56.6)	1.2^{a} (0.0-1.8)	5.8^{ab} (2.1-8.9)	28.0^{bc} (16.9-41.9)	33.4^{c} (14.8-51.1)
Cadaverine	2.6^{a} (0.9-3.8)	9.0^{b} (5.5-16.0)	2.7^{ac} (1.4-3.4)	1.1^{c} (0.3-1.7)	2.5^{a} (1.5-4.3)	5.9^{b} (3.3-13.0)	2.7^{ab} (1.7-3.4)	1.0^{c} (0.4-1.7)
Putrescine	0.6^{a} (0.0-1.0)	28.8^{b} (1.3-41.7)	24.5^{ab} (16.8-29.1)	61.8^{c} (47.6-75.5)	0.7^{a} (0.0-2.9)	18.9^{ac} (9.2-36.3)	25.5^{bc} (14.9-35.5)	53.8^{b} (39.8-87.4)
Spermidine	23.0^{a} (11.3-29.4)	190.0^{b} (71.5-310.5)	11.3^{a} (4.3-18.3)	23.8^{a} (13.2-128.1)	27.6^{a} (19.7-43.0)	84.4^{b} (38.9-169.5)	24.1^{a} (9.9-63.2)	30.0^{a} (21.3-64.3)
Spermine	3.0^{a} (1.9-4.3)	5.1^{ac} (3.2-12.3)	6.4^{bc} (3.9 -8.7)	10.4^{b} (4.7-21.6)	3.4^{a} (1.7-4.3)	3.3^{a} (2.0-6.1)	7.1^{b} (4.5-12.2)	11.0^{b} (6.0-25.8)

Values are expressed as median (minimum-maximum); Cecum: $n_{\text{germfree, conventional, SIHUMI, SIHUMI + Fv}} = 11, 12, 9, 12$; Colon: $n_{\text{germfree, conventional, SIHUMI, SIHUMI + Fv}} = 12, 10, 8, 11$; Medians in the same row not sharing superscript letters differ significantly ($P < 0.05$). Differences among groups were analyzed using the Kruskal-Wallis test (Dunn's Multiple Comparison Test). n.d., not detected; SIHUMI, simplified human microbiota; Fv, *Fusobacterium varium*

In germfree mice, most of the polyamines were present at very low concentrations compared to conventional mice, except for *N*-acetylspermine and spermine, which were similar in both groups (Table 3.5). A comparison of conventional and SIHUMI mice showed that the concentrations of the majority of cecal and colonic polyamines were similar in both groups. However, spermidine and cecal cadaverine were 16.8-fold and 3.3-fold, respectively, lower in SIHUMI mice ($P \leq 0.05$), and colonic spermine concentrations were 2.2-fold higher ($P \leq 0.05$). The presence of *F. varium* in the SIHUMI consortium was accompanied by changes in putrescine and cadaverine concentrations: putrescine concentrations were up to 2.5-fold and 2.8-fold higher compared to SIHUMI and conventional mice, respectively. In contrast, germfree mice had only low concentrations of putrescine in cecal and colonic content. The cadaverine concentrations in SIHUMI + Fv mice displayed up to 2.7-fold and 8.2-fold lower values compared to SIHUMI and conventional mice, respectively.

3.3.4 Short chain fatty acid and lactate concentrations in cecum and colon

SCFA concentrations were also determined because they are known to act as trophic factors [153]. Both SIHUMI groups did not differ in the measured SCFA and lactate concentrations in cecum and colon (Table 3.6). Their SCFA concentrations were between those of germfree and conventional mice. While germfree mice contained hardly any SCFA and lactate in their intestines, conventional mice displayed the highest concentrations, in particular for butyrate and lactate. Conventional mice had 1.6-fold and 2.0-fold higher total SCFA concentrations in cecum and colon, respectively, compared to SIHUMI and SIHUMI + Fv mice.

Table 3.6: Short chain fatty acid (SCFA) and lactate concentrations (mM) in cecal and colonic content (wet weight) of germfree mice, conventional mice and mice colonized with SIHUMI and SIHUMI + Fv

Microbial products	Germfree	Conventional	SIHUMI	SIHUMI + Fv
Cecum:				
Acetate	8.0 (6.5-15.5)^{a}	45.1 (35.2-52.2)^{b}	38.1 (35.6-51.6)^{b}	40.4 (31.0-82.0)^{b}
Propionate	0.1 (0.0-0.1)^{a}	8.1 (3.1-11.0)^{b}	3.5 (3.2-4.7)^{bc}	3.5 (2.5-5.7)^{c}
n-Butyrate	0.0 (0.0-0.1)^{a}	16.0 (6.0-24.9)^{b}	1.4 (0.7-2.6)^{c}	1.4 (0.5-2.7)^{c}
i-Valerate	0.0 (0.0-0.5)^{a}	0.3 (0.2-0.5)^{b}	0.1 (0.1-0.3)^{a}	0.2 (0.0-0.3)^{a}
n-Valerate	0.0 (0.0-0.01)^{a}	0.4 (0.1-0.6)^{b}	n.d.^{a}	n.d.^{a}
Lactate	0.4 (0.0-3.3)^{a}	10.4 (6.8-45.6)^{b}	3.2 (0.0-4.4)^{a}	0.4 (0.0-5.1)^{a}
Colon:				
Acetate	8.5 (7.0-13.8)^{a}	45.5 (17.3-64.9)^{b}	29.4 (24.3-39.6)^{b}	30.5 (20.4-43.2)^{b}
Propionate	0.1 (0.0-0.2)^{a}	6.3 (4.1-10.5)^{b}	2.3 (1.6-3.7)^{c}	2.4 (1.4-3.8)^{c}
n-Butyrate	0.0 (0.0-0.01)^{a}	11.4 (1.5-21.1)^{b}	0.5 (0.0-0.9)^{bc}	0.3 (0.1-1.0)^{ac}
i-Valerate	0.0 (0.0-0.2)^{a}	0.4 (0.1-0.6)^{b}	0.1 (0.0-0.7)^{ab}	0.1 (0.0-0.2)^{a}
n-Valerate	n.d.^{a}	0.4 (0.0-0.9)^{b}	0.0 (0.0-0.01)^{a}	n.d.^{a}
Lactate	0.7 (0.0-7.4)^{a}	13.9 (0.0-46.0)^{b}	1.8 (0.0-9.9)^{ab}	1.9 (0.0-5.2)^{ab}

Values are expressed as median (minimum-maximum); Cecum: $n_{germfree, conventional, SIHUMI, SIHUMI + Fv}$ = 11, 12, 9, 12; Colon: $n_{germfree, conventional, SIHUMI, SIHUMI + Fv}$ = 11, 12, 9, 11; Medians in the same row not sharing superscript letters differ significantly ($P \leq 0.05$). Differences among groups were analyzed using Kruskal-Wallis test (Dunn's Multiple Comparison Test). n.d., not detected; SIHUMI, simplified human microbiota; Fv, *Fusobacterium varium*

3.3.5 Gut lengths and gut weights

To investigate a possible correlation between increased putrescine concentrations in C3H mice and gut morphological parameters, intestinal lengths and weights were determined in all four study groups. The small intestine of SIHUMI + Fv mice was 26.9 % and 24.0 % shorter than that of SIHUMI or germfree mice, respectively, but colonic length did not differ between these mouse groups (Figure 3.2 A). This indicates that the development of small intestinal length is influenced by the microbial status. As no correlation was found between small intestinal length and any of the polyamines measured, it is suggested that bacterial produced polyamines do not contribute to this phenomenon.

The weights of small intestine, cecum and colon did not differ between SIHUMI and SIHUMI + Fv mice (Figure 3.2 B). The small intestinal and colonic weights of both SIHUMI groups were between those of germfree and conventional mice, whereas the cecal weights were comparable to those of the germfree mice. Germfree mice showed higher weights of small intestine and cecum and lower colonic weights compared to conventional mice.

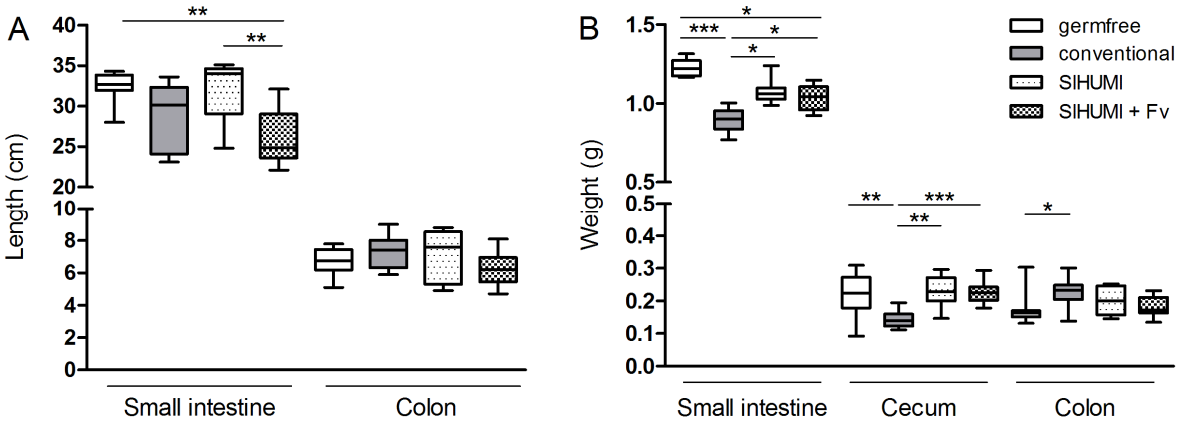

Figure 3.2: Gut length (A) and gut weight (B) of germfree, conventional, SIHUMI and SIHUMI + Fv -colonized mice. Data are expressed as medians. Boxes demonstrate the 25. – 75. percentiles and whiskers represent minima and maxima. n = 12 per group, except for SIHUMI with n = 9. Differences among groups were analyzed using Kruskal-Wallis test (Dunn's Multiple Comparison Test): * $P \leq 0.05$, ** $P \leq 0.01$; *** $P \leq 0.001$

3.3.6 Histology of cecal and colonic tissue

3.3.6.1 Gut morphology

To characterize the impact of an increased putrescine concentration on gut morphology in more detail, gut histological parameters were examined including crypt depth, thickness of epithelial layer, mucosa, submucosa and muscularis externa as well as proliferation and apoptosis.

Histological differences observed between the four mouse groups were similar in cecal and colonic tissue, but more pronounced in the former (Figure 3.4). As expected, germfree mice displayed both 1.5-fold lower crypt depth and mucosal thickness ($P \leq 0.001$) compared to conventional mice. However, the two SIHUMI groups showed no differences in crypt depth, thickness of epithelial layer, mucosa, submucosa and muscularis externa (Figure 3.3, Figure 3.4). Crypt depth and thickness of mucosa in the SIHUMI groups were similar to those of conventional mice and thickness of submucosa

and muscularis externa was more comparable to germfree mice. Hardly any differences between all four groups were observed in the thickness of epithelial layer and submucosa except for cecal submucosa, which was 1.7-fold, 1.7-fold and 1.5-fold thicker in conventional mice ($P \leq 0.001$) compared to germfree, SIHUMI and SIHUMI + Fv mice, respectively.

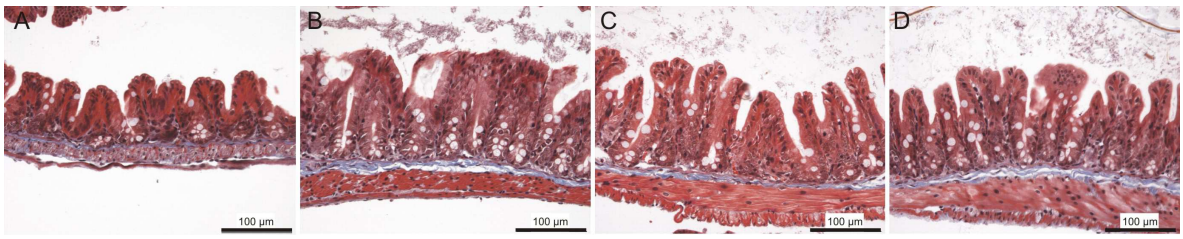

Figure 3.3: Representative image of cecal tissue of germfree (A), conventional (B), SIHUMI (C) and SIHUMI + Fv (D) mice. Cecal tissues were stained with Masson Goldner Staining. Pictures were taken using a 200 x magnification.

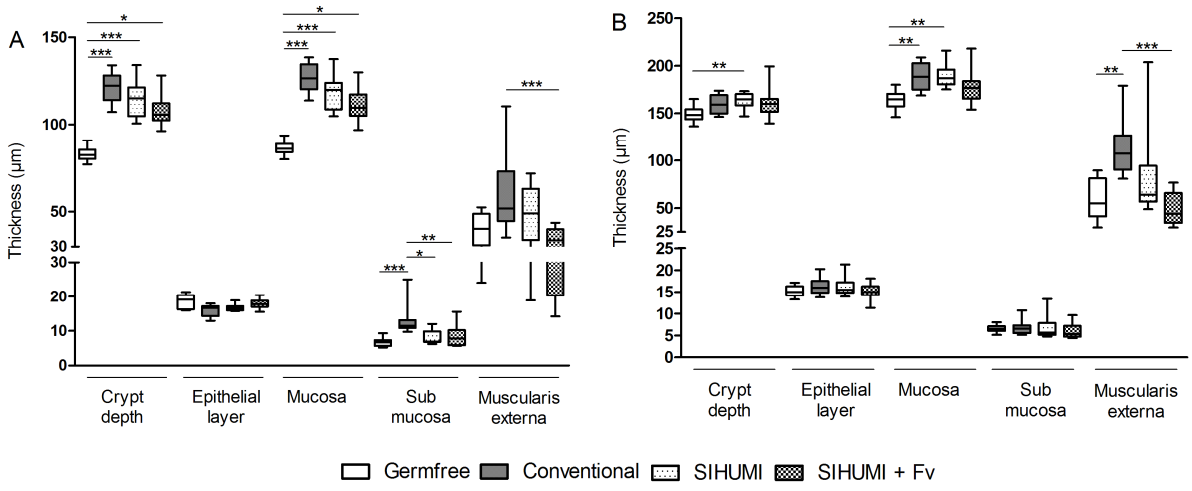

Figure 3.4: Histological analysis of cecal (A) and distal colonic (B) tissue of germfree, conventional, SIHUMI and SIHUMI + Fv mice. Measurements were performed on 50 well-oriented crypts. Data are expressed as medians. Boxes demonstrate the 25. – 75. percentiles and whiskers represent minima and maxima. n = 12 per group, except for SIHUMI with n = 9. Differences among groups were analyzed using the Kruskal-Wallis test (Dunn's Multiple Comparison Test): * $P \leq 0.05$, ** $P \leq 0.01$; *** $P \leq 0.001$

3.3.6.2 Apoptotic and mitotic cells

The number of mitotic cells in the ceca of germfree mice was lower compared to conventional, SIHUMI and SIHUMI + Fv mice ($P \leq 0.001$, Figure 3.5). This phenomenon was also observed in the colon, although it was only a trend ($P = 0.054$). No differences were observed in SIHUMI and SIHUMI + Fv for proliferation and apoptosis in cecal and

distal colonic tissue (Figure 3.5, Figure 3.6). The number of apoptotic cells in cecum and colon of germfree and conventional mice was comparable and slightly higher than those in SIHUMI and SIHUMI + Fv mice (Figure 3.6). As no correlation was observed between any of the measured polyamines and any of the investigated histological parameter, it was concluded that the increased putrescine concentration found in the gnotobiotic C3H mice did not affect the morphological parameters investigated.

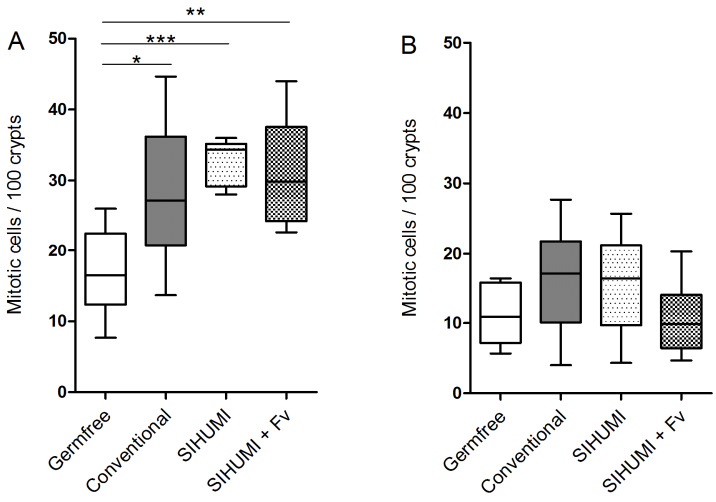

Figure 3.5: Mitosis in cecal (A) and distal colonic (B) tissue of germfree, conventional, SIHUMI and SIHUMI + Fv mice. Measurements were performed on 300 well-oriented crypts. Data are expressed as medians. Boxes demonstrate the 25. – 75. percentiles and whiskers represent minima and maxima. n = 12 per groups, except for SIHUMI with n = 9. Differences among groups were analyzed using the Kruskal-Wallis test (Dunn's Multiple Comparison Test): * $P \leq 0.05$, ** $P \leq 0.01$; *** $P \leq 0.001$

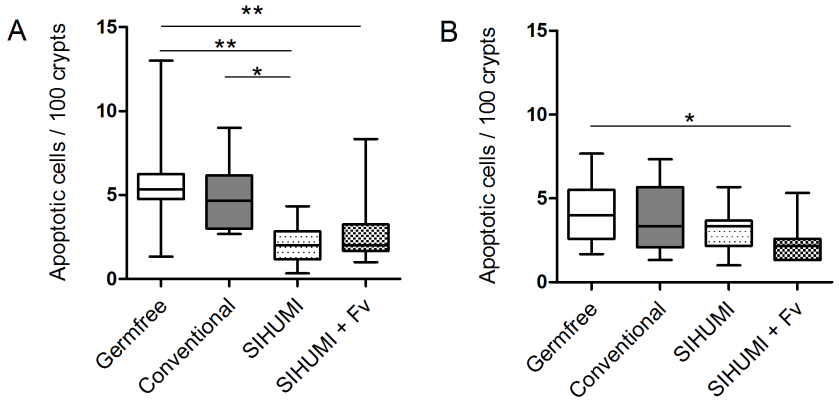

Figure 3.6: Apoptosis in cecal (A) and distal colonic (B) tissue of germfree, conventional, SIHUMI and SIHUMI + Fv mice. Measurements were performed on 300 well-oriented crypts. Data are expressed as medians. Boxes demonstrate the 25. – 75. percentiles and whiskers represent minima and maxima. n = 12 per group, except for SIHUMI with n = 9. Differences among groups were analyzed using the Kruskal-Wallis test (Dunn's Multiple Comparison Test): * $P \leq 0.05$, ** $P \leq 0.01$

3.4 Analysis of the microbial influence on gut length in PRM/Alf mice

Many reports described microbial effects on mucosal parameters [1, 28, 35], but the impact on gut length has rarely been investigated. For such investigations, the PRM/Alf mouse provides an excellent model since this mouse strain exhibits a 30 % longer intestine compared to other mouse strains [2]. It has been proposed that genetic, nutritional and microbiota-derived factors play a role in the development of this phenotype, but experimental evidence is missing. Therefore, a second approach of the present study was to clarify the microbial impact on gut lengthening in PRM/Alf mice and to identify bacterial factors possibly contributing to this phenomenon. Specifically, it was hypothesized that SCFAs produced by the intestinal bacteria play a role, since they exert trophic functions. Although bacterially produced polyamines did not affect gut morphological parameters of C3H mice in the present study, the contribution of polyamines was still investigated in PRM/Alf mice since this mouse strain seems to be an adequate model for gut morphological analysis.

3.4.1 Conventional microbiota

To analyze how different conventional microbiotas contribute to gut lengthening in PRM/Alf mice, germfree PRM/Alf mice were colonized with intestinal bacteria either from conventional PRM/Alf mice (PRM/PRM) or from conventional C3H mice (PRM/C3H). The eight week old offspring of previously colonized mice was used in the study.

3.4.1.1 Gut lengths

In PRM/PRM mice, the total intestines were 23.5 % longer compared to those of PRM/C3H mice ($P \leq 0.001$; Figure 3.7). This was mainly due to the 27.5 % higher length of the small intestine of PRM/PRM mice ($P \leq 0.001$), as the colonic length did not differ between the two mouse groups.

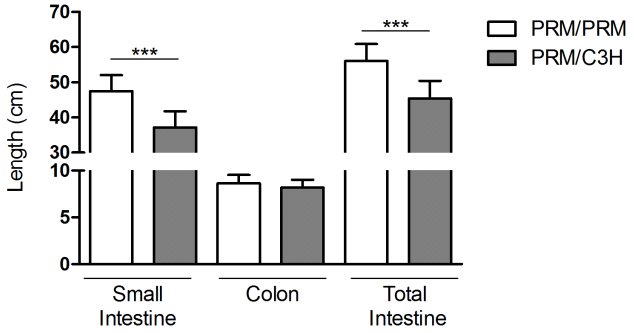

Figure 3.7: Gut length of PRM/Alf mice associated with either a complex PRM/Alf microbiota (PRM/PRM; n = 27) or a complex C3H micobiota (PRM/C3H; n = 27). Data are given as mean ± standard deviation. Differences among groups were analyzed using Student's *t*-test (unpaired). *** $P \leq 0.001$

3.4.1.2 Microbial trophic factors

SCFAs and polyamines are known to act as trophic factors [75, 76]. To investigate possible correlations between those microbial products and an increased gut length, cecal SCFA and polyamine concentrations in PRM/PRM and PRM/C3H mice were compared.

Short-chain fatty acids and lactate

SCFA concentrations did not differ between PRM/PRM and PRM/C3H mice, except for butyrate, which was 1.5-fold ($P \leq 0.01$) increased in PRM/C3H mice (Figure 3.8). Since elevated butyrate concentrations in PRM/C3H mice did not correlate with intestinal length, a special role of butyrate was considered to be unlikely. Lactate concentrations were similar in PRM/PRM and PRM/C3H mice.

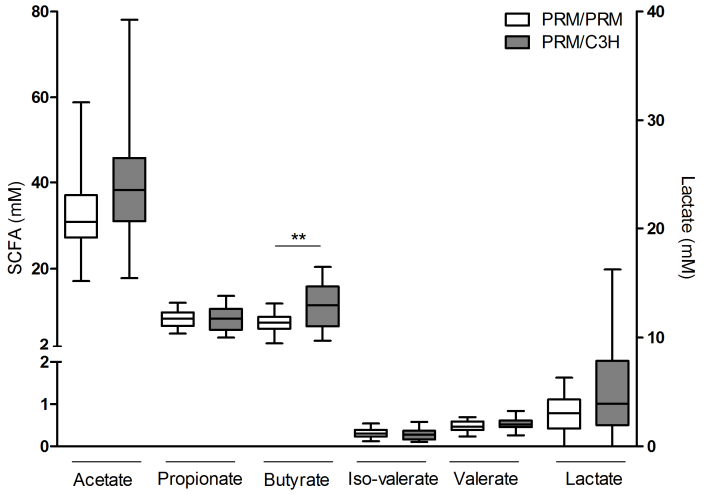

Figure 3.8: Cecal concentrations of short-chain fatty acids (SCFAs) and lactate (mM) in PRM/Alf mice associated either with a complex PRM/Alf microbiota (PRM/PRM; n = 26) or with a complex C3H microbiota (PRM/C3H; n = 27). Data are expressed as medians. Boxes demonstrate the 25. – 75. percentiles and whiskers represent minima and maxima. Differences among groups were analyzed using Mann Whitney test. ** $P \leq 0.01$

Polyamines

The polyamine concentrations did not differ between PRM/PRM and PRM/C3H mice, except for *N*-acetylspermine and *N*-acteylspermidine, which were 7.2-fold ($P \leq 0.001$) increased and 1.2-fold ($P \leq 0.05$) decreased, respectively (Figure 3.9 A). Since the *N*-acetylspermine concentration was significantly correlated with an increased total intestinal length ($R^2 = 0.66$, $P \leq 0.001$; Figure 3.9 B), this polyamine might contribute to gut lengthening.

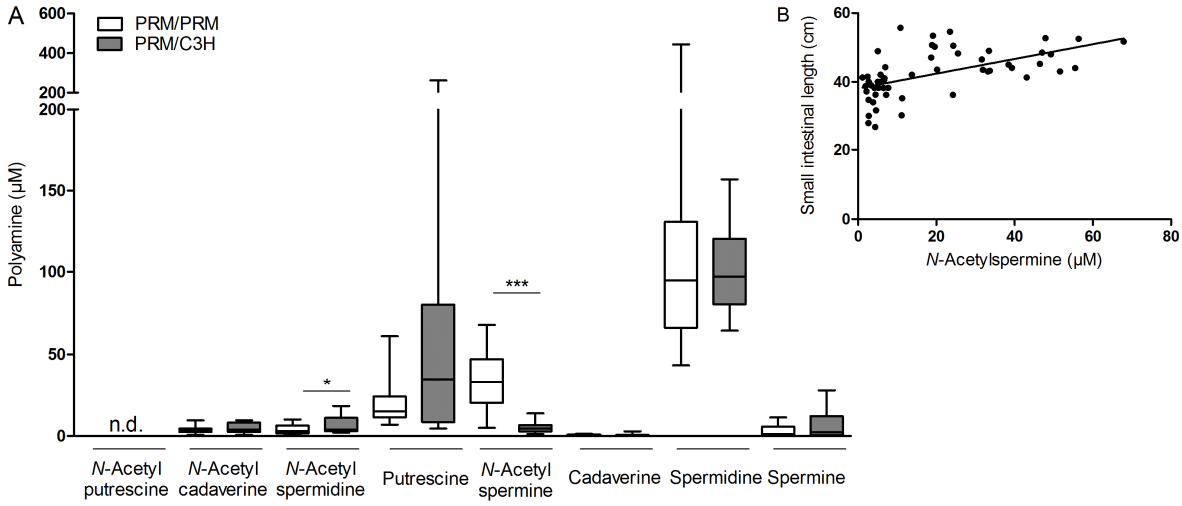

Figure 3.9 (A): Cecal polyamine concentrations (µM) of PRM/Alf mice associated either with a complex PRM/Alf microbiota (PRM/PRM; n = 27) or with a complex C3H microbiota (PRM/C3H; n = 27). Data are expressed as medians. Boxes demonstrate the 25. – 75. percentiles and whiskers represent minima and maxima. Differences among groups were analyzed using Mann Whitney test. * $P \leq 0.05$; *** $P \leq 0.001$; n.d. not detected **(B): Correlation of *N*-acetylspermine concentration (µM) and small intestinal length (cm) in PRM/PRM and PRM/C3H mice.** Data are expressed as medians. Statistical analysis was done with Spearman's rank correlation coefficient.

3.4.2 Simplified human microbiota

The results from the PRM/Alf experiment using different conventional microbiotas suggested that the complex microbiota from PRM/Alf mice contributed to gut lengthening. The next aim was to test whether a defined microbial community induces gut lengthening and whether SCFAs and/or polyamines produced by intestinal bacteria play a role in this induction. For this purpose, PRM/Alf and C3H mice were colonized with the SIHUMI + Fv community to increase the intestinal polyamine exposure. Eight week old offspring of mice previously associated with this community were used for this study.

3.4.2.1 Body weight

At eight weeks of age, SIHUMI + Fv -colonized PRM/Alf mice weighed 27.3 g (21.6 g – 31.6 g). Thus, their body weight was higher than that of C3H mice, which weighed 24.1 g (18.6 g – 28.4 g). These differences in body weight were even more pronounced when germfree and conventional mice were compared. Germfree and conventional PRM/Alf mice weighed 29.1 g ± 2.3 g and 27.2 g ± 2.3 g, respectively, while the body weights of C3H mice were 20.8 g ± 1.1 g and 18.9 g ± 0.6 g, respectively.

3.4.2.2 Gut lengths and gut weights

Small intestine, colon and total intestine of PRM/Alf mice colonized with SIHUMI + Fv were 39.2 %, 21.8 % and 35.9 %, respectively, longer than those of C3H mice ($P \leq 0.001$, Figure 3.10). Nevertheless, the proportion of small intestine and colon lengths relative to total gut lengths did not differ between the two mouse groups (83:17 in PRM/Alf vs. 81:19 in C3H, respectively). In addition, the weights of the full ceca and cecum walls from SIHUMI + Fv -colonized PRM/Alf mice were 3.5-fold ($P \leq 0.001$) and 1.1-fold ($P \leq 0.05$), respectively, higher than those from the PRM/Alf mice.

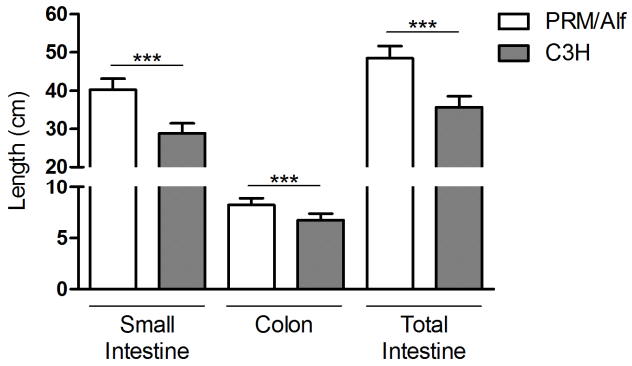

Figure 3.10: Gut length of SIHUMI + Fv -colonized PRM/Alf mice (n = 25) and C3H mice (n = 26). The lengths of the intestinal parts were determined in eight weeks old pups. Data are expressed as mean ± standard deviation. Differences among groups were analyzed using Student's *t*-test (unpaired). *** $P \leq 0.001$

When SIHUMI + Fv -colonized PRM/Alf mice were compared with their germfree or conventional PRM/Alf counterparts, the complete intestine of SIHUMI + Fv –colonized mice was 13.6 % and 20.2 %, respectively, shorter ($P \leq 0.001$, Figure 3.11 A). However, small intestine and colon were not reduced proportionally: The small intestine of SIHUMI + Fv –colonized PRM/Alf mice was 16.4 % and 21.7 % shorter than that of germfree and conventional PRM/Alf mice, respectively, but their colonic length was 2.5 % longer and 12.4 %, respectively, shorter.

When C3H mice differing in their microbial status were compared no differences in total gut length were observed between germfree, conventional and SIHUMI + Fv -colonized mice (Figure 3.11 B). Although length of small intestine and colon differed between the groups ($P \leq 0.05$), the differences were small.

This experiment indicates that the SIHUMI + Fv community contributes to gut shortening in the PRM/Alf mouse. In contrast, no differences in gut length were observed in C3H

mice. Thus, the influence of the microbiota on gut length seems to be specific for the mouse strain.

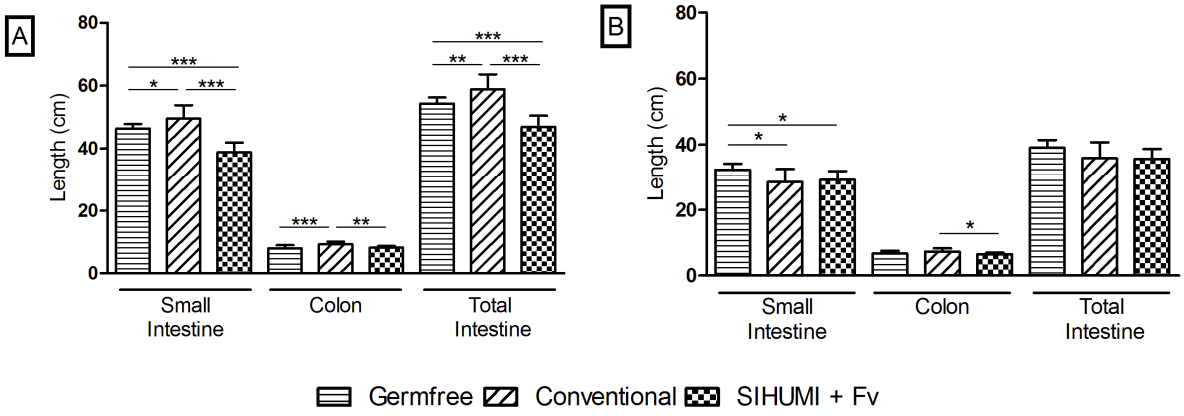

Figure 3.11: Gut length of germfree, conventional and SIHUMI + Fv -colonized PRM/Alf mice (A) and C3H mice (B). The lengths of the gut sections were determined in eight week old female pups. The total intestinal lengths correspond to the sum of the lengths of small intestines and colons. Data are expressed as mean ± standard deviation. Differences among groups were analyzed using one-way ANOVA (Bonferroni), * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; $n_{germfree}$ (PRM/Alf, C3H) = 14, 12, $n_{conventional}$ (PRM/Alf, C3H) = 14, 12, n_{SIHUMI} (PRM/Alf, C3H) = 12, 13

3.4.2.3 Bacterial cell numbers in the cecum

To investigate whether bacterial colonization was similar in both mouse strains, bacterial cell numbers of the members of the SIHUMI + Fv community in PRM/Alf and C3H mice were quantified with qPCR. PRM/Alf mice harbored lower cell numbers of *A. caccae*, *B. thetaiotaomicron*, *B. longum*, *C. butyricum* and *F. varium* ($P \leq 0.01$, Table 3.7). However, the differences were small, except for *A. caccae*, whose cell number was 0.84 $\log_{10}$ lower in PRM/Alf compared to C3H mice.

Table 3.7: Microbial cell numbers in cecal contents of PRM/Alf and C3H mice, both colonized with SIHUMI + Fv. Data are expressed as LOG numbers/g dry gut content.[1]

Organism	PRM/Alf	C3H
A. caccae	8.58 ± 0.20	9.42 ± 0.21 ***
B. thetaiotaomicron	10.80 ± 0.24	11.15 ± 0.28 ***
B. longum	9.56 ± 0.28	9.99 ± 0.24 ***
B. producta	9.35 ± 0.32	9.50 ± 0.17
C. butyricum	8.84 ± 0.42	9.18 ± 0.27 **
C. ramosum	9.73 ± 0.29	9.77 ± 0.23
E. coli	8.67 ± 0.30	9.09 ± 0.35
F. varium	9.81 ± 0.27	10.27 ± 0.16 ***
L. plantarum[2]	n.d.	n.d.
total	11.13 ± 0.26	11.46 ± 0.31 ***

[1] Values are given as mean ± standard deviation; n (PRM/Alf) = 26, n (C3H) = 26; Significant differences were analyzed using Student's *t*-test (unpaired): ** $P \leq 0.01$; *** $P \leq 0.001$

[2] Cell numbers of *L. plantarum* were determined by plating on Rogosa agar (Oxoid, UK).

n.d., not detected

3.4.2.4 Microbial products

SCFAs, lactate and polyamines were quantified in cecal contents of PRM/Alf and C3H mice harboring a SIHUMI + Fv microbiota.

Short-chain fatty acids and lactate

In PRM/Alf mice, both acetate and butyrate concentrations were 1.2-fold lower compared to C3H mice ($P \leq 0.05$, Figure 3.12). However, the molar acetate:propionate:butyrate ratios of 84:11:5 in PRM/Alf mice and 86:9:5 in C3H mice were comparable. Lactate concentrations were 1.5–fold lower in PRM/Alf mice ($P \leq 0.05$). No further correlation was found between SCFAs or lactate concentration and intestinal length, suggesting that SCFAs and lactate do not contribute to intestinal lengthening.

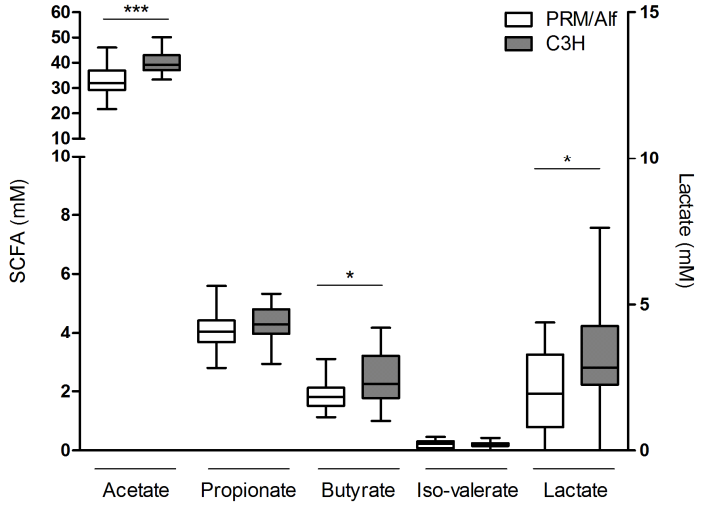

Figure 3.12: Cecal SCFA and lactate concentrations (mM) in SIHUMI + Fv -colonized PRM/Alf mice (n = 27) and C3H mice (n = 26). Data are expressed as medians. Boxes demonstrate the 25. – 75. percentiles and whiskers represent minima and maxima. Differences among groups were analyzed using Mann Whitney test. * $P \leq 0.05$, *** $P \leq 0.001$

Polyamines

In PRM/Alf mice, the concentrations of putrescine, spermidine and spermine were by factors of 1.4, 2.0 and 2.1, respectively, smaller compared to C3H mice ($P \leq 0.001$, Figure 3.13 A). In contrast, concentrations of *N*-acetylcadaverine and cadaverine were 3.1-fold and 1.8-fold, respectively, greater in PRM/Alf mice than in C3H mice. Interestingly, *N*-acetylcadaverine concentrations were positively correlated with total intestinal length in PRM/Alf mice ($R^2 = 0.76$, $P \leq 0.001$; Figure 3.13 B), indicating that this polyamine might contribute to intestinal lengthening in SIHUMI + Fv -colonized mice. No correlation was found for cadaverine ($R^2 = 0.51$, $P \leq 0.001$).

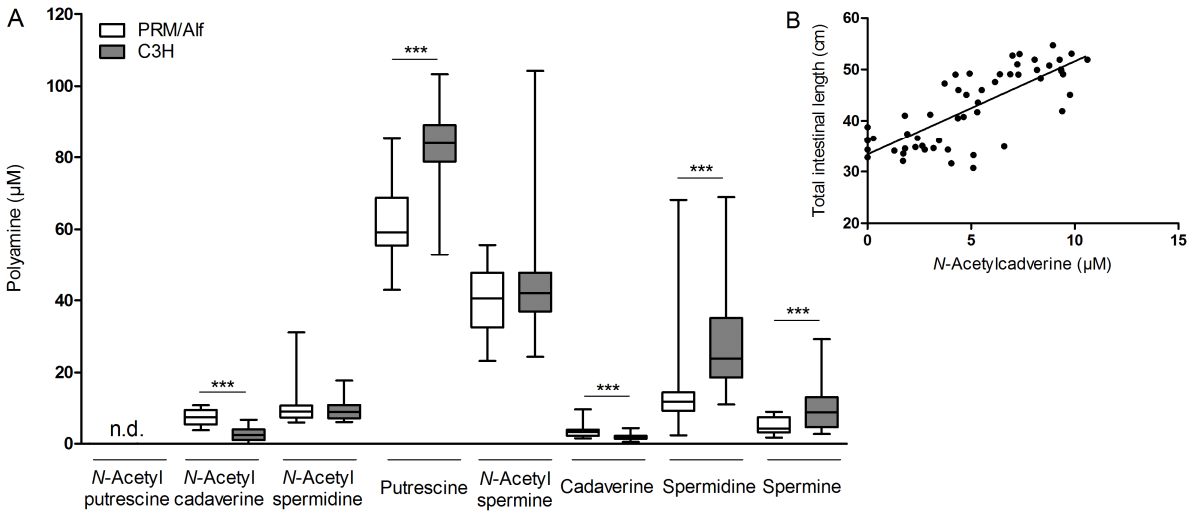

Figure 3.13 (A): Cecal polyamine concentrations (µM) in SIHUMI + Fv -colonized PRM/Alf mice (n = 29) and C3H mice (n = 26). Data are expressed as medians. Boxes demonstrate the 25. – 75. percentiles and whiskers represent minima and maxima. Differences among groups were analyzed using Mann Whitney test. *** $P \leq 0.001$; n.d., not detected **(B): Correlation of *N*-acetylcadaverine concentration (µM) and total intestinal length (cm) in SIHUMI + Fv – colonized PRM/Alf and C3H mice.** Data are expressed as medians. Statistical analysis was done with Spearman's rank correlation coefficient.

3.4.2.5 Bacterial proteome analysis

To identify bacterial factors other than SCFAs and polyamines possibly involved in gut lengthening, proteome analysis was carried out on bacteria isolated from cecum content of PRM/Alf and C3H mice, both colonized with SIHUMI + Fv, using 2D-DIGE (Figure 3.14). Differentially expressed proteins were selected for significant at least 2-fold changes ($P \leq 0.05$) and analyzed using NanoLC-ESI/MS-MS.

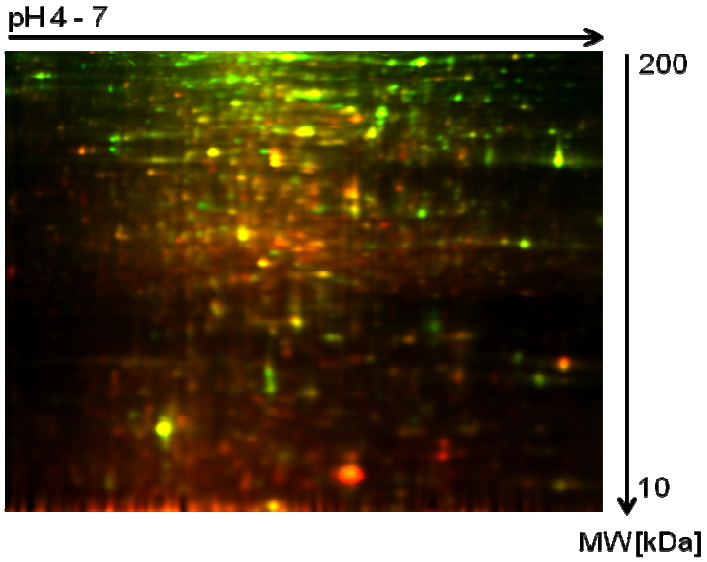

Figure 3.14: Representative 2D-DIGE image of cecal bacterial proteins isolated from SIHUMI + Fv -colonized PRM/Alf and C3H mice (male). Cecal bacterial proteins from PRM/Alf and C3H mice were labeled with CyDye DIGE Fluor Cy3 (red) and Cy5 (green), respectively, and separated by 2D-DIGE. Internal standard was labeled with CyDye DIGE Fluor Cy2 (yellow). MW: Molecular weight

Bacterial proteome analysis revealed 250 differently expressed proteins, 100 of which were up-regulated and 150 of which were down-regulated. However, due to small amounts of protein, only 20 of these proteins were unambiguously identified. Among those, 19 proteins were down-regulated and one protein was up-regulated (Table 3.8). The proteins mostly belong to the bacterial strain *B. thetaiotaomicron* and are mainly involved in glycolysis, glyconeogenesis, ATP synthesis, transcription, and translation. As these proteins were also often reported to be differently expressed in other studies with different aims and approaches [151, 154], it is difficult to see that these proteins may affect gut lengthening in the PRM/Alf mice.

Table 3.8: Differently expressed proteins of bacteria of the SIHUMI + Fv community in PRM/Alf mice compared to C3H mice

Uniprot ID[1]	Name of protein	Organism	Function	Spot no.[2]	Protein expression in PRM/Alf, $P \leq 0.05$
Q8A6P8	60 kDa Chaperonin (HSP 60)	*B. thetaiotaomicron*	Translation	106	-3,25
				116	-2,33
				297	-2,22
Q8A414	Phosphoenolpyruvate-carboxykinase	*B. thetaiotaomicron*	Gluconeogenesis	130	-2,33
				138	-2,45
				114	- 2,03
Q8A287	2,3-bisphosphoglycerate-independent phosphoglycerate-mutase	*B. thetaiotaomicron*	Gluconeogenesis	123	-2,83
				124	-2,99
Q8A9E3	Aspartyl-tRNA synthetase	*B. thetaiotaomicron*	Translation	193	-2,11
Q8G5B7	Elongation factor Tu	*B. longum*	Translation	229	-2,10
Q8A463	Elongation factor Tu	*B. thetaiotaomicron*	Translation	1226	-5,35
				1535	-2,09
Q8A0Z3	Elongation factor Ts	*B. thetaiotaomicron*	Translation	678	-2,00
Q8A9V4	ATP synthase subunit beta	*B. thetaiotaomicron*	ATP Synthesis	377	-2,00
B3DSV2	Enolase	*B. longum*	Glycolysis	402*	2,11*
				1240	-4,18
Q8G6D6	Phosphoglycerate kinase	*B. longum*	Glycolysis	409	-3,46
				409*	-2,42*
Q8A4A2	DNA-directed RNA polymerase subunit alpha	*B. thetaiotaomicron*	Transcription	655	-2,38
Q8A1A2	L-Rhamnose isomerase	*B. thetaiotaomicron*	Fructose metabolism	387	-2,78

[1] Uniprot ID (universal protein identification): correspond to a specific protein in the database
[2] The spot number was chosen from the DeCyder software version 6.5 (GE Healthcare, Munich, Germany).
*Proteins were found to be differently expressed in female mice, whereas all other detected proteins were differently expressed in male mice.

4 DISCUSSION

The intestinal microbiota affects gut morphology by influencing epithelial proliferation, development of the lamina propria, villus length and crypt depth [1]. However, it is as yet unclear how these effects are brought about and which microbial factors are involved. Moreover, the bacterial influence on gut length has rarely been investigated, even though it might be important for patients suffering from short bowel syndrome.

The objective of this work was therefore to characterize the role of the microbiota on gut length and to identify possible bacterial factors involved in mucosal growth. Specifically, it was hypothesized that SCFAs and/or polyamines contribute to intestinal lengthening and mucosal growth, since these microbial metabolites are known to act as trophic factors. Two different approaches were used in this study. First, the role of bacterially-produced polyamines was investigated on mucosal parameters including crypt depth, mucosa thickness and epithelial proliferation using C3H mice. Second, the microbial effect on gut length was established using PRM/Alf mice.

4.1 Mucosal parameters are not influenced by bacterial polyamines in C3H mice

Studies investigating host-microbe interactions are hampered by both the complexity and the inter-individual variability of the gut microbiota [133]. To circumvent these problems, the SIHUMI community was used in this study, which was originally established in rats by Becker *et al.* [133]. The bacterial members were selected according to their incidence in humans, the spectrum of the fermentation products formed and the availability of their genomic sequence [133]. Although the SIHUMI model does not completely reflect the situation in animals with a complex microbiota, there are many similarities between a complex microbiota and the SIHUMI community. First, Becker *et al.* reported that the metabolic activity of the SIHUMI community mimics at large that of the complex microbiota [133]. Second, the present study revealed hardly any differences in intestinal polyamine concentrations between conventional- and SIHUMI-C3H mice except for two polyamines. Third, gut morphological parameters determined in this study did not differ essentially between conventional and SIHUMI mice. Fourth, total bacterial cell numbers of the SIHUMI consortium were comparable to those of a complex microbiota. The SIHUMI community resembles a complex microbiota in many aspects and its use therefore circumvents problems that arise from both the complexity and the inter-individual

variability of a conventional microbiota. Therefore, the SIHUMI provides an excellent model for investigating host-microbe interactions.

In the present study, it was hypothesized that an increased bacterially-produced polyamine exposure influences mucosal parameters such as crypt depth, mucosa thickness and epithelial proliferation. To test this hypothesis, germfree C3H mice were colonized with two different defined microbial communities differing in their ability to produce polyamines. One group was colonized with a simplified human microbiota (SIHUMI) consisting of eight bacterial strain producing low amounts of polyamines. The second group was colonized with SIHUMI plus the polyamine-producing *F. varium* (SIHUMI + Fv) [134, 155]. Germfree and conventional mice served as reference groups.

The addition of *F. varium* to the SIHUMI community resulted in changes of the microbial composition, but total bacterial cell numbers did not change indicating that the SIHUMI represents a stable community. In SIHUMI + Fv mice, *L. plantarum* was not detectable any more. Since the proportion of this strain was <0.001% of total bacteria and it did not produce any polyamines *in vitro*, the absence of *L. plantarum* was not considered to be of major importance in this study.

The present study was designed to investigate the influence of an increased exposure of the gut mucosa to bacterial polyamines. However, effects of SCFAs were also taken into consideration since they are known to act as trophic factors [76, 156]. As SIHUMI and SIHUMI + Fv mice did not display differences in their cecal and colonic SCFA concentrations, confounding effects of SCFAs can be eliminated. Thus, mice colonized with SIHUMI + Fv serve as an excellent model for investigating the effect of bacterially produced polyamines on gut morphology.

SCFA concentrations of SIHUMI and SIHUMI + Fv –colonized C3H mice were up to 2.0-fold lower compared to conventional C3H mice, but their intestinal lengths and weights were similar or even higher. This allows two possible conclusions. First, SCFAs do not influence intestinal length. Second, the SCFA concentrations reached in SIHUMI and SIHUMI + Fv mice were sufficient for intestinal length development and a further increase in SCFA had no additional effects. This would be in agreement with the findings of Bartholome *et al.*, who showed that physiological levels of approximately 9 mM of butyrate are sufficient to increase mucosal growth and produced the same mucosal effects as 60 mM of butyrate [93]. However, in both SIHUMI groups cecal butyrate concentrations were only 1 mM. Moreover, small intestine of SIHUMI + Fv mice was shorter than that of SIHUMI mice. The reason for this finding remains unclear, but it cannot be explained by

the differences in intestinal SCFA concentrations, because they were similar in both groups.

By adding the polyamine-producer *F. varium* to the defined microbial SIHUMI community in C3H mice, the intestinal polyamine concentrations were increased up to 1.7-fold. This was mainly due to the increase in putrescine, which was up to 2.8-fold higher in cecum and colon of SIHUMI + Fv mice compared to SIHUMI and conventional mice. Germfree mice had only low concentrations of putrescine. The elevated intestinal putrescine concentration after the addition of *F. varium* is in line with the findings by Noack *et al.*, who reported a putrescine production of *F. varium in vitro* and *in vivo* [134, 155]. *F. varium* became a dominant member of the SIHUMI + Fv community with a proportion of up to 13 %. Dietary putrescine is rapidly and completely absorbed in the small intestine [157]. Therefore, it may be concluded that the diet was no source of putrescine detected in cecum and colon.

The predominant polyamine in germfree mice was spermidine, suggesting that this polyamine is the main endogenously formed polyamine in mice. This is in agreement with the report of Matsumoto *et al.*, who observed also spermidine as dominant polyamine in the colonic lumen of germfree mice [158]. In contrast, Noack *et al.* reported putrescine to be the dominant polyamine in germfree rats [159]. However, it is conceivable that germfree mice and rats differ in their polyamine metabolism.

In accordance with Noack *et al.* and Matsumoto *et al.* [158, 159], very high spermidine concentrations and relatively low putrescine and spermine concentrations were observed in conventional mice. The cecal and colonic spermidine concentrations in these mice were 8.3-fold and 3.1-fold higher than those in germfree mice. This indicates that the intestinal microbiota plays an important role in the polyamine metabolism, which supports previous studies [158-160]. The luminal spermidine concentrations in SIHUMI and SIHUMI + Fv mice were similar to those of germfree mice, indicating that there was no spermidine-producing bacterium in the SIHUMI community. This is in contrast to the results of the *in vitro* experiments, in which *C. butyricum* formed spermidine (Table 3.3). Furthermore, Noack *et al.* reported that *B. thetaiotaomicron* produces spermidine under both *in vitro* and *in vivo* conditions [134, 155]. This discrepancy might be due to different diets, as bacterial polyamine patterns change dramatically in response to certain proteins or indigestible polysaccharides [155, 161].

In SIHUMI + Fv mice, up to 2.7-fold lower cadaverine concentrations were observed compared to SIHUMI mice. Since none of the SIHUMI bacteria produced cadaverine *in vitro*, the source of cadaverine cannot be definitely identified. It may be speculated that

E. coli is responsible for the lower cadaverine concentration in SIHUMI + Fv mice, since this species is known for its ability to produce cadaverine [119] and it exhibited a 3.4-fold lower cell number in these mice. Interestingly, cadaverine was also detected in germfree mice, even thought it has long been considered to be exclusively produced the intestinal microbiota [155, 162]. However, there are also reports about cadaverine formation by mammalian tissue [163].

Large differences in gut anatomy were observed between germfree and conventional mice. In germfree mice, the crypts were shallower, mucosa and muscularis externa were thinner and cell proliferation was reduced. These results are in agreement with the literature [44, 48, 51]. However, no differences in the number of apoptotic cells were observed between germfree and conventional mice in the present study. This is in contrast to the findings of Willing and Kessel, who reported an increased apoptosis of epithelial cells in conventional pigs compared to germfree pigs [164]. This discrepancy might be due to the use of a different animal species or the application of different methods for the assessment of apoptosis. In the present study, apoptotic cells were quantified with a method that only detects cells in the final phase of apoptosis. The mitosis / apoptosis ratios showed an imbalance towards a higher cell proliferation in all study groups, which may be due to the age of mice, as eight week old mice are not yet adult and therefore still growing.

Despite higher cecal putrescine concentrations in SIHUMI + Fv mice, there were no differences between SIHUMI and SIHUMI + Fv mice in various gut morphological parameters including colonic length, intestinal weights, crypt depth and thickness of the different gut layers as well as in intestinal physiological parameters, including proliferation and apoptosis. One exception was the small intestinal length, which was decreased in SIHUMI + Fv mice. However, a contribution of putrescine to gut lengthening is unlikely, since there was no correlation between putrescine concentration and intestinal length.

Numerous studies describes beneficial effects of putrescine on gut growth [165, 166] and on healing of injured intestinal mucosa [167]. Moreover, putrescine stimulates DNA, RNA and protein synthesis *in vitro* [165], increases cell proliferation [168] and acts as an energy source for epithelial cells [169]. These findings are in agreement with the observations of Osborne and Seidel, who demonstrated that intestinal tissue areas with the highest mucosal thickness displayed the highest putrescine concentrations, whereas those with least mucosal thickness contained the lowest putrescine concentrations [170]. However, most studies have been carried out *in vitro*, under non-physiological conditions or in situations where cell proliferation is required, for example during healing of injured

mucosa. The majority of studies describe beneficial effects of dietary polyamines under physiological conditions [130, 171]. However, there are also some reports that do not support such effects [172-174]. For example, Delzenne *et al.* reported an increased cecal putrescine concentration after oral administration of oligofructose, but this did not affect cell proliferation, crypt depth or villus height in the small intestine of Wistar rats [173]. These observations were confirmed by Sabater-Molina and colleagues, who observed no stimulation of gut maturation following an increase of cecal putrescine in piglets fed fructooligosaccharides [174]. There were also no changes in cell proliferation in response to increased colonic putrescine concentrations in pigs fed a polyamine-rich soy protein diet [172].

Three explanations may be possible why there was no putrescine-mediated stimulation of gut development observed in the present study. First, putrescine may only be beneficial in situations where cell proliferation is required. This is the case in suckling pups [126, 171], old mice [175] and animals with damaged mucosa [176]. Second, putrescine concentrations observed in SIHUMI mice may have been sufficient for gut development. Higher putrescine levels, as in the case of SIHUMI + Fv mice, may not have additional effects. Third, luminal putrescine concentrations may have been too low to stimulate gut maturation. Data from the literature indicate that a putrescine concentration of 415.3 nmol/g cecal dry weight (238.1 – 532.7 nmol/g), as measured in the SIHUMI + Fv mice, is below the minimal effective level. Concentrations of up to 620 nmol/g cecal dry weight did not affect crypt depth, villus height and cell proliferation in rats [173] and piglets [174]. In contrast, a stimulation of mucosal growth as characterized by increased DNA, RNA and protein contents was observed in rats infused with 1 µmol of putrescine / h for 66 h [166]. To find out which of these explanations is true, additional experiments need to be conducted. Exposure of the host to bacterial putrescine might be enhanced by complementing the SIHUMI with additional polyamine-producing bacteria [155]. Such experiments might also take situations into account, that are known to involve an increased cell proliferation, as it is the case in growing animals or animals with an injured mucosa.

In conclusion, the present work clearly showed that putrescine can be excluded as trophic factor stimulating mucosal growth under physiological conditions, since it did not affect intestinal morphology although its intestinal concentration was increased.

4.2 Gut length of PRM/Alf mice is influenced by the intestinal microbiota

The microbial impact on gut length was examined in the PRM/Alf mouse, which is a model for intestinal lengthening. To answer the question whether different conventional microbiotas induce gut lengthening differently, germfree PRM/Alf mice were associated with a complex microbiota either from conventional PRM/Alf (PRM/PRM) or C3H mice (PRM/C3H). Moreover, it was investigated whether a simplified human microbiota is able to induce gut lengthening in PRM/Alf mice. Therefore, germfree PRM/Alf and C3H mice were colonized with the SIHUMI + Fv community and compared with germfree and conventional mice of the respective mouse strain.

The length of the small intestine of PRM/PRM mice was one quarter longer compared to PRM/C3H mice. Furthermore, small intestine and colon length of conventional PRM/Alf mice were also longer compared to those of SIHUMI + Fv -colonized PRM/Alf mice. These findings suggest that the intestinal microbiota influences the intestinal length in the PRM/Alf mouse. Since PRM/Alf mice colonized with a complex PRM/Alf microbiota displayed the longest intestine, the present study confirms the observation by Aubin-Houzelstein *et al.* and Boumahrou *et al.* that intestinal lengthening in PRM/Alf mice is not only dependent on genetics, but also on environmental factors [2, 3]. Moreover, the results of the present study clearly showed that microbiota from different donor mice affect the gut length in PRM/Alf mice. Similar observations were reported for crypt depth and villus length in pigs [177, 178]. Che and colleagues reported deeper crypts and longer villi in the jejunum of pigs associated with a human fecal suspension compared to pigs colonized with a complex pig microbiota [177]. Moreover, Shirkey *et al.* demonstrated differences in crypt depth and villus length between pigs monoassociated with *Lactobacillus fermentum* and those monoassociated with *E. coli* [178].

Independent of the bacterial colonization status, body weights as well as small intestinal and colonic lengths of PRM/Alf mice were higher than those of C3H mice. These data are in accordance to the studies of Aubin-Houzelstein *et al.*, who compared conventional PRM/Alf and C3H mice [2]. However, intestinal lengthening observed in PRM/Alf mice cannot be explained by the concomitantly occurring higher body weight, because intestinal length either corrected by body length or body weight was still higher in PRM/Alf mice compared to C3H mice [2]. Aubin-Houzelstein *et al.* reported a total intestinal length in conventional PRM/Alf mice of 74.8 cm ± 5.3 cm [2], which is 27 % higher compared to conventional PRM/Alf mice of the present study (58.9 cm ± 4.7 cm). This difference may be explained by different ages, as PRM/Alf mice used by Aubin-Houzelstein *et al.* were four weeks older, or by changes of the microbial composition in PRM/Alf mice.

The intestines of germfree PRM/Alf mice were shorter than those of conventional PRM/Alf mice, but longer than those of the SIHUMI + Fv mice. This indicates that the SIHUMI + Fv microbiota contributed to intestinal shortening in PRM/Alf mice. It might be possible that mice harboring the SIHUMI + Fv microbiota either lacks factors that support gut lengthening or even possess factors that inhibit gut lengthening.

While the intestinal length of the PRM/Alf mice was influenced by the intestinal microbiota, the gut length of C3H mice was similar between germfree, conventional and SIHUMI mice. This leads to the assumption that specific interactions between the host-genotype and the microbiota are important to influence intestinal length. Other studies using the mouse-strain ICR/JCL reported a longer intestinal length of germfree mice compared to their conventional counterparts [179, 180]. This discrepancy compared to the results found in PRM/Alf mice might not only be explained by different genotypes, but also by different microbial compositions or different diets. Data from the literature indicate that dietary substances such as fiber and proteins influence gut length [180, 181]. However, whether dietary substances affect gut length directly or indirectly via changing microbial composition remains so far unknown and has to be further investigated.

In conclusion, intestinal bacteria influence intestinal length dependent on their microbial composition and on host-genotype. The present study also confirms that the gut length in PRM/Alf mice depends on genetic as well as on environmental factors influenced by the intestinal microbiota, since the longest intestine was reached in PRM/Alf mice harboring a complex PRM/Alf microbiota.

4.2.1 SCFAs do not contribute to intestinal lengthening in PRM/Alf mice

To clarify the role of SCFAs in intestinal lengthening in the PRM/Alf mouse, SCFAs were measured in cecum of PRM/PRM and PRM/C3H mice as well as in cecum of PRM/Alf and C3H mice, which were both colonized with the SIHUMI + Fv microbiota.

In this study, intestinal lengthening was observed mainly in small intestine, but SCFA concentrations were measured in cecum. This is an appropriate way for measuring, since many research groups reported a stimulation of small intestinal mucosal growth after cecal or colonic infusion of SCFAs [85, 87]. Moreover, cecal infusion increased the amount of jejunal DNA and villus height only in innervated rats, but no trophic effect was observed when rats were denervated, suggesting that SCFAs exert their effects systemically [83].

The small intestine of PRM/PRM mice was about one quarter longer than that of PRM/C3H mice, but the cecal SCFA concentration did not differ, except for butyrate, which was 1.5-fold lower in PRM/PRM mice. This finding is similar to the one observed in SIHUMI + Fv -colonized PRM/Alf and C3H mice: Whereas PRM/Alf mice showed a 35.9 % higher total intestinal length compared to C3H mice, cecal acetate and butyrate concentrations were both 1.2-fold decreased. In addition, SCFA concentrations did not correlate with intestinal length in any of the conducted experiments. Thus, it is concluded that SCFAs do not contribute to intestinal length in this experimental model. The lower acetate and butyrate concentrations in mice showing a longer intestine may be explained as follows: First, the absorption rate might be increased due to an increased intestinal surface in PRM/Alf mice. SCFAs are predominantly absorbed by passive diffusion with no competition between acetate, propionate and butyrate [182]. In addition, there is also an active transport of SCFAs via the carrier transporter protein MCT1 [79, 80]. Second, the SCFA production might have been decreased due to the decreased bacterial cell numbers in PRM/Alf mice colonized with SIHUMI + Fv. In particular, cell numbers of the butyrate-producing *A. caccae* were decreased in comparison to C3H mice.

Few data on the effect of SCFA concentrations on intestinal length are available [98, 179, 183]. The corresponding studies have been carried out using dietary fibers such as pectin and cellulose and they reported an increased intestinal length after dietary fiber intake [98, 179, 183]. Since dietary fibers are fermented by the intestinal microbiota to SCFAs, the authors assume that SCFAs contributed to intestinal lengthening. This is in contrast to the results of the present study, which does not support a role of SCFAs. However, intestinal growth may also be stimulated by the high viscosity induced by fiber [184], which may explain the differences between the present and previous studies.

Although several studies have shown a trophic effect of SCFAs on mucosal growth and cell proliferation [83-86, 185], there was no correlation between intestinal length and SCFA concentrations in the present study. Nevertheless, most of the studies from the literature focused their observations on mucosal parameters such as crypt depth, villus height, amount of DNA, RNA and protein as well as epithelial cell proliferation [83-86, 185]. It should be kept in mind that the effect of SCFAs on intestinal length was only indirectly deduced from increasing fiber intake. No reports are available in the literature describing effects of direct infusion of SCFAs on intestinal length.

In conclusion, the present experiments revealed no stimulatory effect of SCFAs on intestinal length. Therefore, other microbial factors influencing gut morphology must exist.

4.2.2 Contribution of polyamines on gut lengthening in PRM/Alf mice remains doubtful

The second hypothesis was that bacterially produced polyamines stimulate intestinal length. Therefore, free polyamines were measured in cecum of PRM/PRM and PRM/C3H mice as well as in cecum of SIHUMI + Fv colonized PRM/Alf and C3H mice.

Measurements showed 7.2-fold higher *N*-acetylspermine concentrations in the cecum of PRM/PRM mice compared to PRM/C3H mice. Moreover, *N*-acetylspermine concentrations correlated positively with intestinal length in PRM/PRM mice, which points to a contribution of this polyamine to gut lengthening. However, little information is available about the biological importance of *N*-acetylspermine. It is known that spermine is acetylated to *N*-acetylspermine by the acetyl-CoA: spermine N^1-acetyltransferase to facilitate the cellular and urinary secretion of spermine and to regulate the pool of free spermine [115, 186, 187]. Moreover, *N*-acetylspermine serves as an intermediate in the conversion of spermine to spermidine [115]. Thus, the elevated *N*-acetylspermine concentrations in the cecum of PRM/PRM mice may have two possible reasons: First, PRM/PRM mice might have had spermine in excess requiring its removal via the formation of *N*-acetylspermine. Since spermine has the strongest physiological function and also toxicity among the physiologic polyamines [75, 188], its concentration has to be strictly controlled [158]. Most prokaryotes do not express the enzyme spermine synthase [119], indicating that the spermine detected in the cecum of PRM/PRM mice was of eukaryotic origin. Since intestinal tissue contains high amounts of spermine [189], excessive spermine in PRM/PRM mice may have resulted from an increased tissue mass due to gut elongation. Second, PRM/PRM mice may require higher spermidine levels, which are formed from spermine via the intermediate *N*-acetylspermine. In support to this pathway, Dorhout and colleagues reported the transformation of deuterium-labeled spermine into putrescine and spermidine in suckling rats [126]. Spermidine is a growth-promoting compound [115], which stimulates intestinal maturation in neonatal rats by increasing brush border enzyme activities of lactase, sucrose and maltase, and increases mucosal growth in rats by increasing DNA, RNA and protein content [127, 129]. However, effects of spermidine on intestinal length have not yet been investigated. So far, no growth-stimulating functions of *N*-acetylspermine have been described. Therefore, a direct effect of *N*-acetylspermine on intestinal length can be excluded. Nevertheless, it remains speculative, whether *N*-acetylspermine stimulates intestinal length indirectly by forming the growth-promoting spermidine.

In PRM/Alf mice colonized with SIHUMI + Fv, many cecal polyamines were similar or lower compared to C3H mice harboring the same microbial community. Interestingly, the concentrations of *N*-acetylcadaverine and cadaverine were higher in the SIHUMI + Fv – colonized PRM/Alf mice than in the SIHUMI + Fv – associated C3H mice. Since *N*-acetylcadaverine correlated positively with intestinal length, a stimulation of intestinal lengthening by this compound can be assumed. *N*-acetylcadaverine is formed from cadaverine [115]. Whenever there are substantial amounts of cadaverine, *N*-acetylcadaverine is also present [190]. Cadaverine increases DNA synthesis in colonic and small intestinal cell lines [191] and elevated cadaverine levels were found in colonocytes isolated from cancerous areas compared to those of normal tissue [192]. Although a role of *N*-acetylcadaverine and cadaverine as growth factors cannot be excluded, two facts argue against it: First, cadaverine did not correlate with intestinal length in the present study. Second, *N*-acetylcadaverine undergoes cellular and urinary secretion to regulate the pool of free cadaverine [115]. No studies were published describing trophic effects for *N*-acetylcadaverine. Therefore, higher cadaverine and *N*-acetylcadervine concentrations found in SIHUMI + Fv –colonized PRM/Alf mice may be explained by differences in the endogenous cadaverine metabolism between PRM/Alf and C3H mice, because it is formed by both the intestinal microbiota and mammalian tissue [155, 162, 163],

In conclusion, concentrations of *N*-acetylspermine and *N*-acetylcadaverine correlated positively with intestinal length, but their contribution to gut elongation could not be confirmed. Whereas the stimulatory effect on intestinal length seems unlikely for *N*-acetylcadaverine, *N*-acetylspermine may act indirectly via formation of the growth-stimulating spermidine.

5 CONCLUSION AND PROSPECT

The results of the present study clearly showed that the intestinal microbiota not only influenced cecal weight, crypt depth, mucosa thickness and cell proliferation, but also affect intestinal length. However, the microbial influence on gut length seems to be dependent on several factors including microbial composition and host-genotype. A conventional PRM/Alf microbiota induced gut lengthening in PRM/Alf mice. In contrast, a defined microbial community in these mice reduced their intestinal length. The entire intestines of PRM/Alf mice colonized with a complex C3H microbiota was 10 cm longer than those of C3H mice harboring the same microbiota suggesting that the gut elongation in PRM/Alf mice depends on both genetics and microbial factors.

In the present study, it was not possible to gain new insights into the mechanism underlying microbiota mediated gut elongation. However, it was clearly shown that SCFAs do not affect gut lengthening in the animal model used. The contribution of polyamines to gut elongation remains unclear. Although putrescine was not able to increase gut length and mucosal growth in C3H mice, the roles of *N*-acetylspermine and *N*-acetylcadaverine are still unclear. To investigate their possible trophic action, further experiments need to be conducted. Such experiments could take advantage of direct infusions of *N*-acetylspermine and *N*-acetylcadaverine into small and large intestine of PRM/Alf mice. The role of spermidine which is known to induce gut maturation could be clarified by colonizing mice with spermidine-producing bacteria.

A metabolomic analysis may be appropriate to identify further trophic factors of microbial origin influencing intestinal length. Based on this approach, additional hypotheses can be generated and subsequently tested using gnotobiotic mouse models. The identification of intestinotrophic factors controlling gut lengthening may be of relevance to the development of noninvasive, postoperative therapies after small and large bowel resections.

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APPENDIX

A1: Preparation of medium 496 YCFA GSC

(Yeast extract – casitone – fatty acids containing glucose, soluble starch and cellobiose; National Collection of Industrial, Food and Marine Bacteria, Aberdeen, Scotland, UK)

Composition (amount per liter H_2O_{dd})

Casitone	10.0 g
Yeast extract	2.5 g
$NaHCO_3$	4.0 g
Glucose	2.0 g
Cellobiose	2.0 g
Maltose	2.0 g
Mineral solution I	150 ml
Mineral solution II	150 ml
VFA mix	3.1 ml
Haemin solution (0.5 g/l)	20 ml
Vitamin solution	1 ml
Resazurin	1 ml
L-cysteine x HCl	1.445 g (added after boiling)

The medium was boiled up twice in the microwave and cooled down under continuously aeration with a mixture of 80 % N_2 and 20 % CO_2 (v/v). After adding L-cysteine x HCl to the medium, the pH was adjusted to 7.3 and aerated with N_2/CO_2. Finally, the medium was autoclaved for 15 min at 121 °C.

Mineral solution I (g/l)

K_2HPO_4	3 g

Mineral solution II (g/l)

KH_2PO_4	3 g
$(NH_4)_2SO_4$	6 g
NaCl	6 g
$MgSO_4$ x 7 H_2O	1.22 g
$CaCl_2$ x 2 H_2O	0.92 g

VFA mix

Acetic acid	17 ml
Propionic acid	6 ml
n-Valeric acid	1ml
Iso-valeric acid	1 ml
Iso-butyric acid	1ml

Vitamin solution (mg/l)

Biotin	10 mg
Cobalamin	10 mg
4-aminobenzoic acid	30 mg
Folic acid	50 mg
Pyridoxamine	150 mg

LIST OF ORIGINAL COMMUNICATIONS

Full length articles:

Slezak K, Hanske L, Loh G, Blaut M (2013) Increased bacterial putrescine has no impact on gut anatmoy and physiology in gnotobiotic adolescent mice. Benef Microbes May 10: 1-14

Slezak K*, Krupova Z*, Rabot S, Loh G, Levenez F, Lepage P, Doré J, Bellier S, Blaut M. Presence of a simplified human microbiota contributes to gut shortening in the PRM/Alf mice. Benef Microbes, in preparation

Scientific events:

Slezak K, Hankse L, Loh G, Blaut M. (2011) Bacterial polyamines have no impact on gut anatomy of mice. NuGOweek 2011 “Measuring Health: How to apply nutrigenomics for measuring metabolic health”, 6-9th September 2011, Wageningen, The Netherlands

Slezak K, Krupova Z, Hanske L, Descamps A, Rabot S, Loh G, Bellier S, Blaut M. (2011) Impact of gut microbiota on gut elongation in the PRM/Alf mouse model. 11th European Nutrition Conference, fens Madrid 2011, 26-29th October 2011, Madrid, Spain

*equally contributed to the work

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STATEMENT/ ERKLÄRUNG

I hereby declare that I have written this thesis on my own using no other sources and media than specified.

Ich erkläre hiermit, dass ich die vorliegende Arbeit selbstständig angefertigt habe und keine anderen als die angegeben Quellen und Hilfsmittel verwendet habe.

Potsdam, 24th June 2013 ……

Kathleen Slezak