Received: 13 July 2019 | Revised: 23 December 2019 | Accepted: 27 December 2019

DOI: 10.1096/fj.201901758RR

RESEARCH ARTICLE

THE FASEB JOURNAL

Digestive enzyme expression in the large intestine of children with short bowel syndrome in a late stage of adaptation

Jan de Laffolie[1] | Diana Sheridan[2] | Konrad Reinshagen[3] | Lucas Wessel[4] | Christian Zimmermann[1] | Sebastian Stricker[1] | Markus M. Lerch[5] | Markus Weigel[6,7] | Torsten Hain[6,7] | Eugen Domann[6,7] | Silvia Rudloff[1] | Buford L. Nichols[8] | Hassan Y. Naim[9] | Klaus-Peter Zimmer[1]

[1]Department of Paediatrics, Justus-Liebig-University Giessen, Giessen, Germany

[2]Department of Pathology, Justus-Liebig-University Giessen, Giessen, Germany

[3]Department of Pediatric Surgery, UKE: University Hospital Eppendorf, Altona Children's Hospital, Hamburg, Germany

[4]Department of Pediatric Surgery, Medical Faculty Mannheim, University Heidelberg, Mannheim, Germany

[5]Department of Internal Medicine A, Ernst-Moritz-Arndt University, Greifswald, Germany

[6]Institute of Medical Microbiology, Justus-Liebig-University Giessen, Giessen, Germany

[7]German Centre for Infection Research (DZIF), Partner Site Giessen-Marburg-Langen, Justus Liebig University Giessen, Giessen, Germany

[8]Children's Nutrition Research Center, Baylor College of Medicine, Houston, TX, USA

[9]Department of Physiological Chemistry, University of Veterinary Medicine Hannover, Hannover, Germany

Correspondence
Jan de Laffolie, Department of General Pediatrics and Neonatology, University of Giessen, Feulgenstr 12, Giessen 35392, Germany.
Email: jan.delaffolie@paediat.med.uni-giessen.de

Funding information
German Research Foundation (DFG) (SFB621, to HYN); intramural funding from Justus Liebig University Giessen

Abstract

Background and aims: Intestinal adaptation in short bowel syndrome (SBS) includes morphologic processes and functional mechanisms. This study investigated whether digestive enzyme expression in the duodenum and colon is upregulated in SBS patients. *Method*: Sucrase-isomaltase (SI), lactase-phlorizin hydrolase (LPH), and neutral Aminopeptidase N (ApN) were analyzed in duodenal and colonic biopsies from nine SBS patients in a late stage of adaptation as well as healthy and disease controls by immunoelectron microscopy (IEM), Western blots, and enzyme activities. Furthermore, proliferation rates and intestinal microbiota were analyzed in the mucosal specimen. *Results*: We found significantly increased amounts of SI, LPH, and ApN in colonocytes in most SBS patients with large variation and strongest effect for SI and ApN. Digestive enzyme expression was only partially elevated in duodenal enterocytes due to a low proliferation level measured by Ki-67 staining. Microbiome analysis revealed high amounts of *Lactobacillus* resp. low amounts of *Proteobacteria*

Abbreviations: ApN, aminopeptidase N; CD, Crohn's disease; CF, complementary food; DCI, daily caloric intake; DPPIV, dipeptidylpeptidase 4; EGF, epidermal growth factor; FTT, failure to thrive; GLP-2, glucagon-like peptide 2; ICV, ileocoecal valve; IEM, immunoelectron microscopy; HC, healthy control patients; LPH, lactase-phlorizin hydrolase; ma, macroscopically; mi, microscopically; MGAM, maltase-glucoamylase; PN, parenteral nutrition; SBBO, small bowel bacterial overgrowth; SBS, short bowel syndrome; SI, sucrase-isomaltase; Yoa, year of age.

in SBS patients with preservation of colon and ileocecal valve. Colonic expression was associated with a better clinical course in single cases. *Conclusion*: In SBS patients disaccharidases and peptidases can be upregulated in the colon. Stimulation of this colonic intestinalization process by drugs, nutrients, and pre- or probiotics might offer better therapeutic approaches.

KEYWORDS

enteral nutrition, microbiota, parenteral nutrition, pediatric intestinal failure, pediatric short bowel syndrome, prognostic factors

1 | INTRODUCTION

Clinical observations, animal studies, and in vitro experiments have shown that various adaptation processes in the remnant bowel of patients with short bowel syndrome (SBS) compensate for the reduction of absorption.[1]

Morphological processes such as small bowel growth in length and diameter, increase of the total enterocyte number/crypt and villous surface area as well as functional changes, which include increase of glucose absorption, replicative enzymes, carrier proteins, digestive enzymes such sucrase-isomaltase (SI), maltase-glucoamylase (MGAM), lactase-phloizin hydrolase (LPH), aminopeptidase N (ApN), and decrease of intestinal permeability, improve intestinal function of the short bowel.[2-10] The digestive activity of disaccharidases is stimulated by enteral nutrients as well as the proliferative effect of glucagon-like peptide 2 (GLP-2) and epidermal growth factor (EGF).[11,12] The adaptation depends on several factors i.a. enteral nutrients, the length and type of the remnant bowel, and the presence of the ileocecal valve. Colonic preservation reduces the need for parenteral nutrition (PN).[13] If an anastomosis between jejunum and colon has been performed, the fraction of patients needing PN is much lower as in patients with an end-jejunostomy.[14]

The remnant colon is involved in intestinal adaptation, which includes the adoption of small bowel features, which is called intestinalization. Beneath morphological changes like increasing mucosal length, weight, thickness, surface and levels of protein, DNA, RNA functional changes concerning expression of proteins linked to energy metabolism, oligopeptide transporter PepT1, and Na+/H+ exchanger have been described in the colon following small bowel resection.[15-19] Ursodeoxycholic acid increased SI activity significantly in a cat model of SBS.[20] It has also been suggested that intestinal microbiota modulate colonic epithelium renewal.[21,22] Recent studies demonstrate that the fecal microbiome of SBS patients affect their outcome.[23-26]

Prenatally digestive enzymes are expressed in the colon, whose proximal part is a derivative of the midgut.[27] After 8 weeks of pregnancy a simple tube develops into an early gut; enzymes like SI, alkaline phosphatase, Dipeptidylpeptidase 4 (DPPIV), and ApN can be measured and a villous structure builds up.[28] Until the 30th week of pregnancy the colon shows a low but stable expression of SI and LPH.[28,29] After birth those enzymes can only be found at the brush border membrane of small intestine, except in human colon cancer.[30] While the regulation of LPH as well as SI is primarily transcriptional, LPH activity is genetically determined and independent of luminal lactose administration in contrast to SI, which can be upregulated by dietary sucrose increasing the amount of active enzyme per cell.[31] Most studies on digestive enzymes in short bowel syndrome were performed in animal models of early stages of intestinal adaptation. Therefore, a translational research approach has been chosen for a better understanding of the functional state of the intestinal epithelium in order to improve our therapeutic options in SBS.

In the experiments reported here the protein expression levels of SI, LPH, ApN, and DPPIV were assessed as protein amounts (Western blots, IEM) as well as enzyme activities in duodenal and colonic tissue together with the replication rate (Ki-67) and the intestinal microbiota in a cohort of children with SBS in a late stage of adaption. The use of immunoelectron microscopy (IEM) allowed the determination of digestive enzyme concentration in the apical membrane of single enterocytes. Our results show an (ontogenetic) upregulation of disaccharidases and peptidases not only in the small intestine but also in the colon although the latter revealed strong interindividual variations most likely depending on the mucosal pathology and microbiome.

2 | MATERIALS AND METHODS

2.1 | Patients

Eleven biopsy specimens were taken endoscopically from the duodenum and the colon (at least from its proximal part) of nine children suffering from SBS from July 2009 to November 2010; patient 2 and 3 were re-examined after half a year (2b), respectively, 1.5 years (3b). Indications for endoscopy included symptoms of bacterial overgrowth, chronic diarrhea, evaluation

for GI bleeding, and suspicion of peptic disease. In addition, four children underwent endoscopy because of Crohn's disease (CD) and five children because of abdominal pain; the latter showed no mucosal pathology and were taken as healthy control patients (HC). The biopsies were examined for histological criteria of inflammation. The study was approved by the ethics committee of the Justus-Liebig-University Giessen (reference number 113/10); written informed consent was obtained from the parents of all participants.

For each bowel side one biopsy was immediately fixed in paraformaldehyde (PFA) for IEM, another one frozen in liquid nitrogen each for determination of disaccharidase activity and if available for performing a Western blot.

2.2 | Immunoelectron microscopy

After fixation in 5% paraformaldehyde in 50 mM HEPES and cryoprotection by polyvinylpyrolidone and sucrose, tissue specimens were frozen in liquid nitrogen. Sectioning and labelling were carried out using an ultracryomicrotome (Leica EM UC6 Ultracut, Leica, Bensheim, Germany) at –100°C. Ultrathin sections (55 nm) were blocked with cold water fish gelatine (Aurion, Wageningen, The Netherlands) incubated with primary antibodies for 45 minutes as described before.[32] For detection of ApN a monoclonal mouse antibody (Chemicon, Hofheim, Germany) was used in a dilution of 1:10. LPH and sucrase were labelled with mouse monoclonal antibodies kindly provided by B. Nichols (Baylor College of Medicine, Houston, Texas); Mlac 2 and Mlac 10 were diluted to 1:640 and 1:160 and pooled (1:1) before incubation; the sucrase antibody was used in a concentration of 1:1000. The antibody toward DPPIV was a monoclonal mouse antibody derived from the hybridoma cell line HBB3/153 and applied at a dilution of 1:10.

For Ki67 a Clin MIB-1 antibody (monoclonal mouse, Dako) was diluted 1:10 and secondary antibody Alexa Fluor 488 Goat-a-Mouse (Life Technologies) was diluted 1:200.

Sections were then incubated with a goat-anti-mouse antibody bound to 12 nm immunogold (Dianova, Hamburg, Germany) for 45 minutes. After contrasting in uranyl acetate and embedding in 2% methylcellulose containing 0.4% uranyl acetate the sections were examined using a Philips 400 electron microscope (Philipps, Eindhoven, The Netherlands). All pictures were taken in a 17 700× magnification.

2.3 | Quantitation of antibody binding on electron microscopical level

The number of enterocytes per patient and bowel side, which showed labelling of sucrase, LPH, and ApN at the apical membrane, was counted per 100 enterocytes. In addition, a total of about 300 µm of apical membrane from enterocytes for each patient, enzyme, and bowel side (LPH, sucrase, and ApN in the duodenum; and colon and DPPIV in the colon) were used to determine labelling density as number of gold particles (gp) per µm apical membrane. For this randomized electron microscopical pictures were taken at a magnification of ×17 700. Positive labelling was defined as 5 or more gold particles per cell and 1.5 gold particles or more per µm. The evaluation of label per membrane length was done by point counting described by Griffiths.[33] Briefly, the cells were overlayed with a grid holding a 1 µm distance between the square lines. The intersections with the apical membrane as well as the number of gold particles on the apical membrane were counted. The number of gold particles per membrane length was then calculated by the formula: number of gold particles divided by ($\pi/4 \times$ number of intersections $\times$ 1 µm). Results are expressed as medians with ranges. Statistical evaluation between groups (SBS patients, Crohn's disease patients and healthy control patients) was performed by the Mann-Whitney U test.

2.4 | Determination of enzyme activity with photometric emission measurement

Frozen tissue (1 mg) was homogenized on ice in 99 µL 0.12 M potassium chloride solution. Activity of LPH, isomaltase, sucrase, and maltase in the specimen were measured utilizing the substrates lactose, isomaltose, sucrose, and maltose, respectively, in a concentration of 0.056 M. The amount of glucose released was determined using the Gluco-Quant-Kit (Roche-Hitachi, Mannheim, Germany) following the manufacturer's instructions. Results are expressed as medians with ranges.

2.5 | Western blotting

Western blot analysis was performed for sucrase, isomaltase, and LPH using Triton X-100 detergent extracts of the duodenal and colonic biopsies and the antibodies described above. The secondary antibody employed sheep anti-mouse IgG linked to peroxidase (Amersham Biosciences). The detection was performed with a Super Signal ELISA Femto kit (Pierce, PERBIO).

2.6 | Microbiome analysis

16S rDNA sequencing for microbiome profiling was performed in paraformaldehyde fixed biopsies from the colon of eight patients using the Nextera XT kit and the MiSeq Soap (Mothur vs 1.36.1) as described in the electronic supplement.

3 | RESULTS

3.1 | Patient characteristics

At the time of biopsy, the patients in the CD group (three boys, one girl) were 14.2 ± 3.3 years old. HC individuals (two boys, three girls) were 10.8 ± 2.2 years old.

The mean age of the SBS patients (5 boys, 4 girls) at the time of biopsy was 4.6 ± 2.6 years (SD) corresponding in most cases to a neonatal origin of bowel resection, that is, to a late stage of intestinal adaptation. Five of the SBS patients were able to be weaned from PN in the follow-up. In the course of 6.5 to 8 years one patient died, two patients grew satisfying, three sufficiently and the other three patients insufficiently. Details of surgical procedures (Bianchi or STEP), anatomic conditions (bowel length or dilatation, preservation of ileocecal valve), underlying diseases, involvement of SBBO, histological detection of mucosal inflammation, complications (septicaemia, GI bleeding, liver failure i.a.) and extent of enteral nutrition are summarized for the follow up in the püatient data table (Figure S2).

3.2 | Significant amounts of disaccharidases and ApN in the colon epithelium of SBS patients

Immunoelectron microscopy detection of brush border enzymes showed a distinct labelling for ApN, LPH, and sucrase on the apical membrane of colon enterocytes from most of SBS biopsies (Figures 1A, 2A, and 3A), which was not found in HC for LPH and sucrase (Figures 1B and 2B) and only in weak amounts for ApN (Figure 3B). While the colonic labelling for LPH and sucrase was weaker than in duodenal enterocytes of

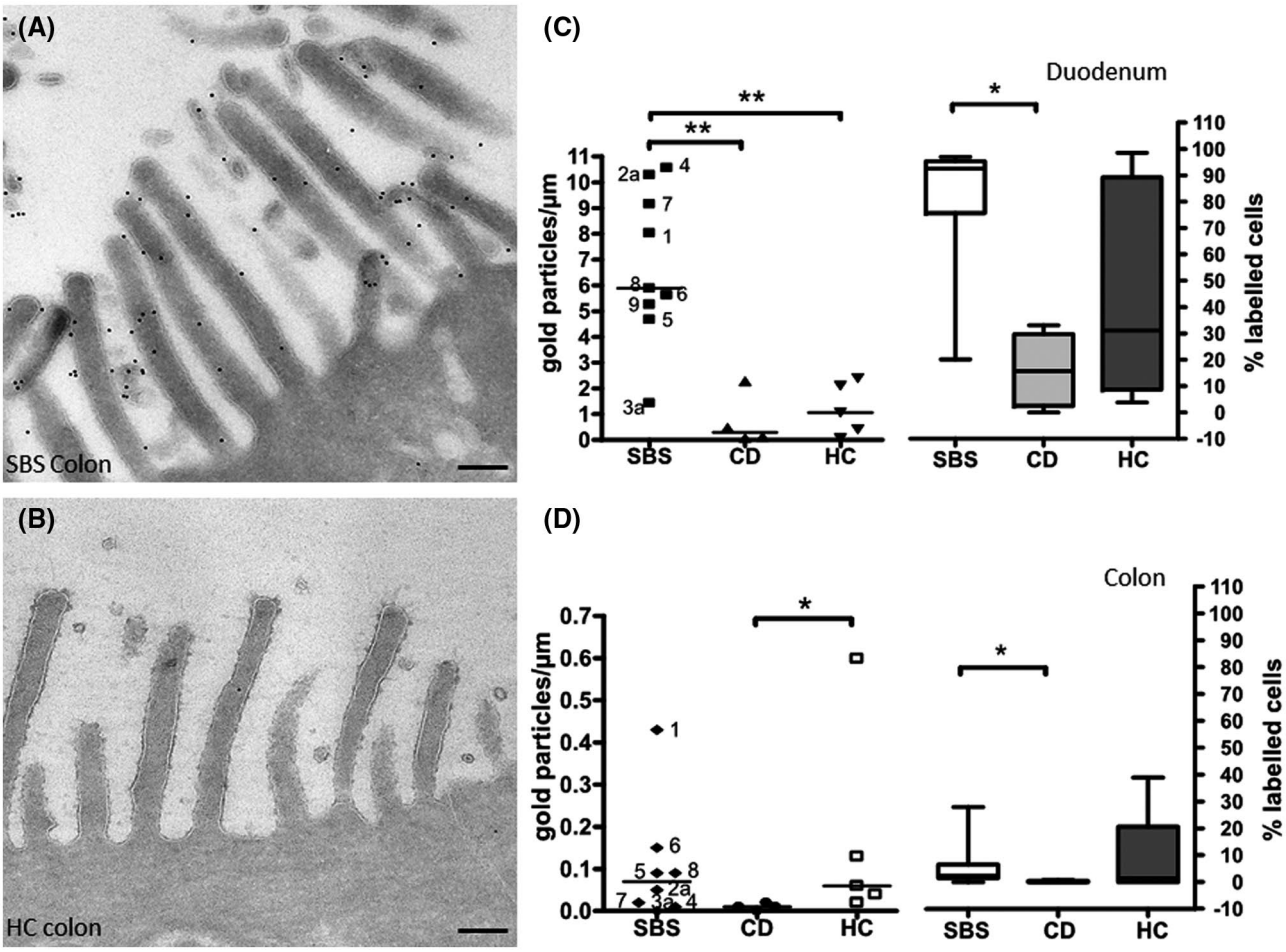

FIGURE 1 Immunoelectron microscopical labelling of lactase (LPH) in SBS (A) and HC (B) colon. Lactase labelling density was increased in SBS duodenum compared to CD and HC (C), while it was lowered in duodenum and colon of CD and HC (C, D). The labelling for lactase is more than 5 times as high in the duodenum of SBS as in the HC group and more than 19 times in CD patients (C) (SBS 5.9; range: 1.4-10.6, CD 0.3; range: 0.1-2.3, HC 1.0; range: 0-2.4 gp/µm). Labelling densities in the colon showed no significant difference between SBS and CD or HC (SBS 0.1; range: 0-0.4, HC 0.1; range: 0-0.6 gp/µm), but a lower density in CD compared to HC. For lactase there was in increase of labelled enterocytes between SBS and CD in duodenum (SBS 92.5%; range: 20.2-97.0% vs CD 15.6%; range: 0-33.1%) as well as in colon (SBS 2.4%; range: 0-28.0%, HC 1.5%; range: 0-39.0%) ***$P < .001$ **$P < .01$ *$P < .05$. Scale = 0.5 µm

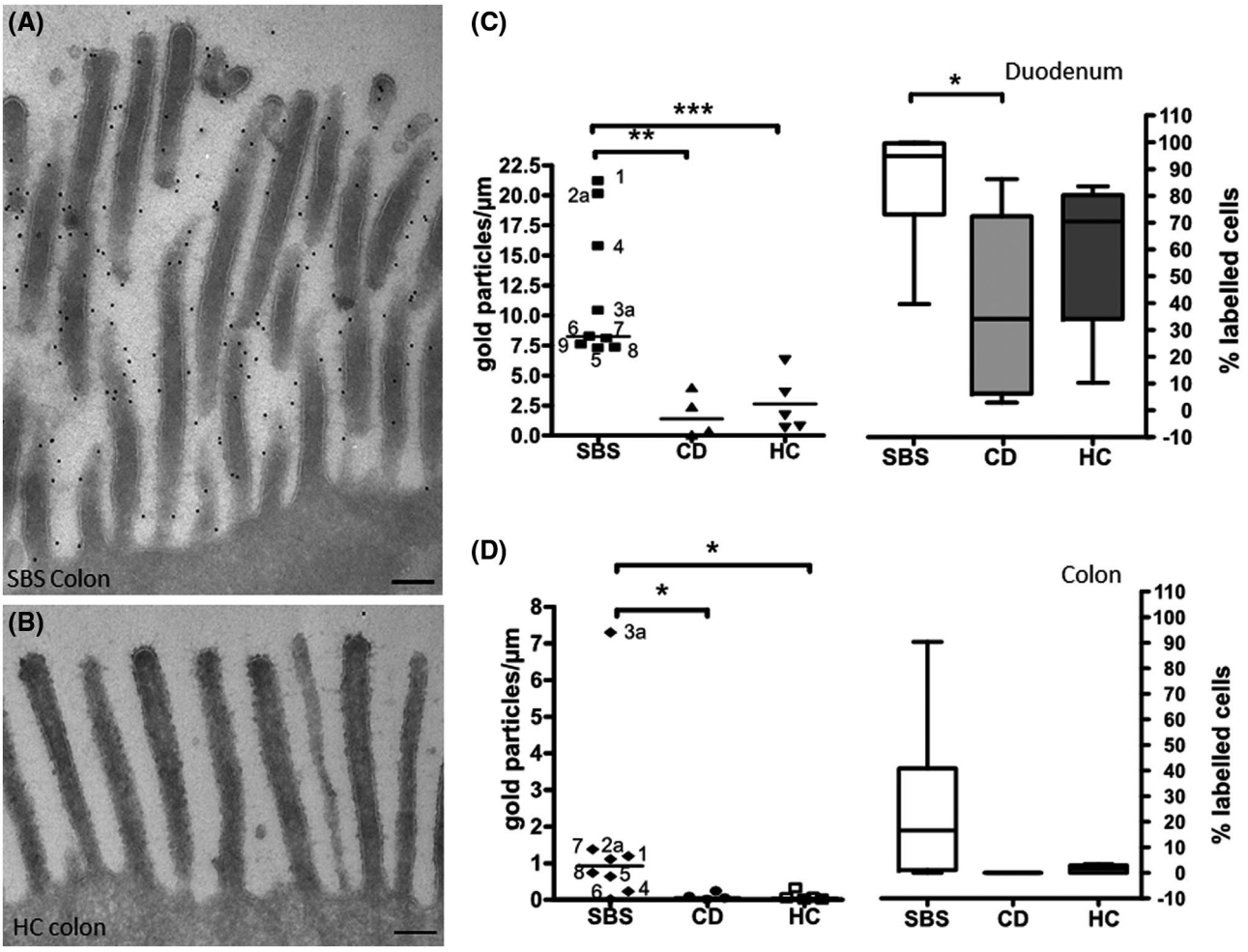

FIGURE 2 Immunoelectron microscopical labelling of sucrase in SBS (A) and HC (B) colon. Sucrase labelling densities were enhanced in SBS duodenum and colon vs CD and HC duodenum and colon (C, D) with an increase of labelled cells in duodenum vs CD (C). Sucrase labelling is more than five times higher in the duodenum of SBS than of CD (SBS 8.2; range: 7.3-21.2, CD 1.4; range: 0.1-4.0, HC 2.7; range: 0.5-6.3 gp/µm) and more than four times in the colon of SBS than of HC (SBS 0.9; range: 0-7.3, HC 0; range: 0-0.3 gp/µm). The percentage of colonocytes labelled with sucrase in SBS patients was 16.7% (range: 0 - 90.4%), whereas HC individuals had 1.5% (range: 0-3.4%) and CD patients none. ***$P < .001$ **$P < .01$ *$P < .05$. Scale = 0.5 µm

SBS patients, ApN staining in colonocytes of these samples was comparable to that in the duodenum. The labelling of LPH was less intense than for sucrase in SBS colon.

Labelling densities on the apical membrane of colonocytes were three times higher for ApN in SBS than in HC (Figure 3D), for SI, which was significantly increased compared to HC (Figure 2D) and for LPH, which was the lowest from the three enzymes analyzed (Figure 1D). In the colon of CD patients there was no distinct labelling for any of the enzymes marked, which suggests downregulation compared to HC (Figures 1D, 2D, and 3D). The variability of the labelling density was striking with maximal values of sucrase for patient 3 (7.3 gp/µm), patient 7 (1.4 gp/µm), and patient 1 (1.2 gp/µm) and of LPH for patient 1 (0.4 gp/µm) and patient 6 (0.2 gp/µm). Patient 3 was also characterized by the highest amount of DPPIV on the apical membrane of colonocytes from SBS patients: 0.5 gp/µm with a median labelling density of 0 (range: 0-0.5) gp/µm (Figure 4A); the labelling density for DPPIV in the duodenum of patient 3 was 0.3 gp/µm. We also found a significant labelling for MGAM in patient 3 (Figure 4B) in contrast to the other SBS patients with a labelling density of 3.0 gp/µm in the duodenum and 0.7 gp/µm in the colon.

In the colon the percentage of labelled enterocytes was much lower in comparison to the duodenum (except for ApN) (Figures 1C, 2C, and 3C), but SBS patients showed tentatively more of them at least for sucrase and ApN compared to HC (Figures 2D and 3D). The number of colonocytes positive for ApN was significantly higher in SBS than in CD patients (Figure 3D); for LPH there was no difference between SBS and HC in the percentage of colonocytes with positive labelling (Figure 1D).

In the colonic specimen of seven SBS patients, there was significant disaccharidase activity which was lower than in

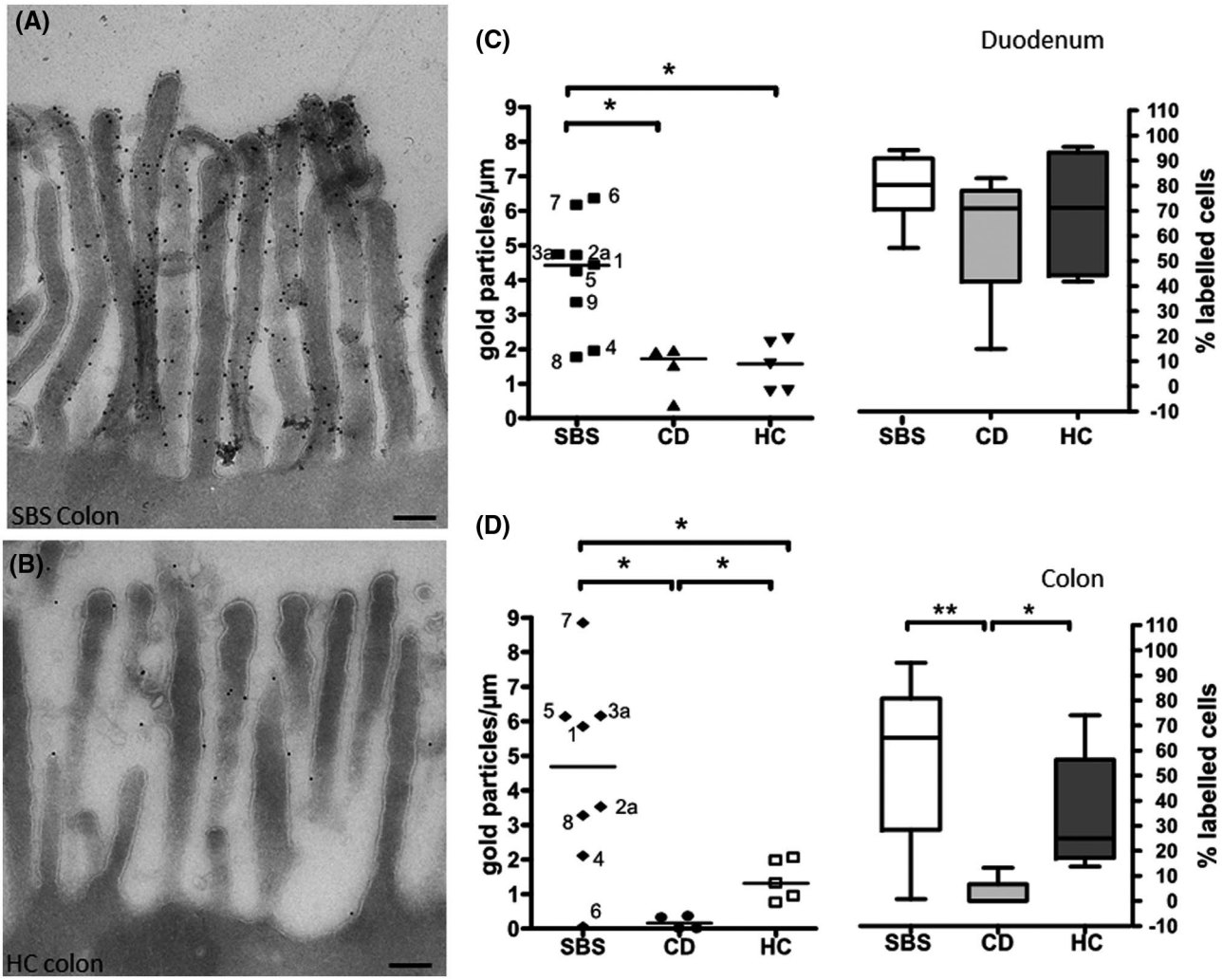

FIGURE 3 Immunoelectron microscopical labelling of Aminopeptidase N (ApN) in SBS (A) and HC (B) colon. ApN was upregulated in SBS duodenum and colon (C, D). There are more than twice as many gold particles per µm (gp/µm) for ApN in the duodenum of SBS as there are in the CD or in the HC group (SBS 4.4; range: 1.8-6.6, CD 1.7; range: 0.4-2.0, HC 1.6; range: 0.8-2.3 gp/µm). Labelling densities in the colon revealed higher values for SBS compared to HC (SBS 4.7; range: 0.1-8.9, HC 1.3; range: 0.8-2.1 gp/µm). The number of colonocytes positive for ApN was significantly higher in SBS than in CD patients (SBS 65.1%; range: 0.8-95.0%, CD 0%; range: 0-13.3%, HC 25.0% (range: 13.8-74.1%). $^{***}P < .001$ $^{**}P < .01$ $^{*}P < .05$. Scale = 0.5 µm

the duodenum (Figure 5). The highest enzyme activities were obtained in the colon of patient 2 (LPH 5, maltase 22, sucrase 5, and isomaltase 9 IU/g), patient 5 (LPH 4, maltase 30, sucrase 8, and isomaltase 12 IU/g), and patient 6 (LPH 14, maltase 24, sucrase 11, and isomaltase 12 IU/g).

Among the SBS patients Western blotting confirmed the presence of isomaltase or sucrase in colonic biopsies from two of five patients by faint but significant bands. The patient who died 5 years after taking his biopsies did not show a band for isomaltase nor lactase (Figure 6A). In patient 4 the bands for isomaltase and sucrase were only present in the sigma, but not in the proximal colon and in patient 2 (Figure 6B) these bands were present in the proximal as well as sigma specimen. We found a band for LPH in colonic biopsy obtained from one of four SBS patients (patient 4 showing a corresponding band in the sigma as well as in the proximal colon).

The colonic values of IEM for sucrase and MGAM but not for LPH were highest in SBS patient 3 (Figures 2, 4, and 5, no material for Western blotting left); this patient showed also a high value for DPPIV within the colon (Figure 4) indicating a relative strong expression of brush border enzymes within the colon possibly on the basis of adult hypolactasia. Colonic biopsies of patient 6 showed low labelling densities for sucrase, LPH, and ApN with a lacking band for isomaltase and LPH in Western blotting and a medium range of colonic isomaltase, sucrase, and LPH activities (Figure 6A). The bands in the Western blot for sucrase (and isomaltase) obtained from the colon descendens and sigma of patient 2 could be reproduced by enzyme activities and IEM (Figure 6B).

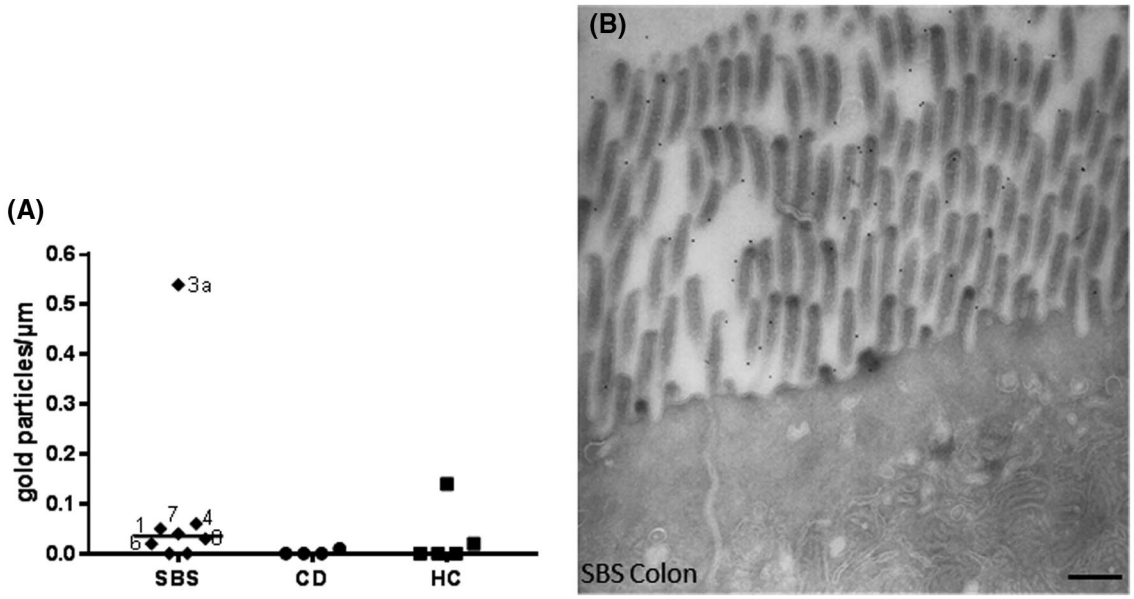

FIGURE 4 Detection of DPPIV and MGAM in the colon epithelium: Labelling densities of DPPIV with an increased amount of patient 3a (A); Immunoelectron microscopical detection of MGAM in the colon which was strongest in patient 3a (B). Patient 3a suffered from SBS due to gastroschisis (150 cm small bowel, 25 cm colon) and developed recurrent septicemias and Crohn-like lesions in the further course. The first biopsy was taken at the age of 8.4 years, while Bianchi procedure was performed. After initial recovery he presented with severe FTT, ileus and SBBO. His duodenum dilated up to 7 cm suspecting CIPO. A second biopsy from STEP at the age of 9.9 was analyzed and showed less expression of Ki67 and sucrase within the duodenum compared to the first biopsy. After perforation due to anastomotic stenosis FTT persisted on parenteral 50% DCI (15 years of age: weight −4.31; height −3.73). Scale = 0.5 µm.

3.3 | Partial increase of disaccharidases and ApN in the duodenal epithelium of SBS patients

Evaluating the antibody labelling by IEM we found a strong labelling for LPH (Figure 1A), sucrase (Figure 2A), and ApN (Figure 3A) on the apical membrane of duodenal enterocytes from SBS patients in contrast to disease and healthy (Figures 1B, 2B, and 3B) controls.

Quantitation of labelling density on the apical membrane of enterocytes revealed that the labelling for LPH is more than 5 times as high in the SBS as in the HC group and more than 19 times in CD patients (Figure 1). Sucrase labelling is more than five times higher in SBS than in CD and more than four times in SBS than in HC (Figure 2). There are more than twice as many gold particles per µm for ApN as there are in the CD or in the HC group (Figure 3).

Determining the percentage of labelled duodenal enterocytes, which also indicates to the expression level of the brush border enzymes, we found 80.2% positive cells for ApN in SBS, whereas in CD there were only 70.9% and in HC 71.3% (Figure 3C). In SBS the LPH containing cells made up for 92.5% whereas in CD there were only 15.6% and in HC there were 31.1% (Figure 1C). The percentage of positive cells for sucrase was almost twice as high in SBS (95.0%) than in CD (34.1%); in HC the value was almost as high as in the SBS patients (70.4%) (Figure 2C). There was a significant decrease of LPH and sucrase in CD in comparison to SBS (Figures 1C and 2C).

The disaccharidase activities in duodenal biopsies of eight SBS patients indicated a lower or medium enzymatic activity compared to HC; only patient 2 showed an increased activity for MGAM, isomaltase, and SI (Figure 5).

Duodenal biopsies for Western blotting were available from five SBS patients for isomaltase, and sucrase, and four SBS patients for LPH detection. The analysis revealed clear protein bands corresponding to sucrase, isomaltase, and LPH in the duodenum and terminal ileum of all patients, albeit to varying expression levels. The highest expression levels for sucrase or isomaltase were found in patient 4 followed by patient 2 (Figure 6B) and the weakest in patients 5 and 6 (Figure 6A). The protein expression pattern varied also when LPH was analyzed with LPH expressed at high levels in patients 7 and 2 (Figure 6B) and low levels in patient 5. Additional biopsies from the residual small bowel (jejunum from patient 4, 5, and 6 or ileum from patient 2) showed equal or even stronger bands for disaccharidases compared to a HC duodenal biopsy (Figure 6A,B).

The results of Western blotting, enzyme activity, and IEM do not correlate completely within the duodenal mucosa. Patient 2, for example, showed very high values for sucrase, LPH, and ApN measured by IEM, while on the enzyme activity level isomaltase, sucrase, and LPH were lower and on

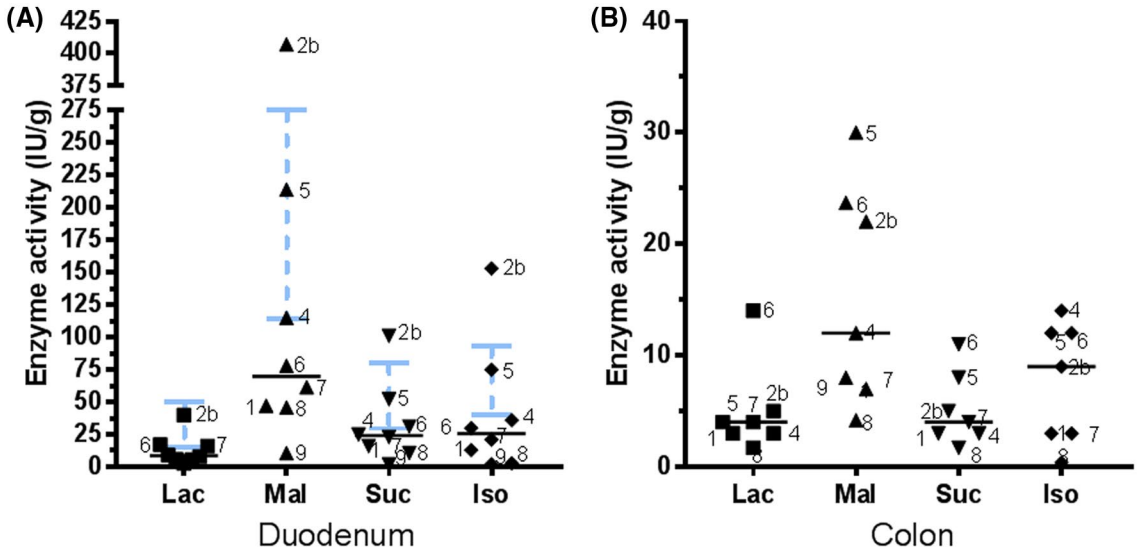

FIGURE 5 Disaccharidase activities of SBS patients: The graph depicts single values and the median. Normal ranges for duodenal values are marked blue (left side).[37] Only patient 2b reveals increased disaccharidase activities for maltase, sucrase, and isomaltase in the duodenum (A). Disaccharidase activity in eight SBS colonic biopsies are lower compared to the duodenum (B): LPH 4.0 (range: 3.0-14.0) IU/g, maltase 12.0 (range: 4.2-30.0) IU/g, sucrase 4.0 (range: 3.0-11.0) IU/g, isomaltase 9.0 (range: 1.8-14.0) IU/g with patients 2b, 5, and 6 showing the highest values for the four enzymes measured and patient 4 for maltase/isomaltase

the Western blot level isomaltase and LPH were similar and sucrase lower compared to HC (Figure 6B). Patient 6 also revealed high values for sucrase, LPH, and ApN by IEM in contrast to low values for isomaltase, sucrase, and LPH in Western blots and enzyme activities (Figure 6A). It is likely that the processing of these enzymes to a mature form under the examined conditions is incomplete. For example, O-glycosylation is one event that regulates the enzymatic activity of sucrase and isomaltase.[34] Addressing this issue was not possible due to tissue limitation.

3.4 | Low proliferation level of crypt and villi enterocytes in SBS patients—Both in the colon and the duodenum

Because increased proliferation of enterocytes was reported from animal SBS models, we performed Ki-67 staining in the analyzed biopsies of our SBS and HC patients to determine the harboring proliferation activity on immunofluorescence level. Villi showed only rarely Ki-67 positive enterocytes in SBS as well as HC specimen, in crypts the number of Ki-67 positive cells was decreased in SBS (Figure S1). SBS patients 3 and 4 who expressed relatively high amounts of brush border enzymes within the duodenum showed the highest proliferation activity in the duodenum (25.7 and 22.1%), while SBS patient 3 characterized by high colonic activity (isomaltase, sucrase) and labelling density (sucrase, ApN) levels revealed the most intense Ki-67 staining in the colon (4.6%). The two SBS patients (patient 2 and 4), in whom we could confirm sucrase and isomaltase colonic expression by Western blotting, exhibited low cell proliferation determined by Ki-67 staining within crypts.

3.5 | Microbiome analysis

The eight colon biopsies showed a diverse composition of the intestinal microbiota (Figure 7). The two patients (1 and 5) with ileocecal valve had more *Lactobacillus* (11.15%, 31.17% vs median 1.3% [P25 0.24%-P75 11.0%]), while *Proteobacteria* dominated in those without ileocecal valve (median 58.46% [P25 51.06%-P75 66.11%] vs 28.2%, 26.47%). Shannon diversity index or pattern of microbiome composition (reads >5% or >1%) was not related to weaning success, growth restriction, death or other patient relevant outcomes in mixed linear models, logistic regression or recursive partitioning models. However, microbiome data from the patient who died were obtained 5 years before death.

4 | DISCUSSION

The impact of the remnant colon in SBS has been addressed in several studies.[35,36] Nutritional factors increase colon fermentation, short chain fatty acids and *Bifidobacterium spp.;* dietary amylose correlates positively with colon length in pigs.[37,38] Animal studies detected different effects on colon epithelium following small bowel resection with

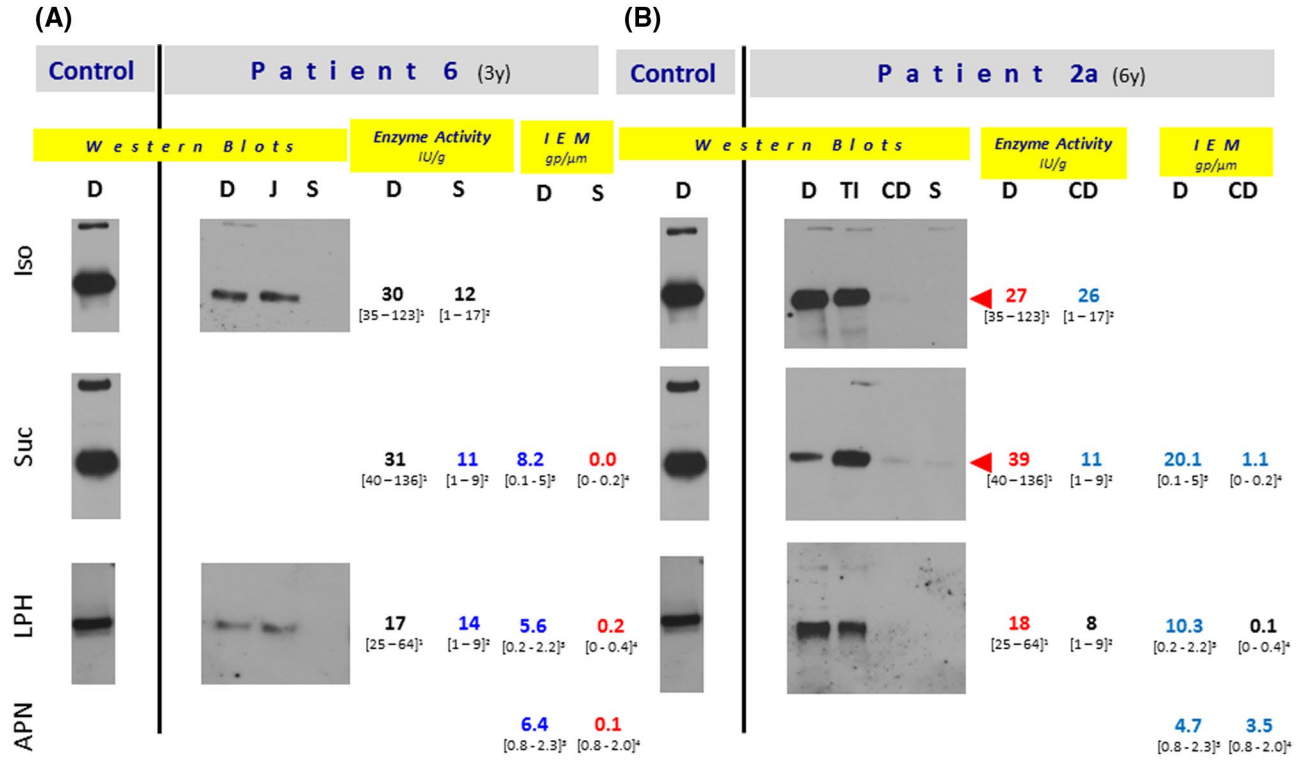

FIGURE 6 A, SBS patient without sucrase-isomaltase expression in the colon comparing experimental data (Western blots, disaccharidase activities, and quantitative immunoelectron microscopy) with clinical course: Duodenal values for isomaltase, sucrase, LPH, and APN varied. Significant enzyme activities were only measured for sucrase and LPH in the colon (sigma) in contrast to the results obtained by western blotting (Iso, LPH) and IEM (Suc, LPH, APN). Patient 6 suffered from SBS due to gastroschisis/volvulus and developed septicemias and Crohn-like lesions in the follow-up. The length of small bowel was 43 cm and of the colon (rectum) 20 cm. He developed SBBO with mucosal inflammation, severe FTT (low intake), liver failure, and ulcer bleeding; and died 5 years after the biopsy was taken. D, duodenum; Iso, isomaltase; J, jejunum; Lac, lactase; S, sigma; Suc, sucrase. 1 Normal Range Duodenum (healthy controls); 2 Mean ± SD Colon (SBS patients); 3 Mean ± SD Duodenum (healthy controls); 4 Mean ± SD Colon (healthy controls). B, Short bowel syndrome patient with sucrase-isomaltase expression in the colon comparing experimental data (Western blots, disaccharidase activities, and quantitative immunoelectron microscopy) with clinical course: Disaccharidases were detected by western blotting, enzyme activities (lower range), and IEM (higher range) in the duodenum. Clear bands for SI were found in western blots in the colon samples (CD, S) which also revealed high values by enzyme activities (SI) and IEM (Suc, APN). Patient 2a suffered from SBS due to gastroschisis and ileal atresia; 60 cm distal colon was left, small bowel length was increased by a Bianchi procedure from 54 to 105 cm at the age of 1 year. The further course was characterized by septicemias (salmonella), Crohn-like lesions, ulcer bleeding (hematochezia) with iron deficiency anemia suggesting food allergy. This child did not receive parenteral nutrition and was fed ad lib in addition with soy-based formula via PEG. Seven years after taking the examined biopsy (at the age of 14.6 years) his weight was 35.8 kg (*z*-score—2.92) and his height 150 cm (*z*-score—2.48). CD, colon descendens; D, duodenum; Iso, isomaltase; S, sigma; Suc, sucrase; TI, terminal ileum. 1 Normal Range Duodenum (healthy controls); 2 Mean ± SD Colon (SBS patients); 3 Mean ± SD Duodenum (healthy controls); 4 Mean ± SD Colon (healthy controls)

morphological and functional adaptation, while human derived data are still scarce.[15-19,21,22]

Especially the expression of digestive enzymes in the colon has been poorly investigated in human biopsy specimen under SBS conditions as part of a mechanism of energy salvage, while morphological adaptation has been described.[36] Maltase expression was found in colonic interpositions of a rat model,[39] but there is evidence that the intestinal adaptation process of rodent models differ from the human adaptive response.[20,40] This translational study in a small group of SBS children clearly demonstrates intestinalization processes in the colon on a functional level analyzed by IEM, Western blotting, and enzyme activities. Induced expression of digestive enzymes within the colon epithelium is pronounced for ApN and to lesser extent for SI as shown in patient 2 and 3 the latter being also positive for DPPIV and MGAM. Patient 2 was suffering from food allergy, did not receive PN during the study period and showed a satisfying growth. The colonocytes of patient 1 and 7, the first being weaned (70 cm small bowel, complete colon, ileocecal valve preserved), the second still receiving 50% of daily caloric intake (DCI) by PN (30 cm small bowel, ½ colon, no ileocecal valve left), revealed also higher amounts of ApN and sucrase; both grew satisfactory. Patient 6 (43 cm small bowel, 20 cm colon, no ileocecal valve left, Z-score—5.58) presented very low digestive enzymes and a peculiar intestinal microbiota

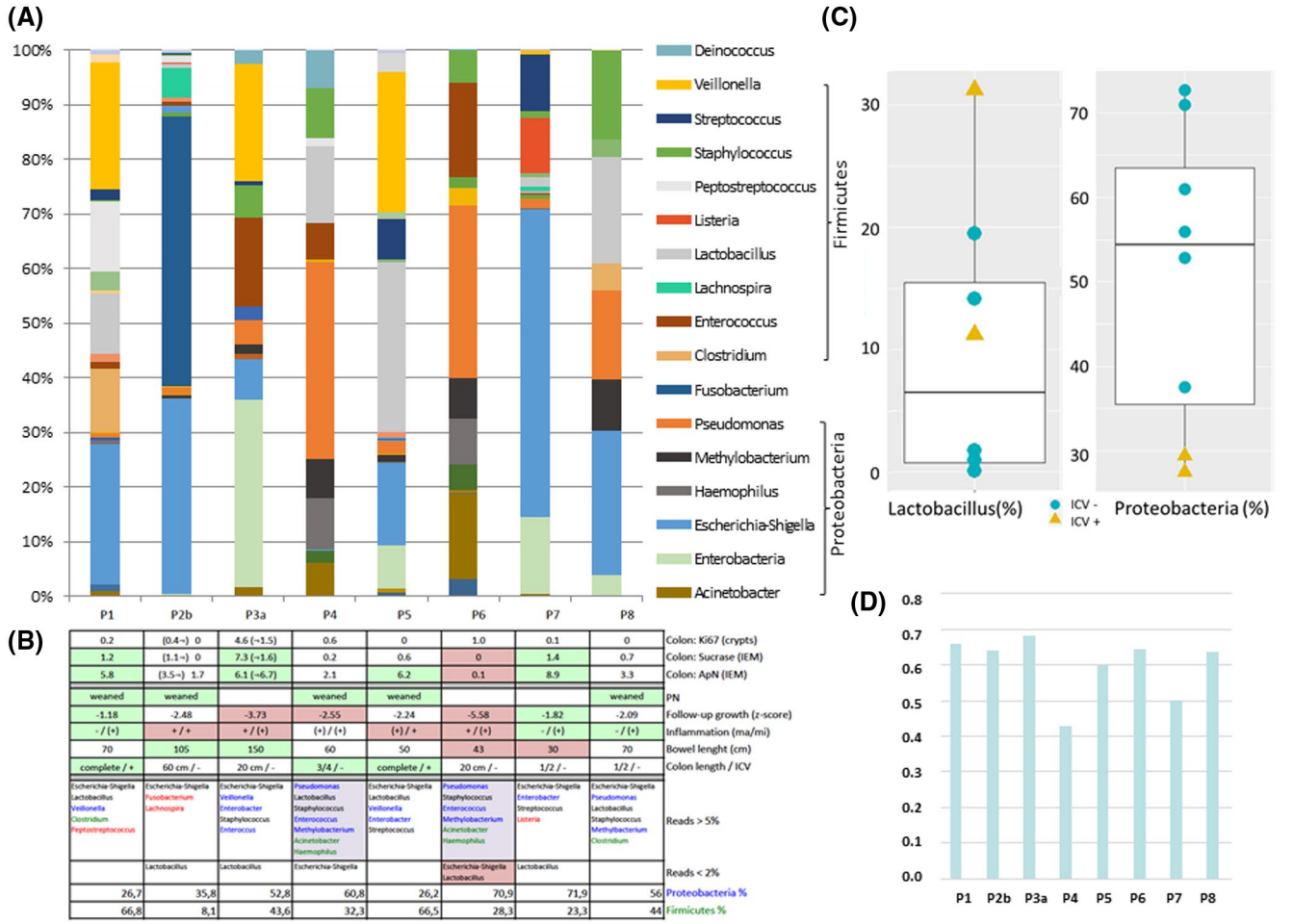

FIGURE 7 Microbiome Analysis: A,B Escherichia-Shigella species are absent in patients 4 and 6 and *Lactobacillus* in patients 2b, 3a, 6, and 7. Among those bacteria with relative abundance (more than 5% of total reads) only two patients revealed Clostridium (1 and 8), Acinetobacter (4 and 6), and Haemophilus (4 and 6); three patient biopsies contained Pseudomonas (4, 6, and 8), Veillonella (1, 3a, and 5), Enterobacter (3a, 5, and 7), Methylbacterium (4, 6, and 8), and Enterococcus (3a, 4, and 6). In higher amounts Listeria was found only in biopsy of patient 7, Peptostreptococcus only in patient 1 and Fusobacterium as well as Lachnospira only in patient 2b; C In patients with ICV there were more Lactobacillus, while without ICV proteobacteria dominated. D Shannon Index did not relate to disease course and clinical data (especially patient 6, who died 5 years after the biopsy was taken, did not show a reduced diversity of the microbiome)

(71% *Proteobacteria* and <5% *Escherichia-Shigella* as well as *Lactobacillus*). A significant alteration in microbiota composition has been previously described as a consequence of SBS and a negative predictor in severe cases also toward liver steatosis.[41,42] However, death form liver steatosis in this patient occurred 5 years after the biopsies were obtained and thus can hardly be linked to dysbiosis alone.

The strong expression of ApN in SBS colonocytes corresponds to the observed upregulation of the oligopeptide transporter PepT1 in the colon of adult SBS patients.[16,18] Because the up-regulation of SI is less strong in comparison to ApN, it could be assumed that the adaptation process in the SBS colon is more focused in terms of protein than of carbohydrate metabolism. LPH is poorly upregulated in the colonocytes of our SBS patients, while we did not exclude adult hypolactasia.

The results of duodenal disaccharidases in our SBS patients were less pronounced than reported after stimulation by ursodeoxycholic acid, IGF I, and GLP-2,[20,43,44] A low proliferation status in our SBS patients as compared to HC patients may be responsible for the moderate expression of disaccharidases measured in the small intestine but also in the colon concomitant with a late stage of intestinal adaption in the SBS patients. Proliferation was highest in the duodenal crypts of patient 3 and 4 who showed also high values for sucrase. In the colonic crypts only patient 3 showed Ki67 values comparable to HC biopsies; this patient is also characterized by increased expression of sucrase, DPPIV, and MGAM in the colon epithelium.

Our data on the analysis of the intestinal microbiota in SBS are in line with observations that the microbiome of patients with complete colon and preserved ileocecal valve

(patient 1 and 5) is enriched in *Lactobacillus* and contains a low amount of *Proteobacteria*, which may increase the risk of D-lactate acidosis and encephalopathy depending on the metabolic activity of the microbiota and individual ability to metabolize D-lactic acid.[35,45,46] The association between an increased abundance of the LPS producers, proteobacteria, with inflammation in the distal small bowel as well as hepatic injury in SBS remains controversial.[42,47] A recent study unravelled an association of *Firmicutes* with a remnant small bowel length >35 cm.[48]

The gut microbiome diversity of the SBS cohort in this study was not reduced as previously reported[25,41] One factor may be the use of biopsies instead of luminal content or stool and the high amount of enteral feeds. Previously, jejunal aspirates in SBS patients, especially with enteral feeds showed a greater diversity than stool samples.[41] Very few studies analyzed biopsies, but epithelial adherent bacteria may be the most important ecological system for intestinal adaptation. In healthy adult volunteers luminal samples revealed great biological diversity as compared to the mucosa-associated microbiota that was relatively more conserved.[49] One frequent influence would be recurrent courses of antibiotic treatment, which occur more frequently in SBS children than healthy controls.[26]

Patient 6, who is characterized by the worst outcome (death 5 years after the examined biopsy), revealed a high amount of *Proteobacteria* (71%) and a loss of *Escherichia-Shigella* and *Lactobacillus*, comparable to severe dysbiosis previously described with worse outcome.[25,26] In contrast, patient 4 showing a similar composition of the microbiome as patient 6 was successfully weaned but without satisfactory growth, raising the question what can be relevant predictors of success or metrics of performance in pediatric SBS. Further exploration of the SBS microbiome will help to understand the expression patterns of the remnant colon.[50,51] It is important to consider the source of information, either from intestinal fresh samples or samples fixed in formaldehyde, which can alter results and the situation (antibiotics, inflammation, metabolic situation) in which they were obtained.

While our results reveal correlations of experimental data and clinical course within single SBS patients, we have to recognize that our patient group is very heterogeneous and characterized by complications, underlying diseases such as CIPO or food allergy, different surgical interventions (STEP = Serial Transverse Enteroplasty Procedure or Bianchi gut lengthening), insufficient adherence to intestinal rehabilitation therapies such as low intake of calories, and a very restricted indication for diagnostic endoscopy. Therefore, our study does not allow to identify distinct general prognostic factors for the clinical course of SBS or therapeutic implications. Nevertheless, it provides strong evidence that the human remnant colon is capable to take over digestive functions of the small intestine as part of the intestinal adaption after bowel resection, which is ultimately associated with a more favorable outcome. Further prospective studies in larger and more delineated patient groups (early vs late stage of adaptation) could lead to the identification of drugs, nutrients as well as pre- or probiotics, which allow the targeted use of colonic intestinalization expression mechanisms. For intestinal microbiota analysis this would ideally include intestinal biopsies in addition to stool samples.

ACKNOWLEDGMENTS

The authors would like to thank Larissa Thiessen, Bettina Goritzka, Nadina Böcher, and Gabi Wetzel for technical and laboratory assistance. Stephanie Asmuth for initial data collection and validation, Hanna Gurmai for statistical and visualization assistance and Silke Zechel-Gran for conducting 16sDNA sequencing and microbiome profiling.

CONFLICT OF INTEREST

None considering this study.

AUTHOR CONTRIBUTIONS

JdL participated in study design, data collection and analysis, manuscript preparation and review. SD provided histological expertise, participated in manuscript preparation and review, KR and LW provided surgical perspective in design and preparation of the manuscript and participated in discussion and review, CZ performed data collection and experimental work which was later taken up by SS, who also actively participated in the review process, MML participated in design, manuscript preparation and review, MW, TH, and ED performed and analyzed microbiology experiments and microbiota analysis and participated in manuscript preparation and review. SR participated in the data analysis and provided nutritional expertise as well as active involvement in manuscript preparation and review. BN provided disaccharidases antibodies, helped design and improve the study concept and was involved in preparation and review of the manuscript. HYN performed experiments on enzyme activities as well as Western blotting and was actively involved in study design, analysis and manuscript review. KPZ initiated this translational project including the experimental design, supervised and provided key insights during design, data collection and analysis, drafted the manuscript and was highly involved in review and finalization.

REFERENCES

1. Thiesen A, Drozdowski L, Iordache C, et al. Adaptation following intestinal resection: mechanisms and signals. *Best Pract Res Clin Gastroenterol*. 2003;17(6):981-995.
2. Dou Y, Lu X, Zhao J, Gregersen H. Morphometric and biomechanical remodelling in the intestine after small bowel resection in the rat. *Neurogastroenterol Motil*. 2002;14(1):43-53.
3. Sukhotnik I, Mogilner JG, Pollak Y, Blumenfeld S, Bejar J, Coran AG. PDGF-alpha stimulates intestinal epithelial cell turnover after massive small bowel resection in a rat. *Am J Physiol Gastrointest Liver Physiol*. 2012;302(11):G1274-G1281.

4. Sukhotnik I, Mor-Vaknin N, Drongowski RA, Coran AG, Harmon CM. Effect of dietary fat on fat absorption and concomitant plasma and tissue fat composition in a rat model of short bowel syndrome. *Pediatr Surg Int*. 2004;20(3):185-191.
5. Rossi L, Kadamba P, Hugosson C, De Vol EB, Habib Z, Al-Nassar S. Pediatric short bowel syndrome: adaptation after massive small bowel resection. *J Pediatr Gastroenterol Nutr*. 2007;45(2):213-221.
6. Pereira-Fantini PM, Thomas SL, Wilson G, Taylor RG, Sourial M, Bines JE. Short- and long-term effects of small bowel resection: a unique histological study in a piglet model of short bowel syndrome. *Histochem Cell Biol*. 2011;135(2):195-202.
7. Turner JM, Wales PW, Nation PN, et al. Novel neonatal piglet models of surgical short bowel syndrome with intestinal failure. *J Pediatr Gastroenterol Nutr*. 2011;52(1):9-16.
8. Wang W, Xiao W, Sun L, Zhang C, Chen G, Yang H. Inhibition of ACE activity contributes to the intestinal structural compensation in a massive intestinal resection rat model. *Pediatr Surg Int*. 2012;28(5):533-541.
9. Buccigrossi V, Armellino C, Tozzi A, et al. Time- and segment-related changes of postresected intestine: a 4-dimensional model of intestinal adaptation. *J Pediatr Gastroenterol Nutr*. 2013;56(1):40-45.
10. Sigalet DL, Bawazir O, Martin GR, et al. Glucagon-like peptide-2 induces a specific pattern of adaptation in remnant jejunum. *Dig Dis Sci*. 2006;51(9):1557-1566.
11. Liu X, Nelson DW, Holst JJ, Ney DM. Synergistic effect of supplemental enteral nutrients and exogenous glucagon-like peptide 2 on intestinal adaptation in a rat model of short bowel syndrome. *Am J Clin Nutr*. 2006;84(5):1142-1150.
12. Dunn JC, Parungo CP, Fonkalsrud EW, McFadden DW, Ashley SW. Epidermal growth factor selectively enhances functional enterocyte adaptation after massive small bowel resection. *J Surg Res*. 1997;67(1):90-93.
13. Nightingale JM, Lennard-Jones JE, Gertner DJ, Wood SR, Bartram CI. Colonic preservation reduces need for parenteral therapy, increases incidence of renal stones, but does not change high prevalence of gall stones in patients with a short bowel. *Gut*. 1992;33(11):1493-1497.
14. Messing B, Crenn P, Beau P, Boutron-Ruault MC, Rambaud JC, Matuchansky C. Long-term survival and parenteral nutrition dependence in adult patients with the short bowel syndrome. *Gastroenterology*. 1999;117(5):1043-1050.
15. Jiang HP, Chen T, Yan GR, Chen D. Differential protein expression during colonic adaptation in ultra-short bowel rats. *World J Gastroenterol*. 2011;17(20):2572-2579.
16. Ziegler TR, Fernández-Estívariz C, Gu LH, et al. Distribution of the H+/peptide transporter PepT1 in human intestine: up-regulated expression in the colonic mucosa of patients with short-bowel syndrome. *Am J Clin Nutr*. 2002;75(5):922-930.
17. Musch MW, Bookstein C, Rocha F, et al. Region-specific adaptation of apical Na/H exchangers after extensive proximal small bowel resection. *Am J Physiol Gastrointest Liver Physiol*. 2002;283(4):G975-G985.
18. Joly F, Mayeur C, Messing B, et al. Morphological adaptation with preserved proliferation/transporter content in the colon of patients with short bowel syndrome. *Am J Physiol Gastrointest Liver Physiol*. 2009;297(1):G116-G123.
19. Yang Q, Kock ND. Intestinal adaptation following massive ileocecal resection in 20-day-old weanling rats. *J Pediatr Gastroenterol Nutr*. 2010;50(1):16-21.
20. Kouti V, Papazoglou L, Flaskos J, Angelopoulou K, Karkavelas G, Rallis T. Ursodeoxycholic acid promotes intestinal adaptation in a cat model of short bowel syndrome. *Fundam Clin Pharmacol*. 2011;25(6):734-742.
21. Rul F, Ben-Yahia L, Chegdani F, et al. Impact of the metabolic activity of *Streptococcus thermophilus* on the colon epithelium of gnotobiotic rats. *J Biol Chem*. 2011;286(12):10288-10296.
22. Cherbuy C, Honvo-Houeto E, Bruneau A, et al. Microbiota matures colonic epithelium through a coordinated induction of cell cycle-related proteins in gnotobiotic rat. *Am J Physiol Gastrointest Liver Physiol*. 2010;299(2):G348-G357.
23. Piper HG, Fan DI, Coughlin LA, et al. Severe gut microbiota dysbiosis is associated with poor growth in patients with short bowel syndrome. *JPEN J Parenter Enteral Nutr*. 2017;41(7):1202-1212.
24. Huang Y, Guo F, Li Y, Wang J, Li J. Fecal microbiota signatures of adult patients with different types of short bowel syndrome. *J Gastroenterol Hepatol*. 2017;32(12):1949-1957.
25. Engstrand Lilja H, Wefer H, Nystrom N, Finkel Y, Engstrand L. Intestinal dysbiosis in children with short bowel syndrome is associated with impaired outcome. *Microbiome*. 2015;3:18.
26. Davidovics ZH, Carter BA, Luna RA, Hollister EB, Shulman RJ, Versalovic J. The fecal microbiome in pediatric patients with short bowel syndrome. *JPEN J Parenter Enteral Nutr*. 2016;40(8):1106-1113.
27. Gao S, Yan L, Wang R, et al. Tracing the temporal-spatial transcriptome landscapes of the human fetal digestive tract using single-cell RNA-sequencing. *Nat Cell Biol*. 2018;20(6):721-734.
28. Menard D, Pothier P. Differential distribution of digestive enzymes in isolated epithelial cells from developing human fetal small intestine and colon. *J Pediatr Gastroenterol Nutr*. 1987;6(4):509-516.
29. Tremblay E, Ferretti E, Babakissa C, et al. Gene-expression profile analysis in the mid-gestation human intestine discloses greater functional immaturity of the colon as compared with the ileum. *J Pediatr Gastroenterol Nutr*. 2011;52(6):670-678.
30. Zweibaum A, Hauri H-P, Sterchi E, et al. Immunohistological evidence, obtained with monoclonal antibodies, of small intestinal brush border hydrolases in human colon cancers and foetal colons. *Int J Cancer*. 1984;34(5):591-598.
31. Ferraris RP, Villenas SA, Diamond J. Regulation of brush-border enzyme activities and enterocyte migration rates in mouse small intestine. *Am J Physiol*. 1992;262(6 Pt 1):G1047-G1059.
32. Zimmer K-P, Fischer I, Mothes T, et al. Endocytotic segregation of gliadin peptide 31–49 in enterocytes. *Gut*. 2010;59(3):300-310.
33. Griffiths G, Hoppeler H. Quantitation in immunocytochemistry: correlation of immunogold labeling to absolute number of membrane antigens. *J Histochem Cytochem*. 1986;34(11):1389-1398.
34. Wetzel G, Heine M, Rohwedder A, Naim HY. Impact of glycosylation and detergent-resistant membranes on the function of intestinal sucrase-isomaltase. *Biol Chem*. 2009;390(7):545-549.
35. Mayeur C, Gratadoux J-J, Bridonneau C, et al. Faecal D/L lactate ratio is a metabolic signature of microbiota imbalance in patients with short bowel syndrome. *PLoS ONE*. 2013;8(1):e54335.
36. Norsa L, Lambe C, Abi Abboud S, et al. The colon as an energy salvage organ for children with short bowel syndrome. *Am J Clin Nutr*. 2019;109(4):1112-1118.
37. Fouhse JM, Ganzle MG, Regmi PR, van Kempen TA, Zijlstra RT. High amylose starch with low in vitro digestibility stimulates hindgut fermentation and has a bifidogenic effect in weaned pigs. *J Nutr*. 2015;145(11):2464-2470.

38. Topping DL, Gooden JM, Brown IL, et al. A high amylose (amylomaize) starch raises proximal large bowel starch and increases colon length in pigs. *J Nutr*. 1997;127(4):615-622.
39. King DR, Anvari M, Jamieson GG, King JM. Does the colon adopt small bowel features in a small bowel environment? *Austr New Zealand J Surg*. 1996;66(8):543-546.
40. Gillard L, Mayeur C, Robert V, et al. Microbiota is involved in post-resection adaptation in humans with short bowel syndrome. *Front Physiol*. 2017;8:224.
41. Zeichner SL, Mongodin EF, Hittle L, Huang SH, Torres C. The bacterial communities of the small intestine and stool in children with short bowel syndrome. *PLoS ONE*. 2019;14(5):e0215351.
42. Korpela K, Mutanen A, Salonen A, Savilahti E, de Vos WM, Pakarinen MP. Intestinal microbiota signatures associated with histological liver steatosis in pediatric-onset intestinal failure. *JPEN J Parenter Enteral Nutr*. 2017;41(2):238-248.
43. Vanderhoof JA, McCusker RH, Clark R, et al. Truncated and native insulinlike growth factor I enhance mucosal adaptation after jejunoileal resection. *Gastroenterology*. 1992;102(6):1949-1956.
44. Vegge A, Thymann T, Lund P, et al. Glucagon-like peptide-2 induces rapid digestive adaptation following intestinal resection in preterm neonates. *Am J Physiol Gastrointest Liver Physiol*. 2013;305(4):G277-G285.
45. Mayeur C, Gillard L, Le Beyec J, Bado A, Joly F, Thomas M. Extensive intestinal resection triggers behavioral adaptation, intestinal remodeling and microbiota transition in short bowel syndrome. *Microorganisms*. 2016;4(1):e16.
46. Domann E, Fischer F, Glowatzki F, et al. Genome sequence of *Lactobacillus* delbrueckii strain #22 isolated from a patient with short bowel syndrome and previous d-lactic acidosis and encephalopathy. *Genome announcements*. 2016;4(4).
47. Ziegler TR, Luo M, Estívariz CF, et al. Detectable serum flagellin and lipopolysaccharide and upregulated anti-flagellin and lipopolysaccharide immunoglobulins in human short bowel syndrome. *Am J Physiol Regul Integr Comp Physiol*. 2008;294(2):R402-R410.
48. Engelstad HJ, Barron L, Moen J, et al. Remnant small bowel length in pediatric short bowel syndrome and the correlation with intestinal dysbiosis and linear growth. *J Am Coll Surg*. 2018;227:439-449.
49. Li G, Yang M, Zhou K, et al. Diversity of duodenal and rectal microbiota in biopsy tissues and luminal contents in healthy volunteers. *J Microbiol Biotechnol*. 2015;25(7):1136-1145.
50. Maldonado-Gómez María X, Martínez I, Bottacini F, et al. Stable engraftment of bifidobacterium longum AH1206 in the human gut depends on individualized features of the resident microbiome. *Cell Host Microbe*. 2016;20(4):515-526.
51. Neelis E, Koning B, Rings E, et al. The Gut microbiome in patients with intestinal failure: current evidence and implications for clinical practice. *J Parenter Enteral Nutr*. 2018;43:194-205.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: de Laffolie J, Sheridan D, Reinshagen K, et al. Digestive enzyme expression in the large intestine of children with short bowel syndrome in a late stage of adaptation. *The FASEB Journal*. 2020;34:3983–3995. https://doi.org/10.1096/fj.201901758RR