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Bovine Colostrum Modulates Myeloablative Chemotherapy–Induced Gut Toxicity in Piglets[1–3]

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Abstract

Background: Intensive chemotherapy frequently results in gut toxicity, indicated by oral and intestinal mucositis, resulting in poor treatment outcomes and increased mortality. There are no effective preventive strategies against gut toxicity and the role of diet is unknown.

Objective: We hypothesized that the severity of chemotherapy-induced gut toxicity in early life is diet-dependent, and that intake of bovine colostrum (BC) provides better gut protection than an artificial milk replacer (MR).

Methods: A total of 37 3-d-old pigs received for 6 d either intravenous saline control or myeloablative treatment with busulfan and cyclophosphamide, and were fed either BC or MR, resulting in the following 4 treatments (n = 8–10/group): bovine colostrum plus saline control (Ctr-BC), milk replacer plus saline control (Ctr-MR), bovine colostrum plus busulfan and cyclophosphamide chemotherapy (BUCY-BC), and milk replacer plus busulfan and cyclophosphamide chemotherapy (BUCY-MR). The gut was collected for analysis 11 d after the start of chemotherapy.

Results: Relative to the control groups, both busulfan and cyclophosphamide chemotherapy (BUCY) groups showed signs of gut toxicity, with oral ulcers, reduced intestinal dimensions, and hematologic toxicity. Diet type did not affect mucosal structure on day 11, but BUCY-BC pigs had less vomiting than BUCY-MR pigs (1 of 10 vs. 10 of 10, $P < 0.05$). Markers of intestinal function were higher (up to 20-fold greater galactose absorption and 2–3-fold greater brush border enzyme activity, all $P < 0.05$), and tissue inflammatory cytokine concentrations and serum liver enzyme values were lower in BUCY-BC than in BUCY-MR pigs (30–50% reductions in interleukin 6 and 8, aminotransferase, and bilirubin concentrations, $P < 0.05$). Gut colonization was not significantly affected except that BUCY pigs had lower microbial diversity with a higher abundance of *Lactobacilli*.

Conclusion: BC may reduce gut toxicity during myeloablative chemotherapy in piglets by preserving intestinal function and reducing inflammation. Whether similar effects occur in children remains to be tested. *J Nutr* 2015;145:1472–80.

Keywords: mucositis, gastrointestinal toxicity, bovine colostrum, chemotherapy, piglets

Introduction

The introduction of intense chemotherapy regimens based on risk stratification and hematopoietic stem cell transplantation (HSCT)[9] has led to improved disease control and higher survival rates in children with acute leukemia. Aggressive treatments may also lead to increased toxicity and more complications, together with increased risk of graft-versus-host disease, after HSCT (1, 2). The treatment-related mortality in childhood acute lymphoblastic leukemia, acute myeloid leukemia, and HSCT is 2–5%, 10–15%, and 10–30%, respectively, and infections play a major role (3–5). Among the factors that are believed to contribute to this increased infection risk is mucosal barrier injury in the alimentary tract, potentially leading to increased translocation of bacteria and endotoxins from the gut (6). Gastrointestinal

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[3] Supplemental Figures 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

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[9] Abbreviations used: BC, bovine colostrum; BUCY, busulfan and cyclophosphamide chemotherapy; BUCY-BC, bovine colostrum plus busulfan and cyclophosphamide chemotherapy; BUCY-MR, milk replacer plus busulfan and cyclophosphamide chemotherapy; Ctr-BC, bovine colostrum plus saline control; Ctr-MR, milk replacer plus saline control; HSCT, hematopoietic stem cell transplantation; MR, milk replacer; NEC, necrotizing enterocolitis; OTU, operational taxonomic unit; PCoA, principal coordinate analysis.

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toxicity is a common complication of cytotoxic therapy and the incidence among pediatric leukemia patients is 40–100%, depending on disease, type of cytotoxic drug, dose, dosing schedule, and individual factors (7). Patients with mucositis also suffer from severe pain and reduced quality of life. Unfortunately, it has been difficult to reduce gastrointestinal toxicity without reducing the intensity of treatment, thereby increasing the risk of relapse (8). Children tolerate relatively intense treatment with chemotherapy but it is not clear whether developing organs, including the gastrointestinal tract, are more or less sensitive to chemotherapy-induced damage in early life, and how dietary factors may modulate this sensitivity (9, 10).

Bovine colostrum (BC) contains high concentrations of many bioactive compounds, such as TGF-β and insulin-like growth factors 1 and 2, that may stimulate gut growth and function and induce mucosal protection via immunomodulatory effects (11–13). Preclinical animal studies show that gastrointestinal toxicity may be affected by nutrition, suggesting that dietary treatment may be a possible adjunct strategy to limit gastrointestinal toxicity (14). In preterm pigs, BC is known to reduce the bacteria-dependent, inflammatory lesions that are characteristic of necrotizing enterocolitis (NEC) in preterm infants (15, 16). Cytotoxic chemotherapy may also change the gut microbiota in both humans (17) and animal models (18). In cancer patients, microbiota changes have been correlated with gut toxicity (19) and, in some experimental rat models, alleviation of chemotherapy-induced symptoms is observed after administration of probiotics (20–22). Under germ-free conditions in mice, the response to chemotherapy is either reduced (23) or increased (24). The gut microbiota is relatively unstable in early life but it remains unclear if and how this may affect the response to chemotherapy and to diets with different antibacterial or anti-inflammatory activities in the gut.

We hypothesized that BC would reduce gastrointestinal damage caused by cytotoxic myeloablative chemotherapy. We used young milk-fed piglets because we specifically wished to study the intestinal response to chemotherapy in early life. However, liquid milk-based diets are often required for all age groups of patients with severe chemotherapy-induced oral mucositis or gastrointestinal toxicity. We investigated the response in piglets after a clinically applied regimen consisting of busulfan and cyclophosphamide chemotherapy (BUCY). Our key endpoints were hematology, liver enzymes, gut microbiota, and the structure, function, and inflammatory status of the small intestine.

Methods

Experiment setup. Thirty-seven 3-d-old crossbred pigs (Large White × Danish Landrace) were allocated to be fed either BC or milk replacer (MR), and further allocated to receive either intravenous saline or chemotherapy with busulfan plus cyclophosphamide. Accordingly, the study included the following 4 treatments: bovine colostrum plus busulfan and cyclophosphamide chemotherapy (BUCY-BC, $n = 10$), milk replacer plus busulfan and cyclophosphamide chemotherapy (BUCY-MR, $n = 10$), bovine colostrum plus saline control (Ctr-BC, $n = 8$), and milk replacer plus saline control (Ctr-MR, $n = 9$). An overview of the study design is shown in **Supplemental Figure 1**. The BUCY pigs received 4 d of busulfan treatment (total dose 12.8 mg/kg) and 2 d of cyclophosphamide treatment (total dose 120 mg/kg) over 6 consecutive days. Diet intervention was initiated on day 0, whereas the cytotoxic chemotherapy regimen was initiated on day 1 and pigs were killed and sampled on days 10–11, or earlier if predefined humane endpoints required killing before the end of the protocol. The humane endpoints were defined as severe pain that could not be relieved by paracetamol and buprenorphine, signs of severe toxicity (continued shedding of blood or mucosa per rectum), and signs of sepsis (cyanosis, progressing respiratory distress, and lethargy). On days 10–11, pigs were anesthetized, blood subsequently was collected by cardiac puncture, and pigs were killed by intracardial injection of sodium pentobarbital, 200 mg/kg. Pigs that died or were killed before day 9 were excluded and not sampled. A detailed description of the BUCY-BC protocol used in this study has been published previously (25), with an explanation of surgical and anesthetic procedures, supporting drugs, hyperhydration, and uromitexan treatment to prevent cyclophosphamide-induced hemorrhagic cystitis and toxic response to BUCY.

Housing and feeding. Pigs were kept in individual cages and fed 11 times/d (15 mL/kg, total 165 mL · kg^{-1} · d^{-1}). Pigs were allowed voluntary feeding from individual troughs, but were supplemented with orogastric feeding if appetite was reduced to reach the predetermined daily volume. If a pig vomited during or after feeding, the feeding volume was reduced to 50% and gradually increased to reach the target volume over the following 6–12 h. BC was collected from the first milking within 24 h after parturition at a local Danish dairy farm (Danish Red cows), stored at −20°C, and sterilized by γ-irradiation (10 kilograys; Sterigenics). The macronutrient composition of the colostrum product was 5340 kJ/L energy, 103 g/L protein, 36 g/L carbohydrate, and 55 g/L fat, consistent with earlier reported values for BC (13, 26). The MR was prepared from the products Pepdite (60 g/L, powdered enteral nutrition with nonmilk-derived low molecular weight peptides, essential amino acids, carbohydrate, fat, vitamins, and minerals; Nutricia), Lacprodan (50 g/L, whey protein; Arla Foods Ingredients), Miprodan (50 g/L, casein; Arla Foods Ingredients), Calogen (50 g/L, long-chain TG fat emulsion; Nutricia), Liquigen (80 g/L, medium-chain TG from fractionated coconut oil; Nutricia), and Seravit-SHS (12 g/L, vitamins and trace elements; Nutricia). Similar products and ingredients are commonly used for hospitalized children requiring enteral nutrition formulas. The macronutrient composition in the final MR was 5291 kJ/L energy, 96 g/L protein, 40 g/L carbohydrate, and 79 g/L fat.

Clinical endpoints. Body weight and clinical signs of toxicity (presence of vomiting or diarrhea) were recorded daily. Oral ulcers representing oral mucositis were recorded at tissue collection. Blood samples were drawn before the first infusion of busulfan on day 1 and again on days 4, 7, 9, and 11 for complete hematologic blood count. A biochemical profile was analyzed in serum on days 1, 7, and 11. Blood hematology and biochemical profile were analyzed with the use of the Advia 2120 Hematology System (Siemens).

Intestinal nutrient absorption, permeability, and serum citrulline. To evaluate the in vivo nutrient absorption capacity of the apical sodium-glucose linked transporter 1, a galactose absorption test was performed on days 1, 7, and 10 as described elsewhere (27). To evaluate intestinal permeability, an enteral bolus of lactulose (5%, vol:vol) and mannitol (5%, vol:vol) was administered 3–5 h before tissue collection. Urine was sampled directly from the bladder before tissue collection. Concentrations of lactulose and mannitol were measured as previously described (27).

Circulating concentrations of the amino acid citrulline were measured to evaluate enterocyte mass as a marker of small intestinal toxicity during the study. Citrulline has been shown to correlate with intestinal mass (28), and low concentrations of citrulline correlate with clinical scores of intestinal toxicity (1). At days 1, 7, and 11, serum was collected and concentration of citrulline was quantified as previously described (29), with some modifications. Briefly, the serum samples were processed with the use of a Sirocco protein precipitation plate (Waters) and Oasis HLB Extraction Plate (Waters) then separated on a graphitic carbon column (Thermo Scientific) in an ultra-performance LC tandem quadrupole detector MS (Waters) with L-citrulline-4,4,5,5-d4 (Sigma-Aldrich) as internal standard. Quantification of citrulline was carried out with the use of QuanLynx (Waters).

Organ dimensions, histology, and tissue analyses. The small intestine, from the pyloric sphincter to the ileocecal junction, was removed and its length was recorded. The intestine was divided into 3 segments of equal length, designated proximal, middle, and distal regions, and each region was weighed. Samples from each region were collected for

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histologic evaluation, with villus height and crypt depth in formaldehyde fixated tissue samples measured, mounted in paraffin, sliced, and stained with hematoxylin and eosin as described elsewhere (25). A 10 cm section from the middle of the distal region was removed for measurement of the mucosal proportion, as described previously (30). Other organs (stomach, colon, liver, kidneys, spleen, heart, and lungs) were removed and their wet weights recorded. Finally, a piece of sternum containing bone marrow was collected and bone marrow cellularity was evaluated by a blinded pathologist as described previously (25).

Snap-frozen intestinal samples were later homogenized in 1% Triton X-100 and assayed for intestinal activity of disaccharidases (sucrase, maltase, and lactase) and peptidases (aminopeptidase N, aminopeptidase A, and dipeptidyl peptidase IV), with the use of specific substrates as previously described (30, 31). For cytokine analyses, a protease inhibitor cocktail was added to the homogenization solution. Cytokine concentrations were measured with the use of ELISA kits (R & D Systems) targeted against IL-8, IL-6, IL-1β, and TNF-α as described elsewhere (25).

Gut microbiota. From luminal content collected from the distal small intestine, DNA was purified with the use of the Maxwell 16 Instrument (Promega Corporation), following the manufacturer's protocol for the low elution volume blood DNA system, but with additional lysozyme treatment and bead beating with stainless steel beads for 2 min/20 Hz in a tissue lyser (Qiagen). The V4 and V5 regions of the 16S ribosomal RNA gene were PCR amplified from each sample with the use of barcoded primers: 519F (5′-CAGCAGCCGCGGTAATAC-3′) and 926R (5′-CCGTCAATTCCTTTGAGTTT-3′) and analyzed for quantity and quality in an Agilent 2100 Bioanalyzer (Agilent Technologies) with the use of an Agilent RNA 1000 Nano Kit. An amount of 50 ng DNA from each sample was pooled for library preparation with molecular identifier for 1-region 454 sequencing on GS FLX Titanium PicoTiterPlates (70675) with the use of a GS FLX Titanium Sequencing Kit XLR70 (Roche Diagnostics). Library construction and 454 pyrosequencing was carried out at the National High-Throughput DNA Sequencing Centre, University of Copenhagen.

Data were analyzed with the use of the Quantitative Insight Into Microbial Ecology open source software package (32). Raw data underwent denoising, chimera filtering, and operational taxonomic unit (OTU) picking, as previously described (33), and the remaining high-quality sequences were clustered at 97% relatedness with the use of UCLAST (34). Representative sequences from each cluster were aligned with PyNAST (35), subjected to the Ribosomal Database Project–based 16S ribosomal RNA gene annotation. Alpha diversity was calculated (observed species) for OTU tables unified to 2000 sequences per sample. Principal coordinate analysis (PCoA) plots were generated with the jackknifed beta diversity workflow based on 10 distance metrics calculated with the use of 10 subsampled OTU tables and 85% of sequences taken for each jackknifed subset. Group differences in the PCoA plots were evaluated by analysis of similarities with the use of weighted and unweighted UniFrac distance metrics based on rarefied OTU tables (1000 reads/sample). Group differences in relative taxa abundances at phylum and genus level were calculated and verified with Metastats (36) based on 1000 permutations of random combinations of group pairs. Differences in the most dominating OTUs were tested with the use of the lmer function of mixed model analysis in R version 3.0.1 with treatment, diet, and presence of mucositis as fixed variables.

Statistical methods. Categorical outcomes (vomiting, diarrhea, and oral mucositis) were analyzed with the use of logistic regression on the overall incidence. The daily growth rates were calculated as weight gained (grams) over 2 d, divided by body weight (kilograms) on the first of the 2 d. Continuous data were analyzed with the use of 2-factor ANOVA, with treatment (BUCY or control) and diet (BC or MR) as independent categorical variables. Repeated measurements (growth rate, blood hematology, serum biochemistry, plasma galactose, serum citrulline, villus height, crypt depth, and brush border enzymes) were analyzed with the use of mixed models, with day or intestinal segment as additional fixed effects and pig as a random effect. Normality was assessed by means of standardized residuals and Box-Cox transformed to account for variance heterogeneity, when necessary. Cytokine IL-6 and IL-1β concentrations were analyzed with the use of a Tobit model to account for measurements below the detection limit of the assay. Spearman's rank correlation coefficient was used to investigate associations between plasma concentrations of citrulline and relative intestinal weight at the end of the study. Differences between groups were evaluated with the use of post hoc *t* tests without multiplicity adjustment of *P* values. $P < 0.05$ were considered significant. All values presented are arithmetic means ± SEMs unless otherwise stated. All statistical analyses were performed in Stata/IC 12.1 (StataCorp).

Results

Clinical endpoints. Forty percent (8 of 20) of the BUCY pigs had to be killed before the predetermined end of the protocol on days 10–11, 2 BUCY-BC and 6 BUCY-MR pigs. Mean growth rates during the first 6 d were similar among the groups. After the BUCY treatment (days 7–11), growth rates decreased, resulting in negative growth rates in the 2 BUCY groups, most pronounced in

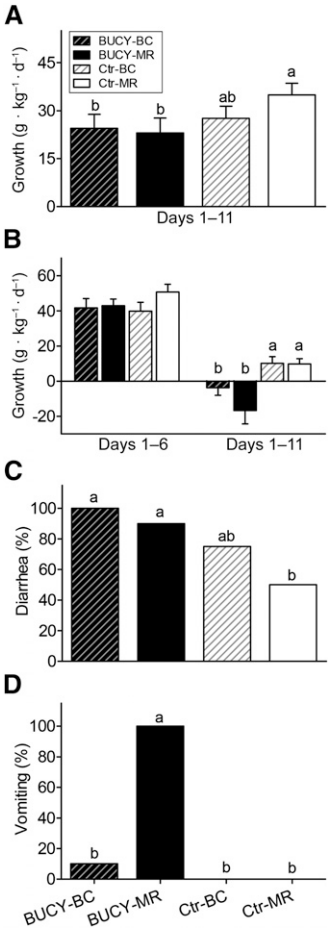


FIGURE 1 Clinical variables in piglets treated with intravenous BUCY or saline and fed either BC or MR throughout the study. Growth rates throughout the entire study (A) and growth rates during and after the BUCY treatment, days 1–6 and days 7–11, respectively (B), and the overall incidence of diarrhea (C) and vomiting (D). Growth rates are means ± SEMs and incidences are overall percentage of affected pigs in each group throughout the study; BUCY-BC, $n = 10$; BUCY-MR, $n = 10$; Ctr-BC, $n = 8$; and Ctr-MR, $n = 9$. Labeled means (at a time) without a common letter differ, $P < 0.05$. BC, bovine colostrum; BUCY, busulfan and cyclophosphamide chemotherapy; BUCY-BC, bovine colostrum plus busulfan and cyclophosphamide chemotherapy; BUCY-MR, milk replacer plus busulfan and cyclophosphamide chemotherapy; Ctr-BC, bovine colostrum plus saline control; Ctr-MR, milk replacer plus saline control; MR, milk replacer.

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the BUCY-MR pigs. Calculated for the entire 1–11 d period, growth rates in the 2 BUCY groups were lower than in the Ctr-MR pigs, and there was no effect from BC on growth rates in BUCY-treated pigs (**Figure 1**A and B).

The overall diarrhea incidence was similar among BUCY-BC and BUCY-MR pigs, and higher than in Ctr-MR pigs, with intermediate values in Ctr-BC pigs (Figure 1C). Vomiting was observed only in the chemo groups, and it was most pronounced in BUCY-MR pigs (Figure 1D). Among pigs that were alive after day 8, oral ulcers were observed in 4 of 5 BUCY-MR pigs vs. 4 of 8 BUCY-BC pigs, whereas oral ulcers were absent in all control pigs.

Organ toxicities and blood chemistry and hematology. The BUCY treatment resulted in bone marrow aplasia in all BUCY pigs, with no effect from diet on mean cellularity (>10% for the 2 BUCY groups vs. 76% for the 2 control groups, $P < 0.001$). Supporting these findings, relative spleen weight was lower in the 2 BUCY groups (1.8–2.2 g/kg) than in Ctr-MR (3.3 ± 0.3 g/kg) and Ctr-BC (4.3 ± 0.2 g/kg) pigs (both $P < 0.05$), and there was no effect from diet in the 2 BUCY groups. Correspondingly, the number of circulating blood cells (neutrophils, lymphocytes, platelets, and reticulocytes) was reduced in all BUCY pigs compared with control (**Supplemental Figure 2**A–D). BC delayed the reduction in platelet counts in BUCY pigs (day 9, $P < 0.05$) (Supplemental Figure 2D) but had no effect on leukocytes and reticulocytes. In controls, BC increased the lymphocyte counts by the end of the experiment (day 11, $P < 0.05$) (Supplemental Figure 2B). Mean red blood cell counts were similar among the groups throughout the study (data not shown).

Indications of chemotherapy-induced liver damage were evaluated by serum concentrations of transaminases and bilirubin. On

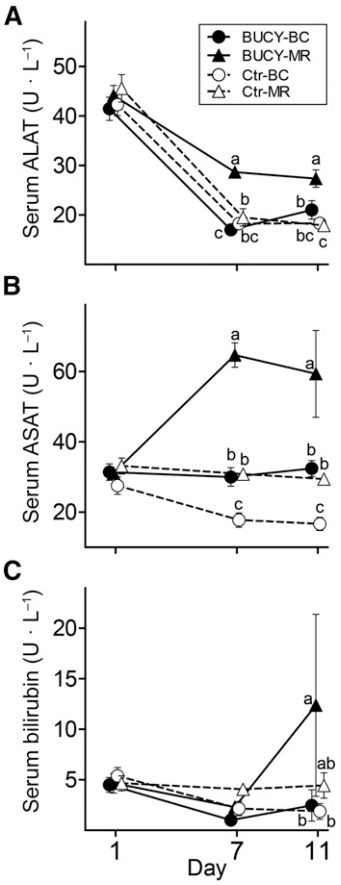


FIGURE 2 Longitudinal serum biochemistry values in piglets treated with intravenous BUCY or saline and fed either BC or MR. ALAT (A), ASAT (B), and bilirubin (C). Values are means ± SEMs; BUCY-BC, n = 6–10; BUCY-MR, n = 3–10; Ctr-BC, n = 6–8; and Ctr-MR, n = 5–8. A minor horizontal jitter has been applied to prevent overlapping and improve visual appearance and identification of group values. Labeled means (at a time) without a common letter differ, $P <$ 0.05. ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; BC, bovine colostrum; BUCY, busulfan and cyclophosphamide chemotherapy; BUCY-BC, bovine colostrum plus busulfan and cyclophosphamide chemotherapy; BUCY-MR, milk replacer plus busulfan and cyclophosphamide chemotherapy; Ctr-BC, bovine colostrum plus saline control; Ctr-MR, milk replacer plus saline control; MR, milk replacer.

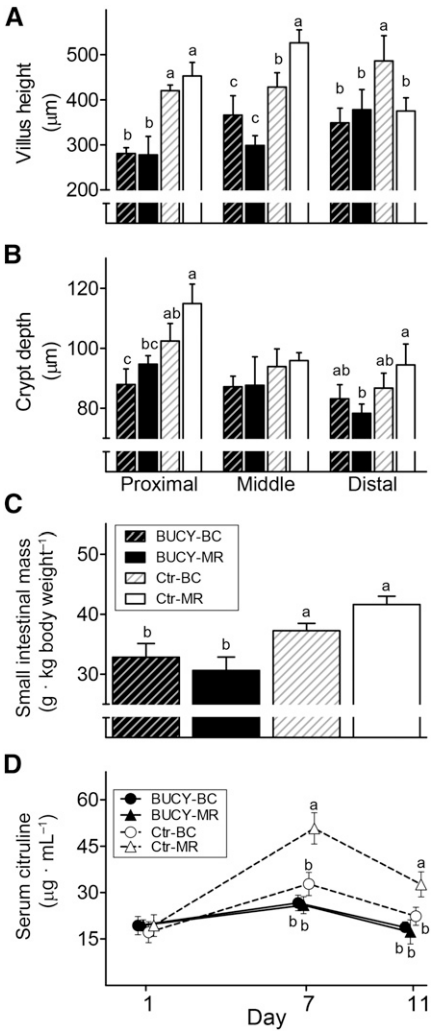


FIGURE 3 Intestinal morphology and mass in piglets treated with intravenous BUCY or saline and fed either BC or MR. Villus height (A) and crypt depth (B) in samples from the proximal, middle, and distal small intestine, total small intestinal weight relative to body weight (C), and serum citrulline concentrations measured longitudinally during the 11 d protocol as a marker of enterocyte mass (D). Values are means ± SEMs; BUCY-BC, n = 4–9; BUCY-MR, n = 4–6; Ctr-BC, n = 4–8; and Ctr-MR: n = 6–8. A horizontal jitter has been applied to prevent overlapping and improve visual appearance and identification of group values for the longitudinal data. Labeled means (at a time or in a region) without a common letter differ, $P < 0.05$. BC, bovine colostrum; BUCY, busulfan and cyclophosphamide chemotherapy; BUCY-BC, bovine colostrum plus busulfan and cyclophosphamide chemotherapy; BUCY-MR, milk replacer plus busulfan and cyclophosphamide chemotherapy; Ctr-BC, bovine colostrum plus saline control; Ctr-MR, milk replacer plus saline control; MR, milk replacer.

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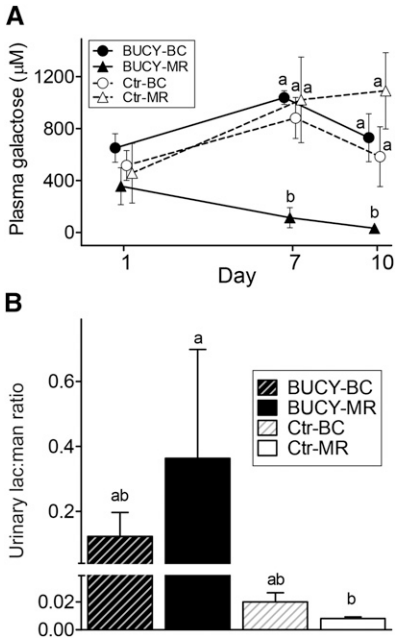


FIGURE 4 Intestinal function in vivo in piglets treated with intravenous BUCY or saline and fed either BC or MR. Absorption of galactose measured in plasma 20 min after an enteral galactose bolus on days 1, 7, and 11 (A) and intestinal permeability assessed with the urinary lactulose-to-mannitol ratio sampled 3h after an enteral bolus solution with lactulose and mannitol (B). Values are means ± SEMs; BUCY-BC, n = 2–5; BUCY-MR, n = 3–7; Ctr-BC, n = 3–5; and Ctr-MR, n = 4–7. A horizontal jitter has been applied to prevent overlapping and improve visual appearance and identification of group values for the longitudinal data. Labeled means (at a time) without a common letter differ, $P < 0.05$. BC, bovine colostrum; BUCY, busulfan and cyclophosphamide chemotherapy; BUCY-BC, bovine colostrum plus busulfan and cyclophosphamide chemotherapy; BUCY-MR, milk replacer plus busulfan and cyclophosphamide chemotherapy; Ctr-BC, bovine colostrum plus saline control; Ctr-MR, milk replacer plus saline control; lac, lactulose; man, mannitol; MR, milk replacer.

days 7 and 11, alaninine aminotransferase and aspartate aminotransferase concentrations were markedly elevated in the BUCY-MR pigs relative to the BUCY-BC pigs and controls (**Figure 2**A and B). A later and more moderate BUCY-related elevation of these liver enzymes was observed in the BUCY-BC group compared with the Ctr-BC pigs. Likewise, BUCY pigs showed greatly elevated bilirubin concentrations on day 11 (Figure 2C), and concentrations were even higher in BUCY-MR pigs than in BUCY-BC pigs.

Intestinal structure, digestive function, and inflammatory markers. Chemotherapy generally reduced the villus height and crypt depth (especially in the proximal region), but these variables were not affected by diet (**Figure 3**A and B), despite the fact that small intestinal mass tended to be higher in Ctr-MR pigs than in Ctr-BC pigs (P = 0.06). Supporting the histomorphologic findings, relative intestinal weight was reduced in both BUCY groups compared with controls (Figure 3C). Colostrum did not affect intestinal length, colon weight, or intestinal mucosal proportions (data not shown). Serum citrulline concentrations were equal among all groups on day 1 (18.9 ± 1.5 μg/mL) and showed limited change with time or treatment, except that values for the Ctr-MR pigs were higher than for the other 3 groups from day 7 to day 11 (Figure 3D). For BUCY pigs, a positive correlation was found between citrulline and relatively small intestinal weight on day 11 (ρ = 0.83, $P < 0.01$).

Active intestinal absorption of hexose via sodium-glucose linked transporter 1 was investigated by measuring plasma galactose after an oral bolus of galactose. In MR pigs, chemotherapy treatment led to a pronounced decrease in galactose absorption, whereas in pigs treated with BC, absorption of galactose was maintained despite chemotherapy (**Figure 4**A). After a galactose bolus on days 7 and 10, BUCY-BC pigs reached 5–7-fold higher concentrations of galactose than BUCY-MR pigs, and values were similar to those in control pigs. Intestinal permeability was assessed with the lactulose/mannitol test. The lactulose-to-mannitol ratio tended to be increased in BUCY-treated pigs vs. control pigs (P = 0.05), but there was no effect from diet (Figure 4B). This may be explained in part by the low sample size for analyses. There were difficulties in sampling urine from many of the pigs at autopsy and only 17 of 37 pigs had sufficient urine for analysis. Similar difficulties in collecting sufficient colon content and blood also influenced the number of represented pigs for other analyses.

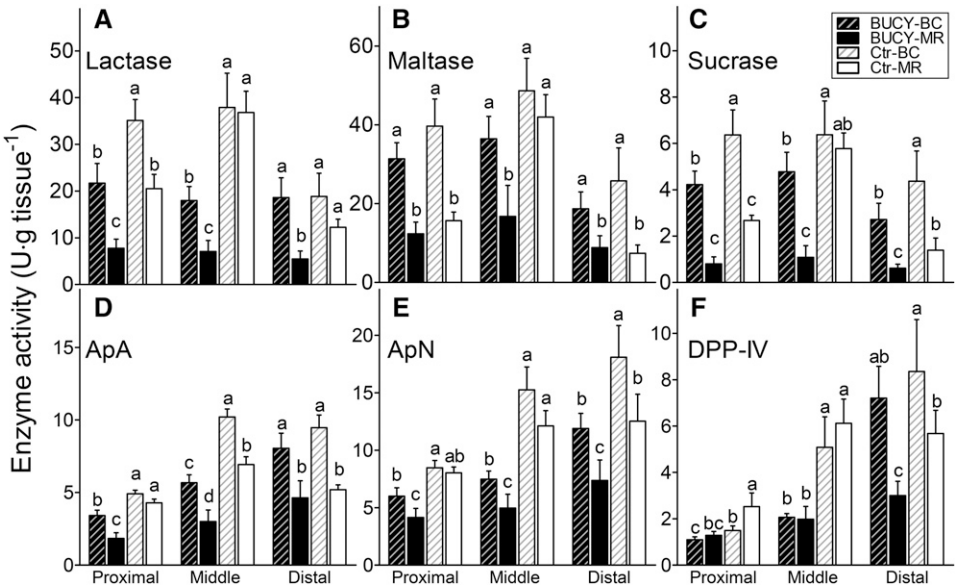


FIGURE 5 Activity of brush border enzymes in the proximal, middle, and distal regions of small intestinal tissue from piglets treated with intravenous BUCY or saline and fed either BC or MR. Lactase (A), maltase (B), sucrase (C), ApA (D), ApN (E), and DPP-IV (F). Values are means ± SEMs; BUCY-BC, n = 9; BUCY-MR, n = 5; Ctr-BC, n = 8; and Ctr-MR, n = 8. Labeled means (in a region) without a common letter differ, $P < 0.05$. ApA, aminopeptidase A; ApN, aminopeptidase N; BC, bovine colostrum; BUCY, busulfan and cyclophosphamide chemotherapy; BUCY-BC, bovine colostrum plus busulfan and cyclophosphamide chemotherapy; BUCY-MR, milk replacer plus busulfan and cyclophosphamide chemotherapy; Ctr-BC, bovine colostrum plus saline control; Ctr-MR, milk replacer plus saline control; DPP-IV; dipeptidyl peptidase IV; MR, milk replacer.

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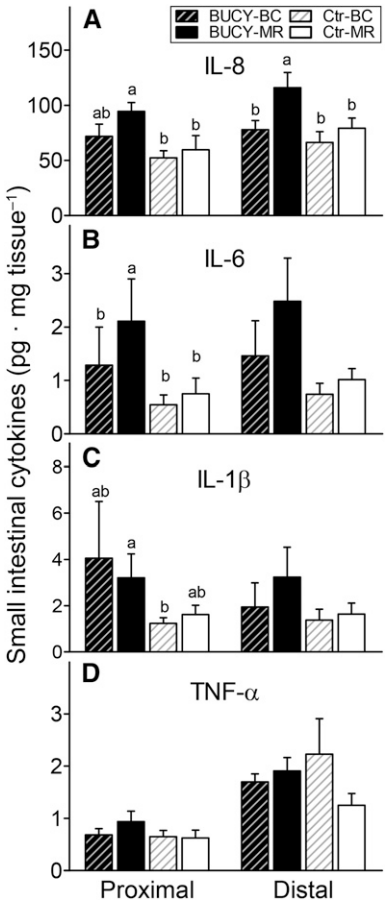


FIGURE 6 Proinflammatory cytokine concentrations in the proximal and distal regions of small intestinal tissue from piglets treated with intravenous BUCY or saline and fed either BC or MR. IL-8 (A), IL-6 (B), IL-1β (C), and TNF-α (D). Values are means ± SEMs; BUCY-BC, $n = 9$; BUCY-MR, $n = 5$; Ctr-BC, $n = 8$; and Ctr-MR, $n = 8$. Values below the detection limit were set to the lowest value of detection and analyzed with the Tobit method for IL-6 and IL-1β. Labeled means (in a region) without a common letter differ, $P < 0.05$. BC, bovine colostrum; BUCY, busulfan and cyclophosphamide chemotherapy; BUCY-BC, bovine colostrum plus busulfan and cyclophosphamide chemotherapy; BUCY-MR, milk replacer plus busulfan and cyclophosphamide chemotherapy; Ctr-BC, bovine colostrum plus saline control; Ctr-MR, milk replacer plus saline control; MR, milk replacer.

The digestive function of enterocytes was assessed by measuring the activity of brush border enzymes. Generally, the enzymes examined showed reduced activity in pigs treated with chemotherapy and the reduction was less pronounced in BC pigs. Thus, disaccharidase activities (sucrase, lactase, and maltase) were 200–300% higher in the 2 groups of BC pigs (control and BUCY) than in MR pigs (**Figure 5**A–C). Aminopeptidase activities (aminopeptidase N and aminopeptidase A) showed the same pattern, although the differences between BUCY-BC and BUCY-MR pigs were not as pronounced. Activity of dipeptidyl peptidase IV was increased 100% in BUCY-BC vs. BUCY-MR pigs in the distal region (Figure 5D–F).

To examine the effect of colostrum on chemotherapy-induced mucosal inflammation, inflammatory cytokines were measured in the intestinal tissue. Generally, chemotherapy tended to increase the tissue concentrations of the cytokines, especially IL-8 and IL-6, albeit to different degrees. Thus, concentrations of IL-8 in the distal intestine, and of IL-6 in the proximal intestine, were higher in BUCY-MR pigs than in the other groups ($P < 0.05$, **Figure 6**A and B). The same pattern was seen for IL-8 in the proximal region and IL-6 in the distal region, although these differences did not reach statistical significance (Figure 6A and B). Concentrations of IL-1β and TNF-α were generally low, and no differences were found between groups (Figure 6C and D).

Gut microbiota. Adequate DNA yield from the distal small intestinal content for subsequent 454 pyrosequencing was obtained from 29 pigs (BUCY-BC, $n = 8$; BUCY-MR, $n = 5$; Ctr-BC, $n = 8$; and Ctr-MR, $n = 8$). After quality control, a total of 247,710 sequences remained with a mean of 8542 ± 1527 sequences per sample (ranging from 721 to 38,310 sequences per sample). Seven phyla were identified, along with a total of 63 OTUs (genus level), with a mean of 13.4 ± 0.8 different OTUs per sample. The data set was strongly dominated by *Lactobacillus*, representing 84%, and *Escherichia*, representing 9% of the total sequences. Chemotherapy or diet did not significantly modify the microbiota composition in the distal small intestine as tested by PCoA and analysis of similarities. However, the relative distribution of the most dominating OTUs (**Figure 7**A) showed that *Lactobacillus* was substantially more abundant in BUCY-pigs than in control pigs, whereas diet had no significant effect. *Escherichia*, the second most dominating OTU, was not affected by chemotherapy or diet, whereas *Lactococcus*, the third most dominating OTU, was significantly more abundant in MR than in BC pigs ($P < 0.05$, Figure 7A). Microbial diversity was significantly lower in the BUCY groups than in control ($P < 0.05$, Figure 7A), with a trend to higher diversity in MR than in BC pigs ($P = 0.08$). No significant differences were found between BUCY-BC and BUCY-MR pigs.

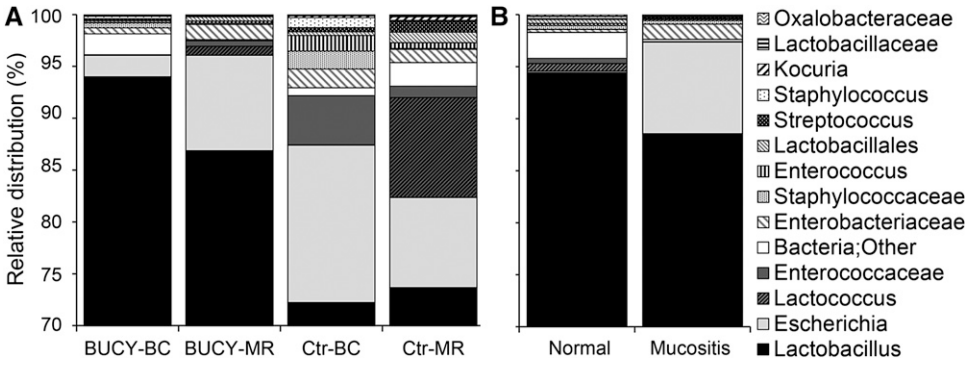


FIGURE 7 Relative distribution of dominating bacteria (genus level) in the colon content of piglets treated with intravenous BUCY or saline and fed either BC or MR. Distribution among all 4 treatment groups (A) and distribution between BUCY pigs with and without oral mucositis (B). The data are based on 454 pyrosequencing, in which *Lactobacillus* accounts for >70% and >85% of the sequences in the control pigs and BUCY pigs, respectively. BUCY-BC, $n = 8$; BUCY-MR, $n = 5$; Ctr-BC, $n = 8$; and Ctr-MR, $n = 8$. BC, bovine colostrum; BUCY, busulfan and cyclophosphamide chemotherapy; BUCY-BC, bovine colostrum plus busulfan and cyclophosphamide chemotherapy; BUCY-MR, milk replacer plus busulfan and cyclophosphamide chemotherapy; Ctr-BC, bovine colostrum plus saline control; Ctr-MR, milk replacer plus saline control; MR, milk replacer.

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Oral mucositis did not affect the relative bacterial distribution of the small intestine, although BUCY pigs with oral mucositis tended to have more *Escherichia* than BUCY pigs with no signs of oral mucositis (8.8% vs. 0.15%, $P = 0.05$, Figure 7B).

Discussion

Using piglets as a model for children subjected to chemotherapy, we have shown that BC improves the intestinal response to myeloablative chemotherapy vs. a diet based on commercially available products for enteral nutrition formulas. This is important because gastrointestinal toxicity after myeloablative chemotherapy is closely associated with treatment-related morbidity and mortality (37). Preventive strategies against mucositis are often limited to pain management, improved oral hygiene, and prophylaxis against bacterial and fungal infections (38). The pathophysiology of gut toxicity is poorly understood but may involve apoptosis, impaired proliferation, inflammation, and increased intestinal permeability (39), together with effects on mucosal metabolic pathways (40), brush border enzyme activities, and nutrient absorption (41). Gastrointestinal toxicity may be modulated by diet (42), but because of the limited availability of tissues from humans, how diet affects acute gut toxicity remains largely unknown. Further, there is limited or no information available from animal models that mimic the developing state of the intestine in infants and children.

In this study, milk-based diets were fed over the entire period from before treatment to after the peak of cytotoxic damage that typically occurs within 3–6 d after initiation of chemotherapy (43). Using this protocol, we demonstrated that piglets subjected to a standard BUCY treatment had intestinal atrophy, increased cytokine concentrations, and impaired intestinal function (brush border enzymes and nutrient absorption) at days 10–11, in addition to the clinical signs of toxicity (weight loss vomiting, and adverse hematology). The colostrum diet had significant positive effects on markers of intestinal function in BUCY pigs (brush border enzymes and hexose absorptive capacity), whereas the effects on structural markers were limited or absent (villus structure, intestinal weight, and plasma citrulline). Colostrum also tended to reduce concentrations of proinflammatory cytokines (IL-8 and IL-6). Finally, our results showed that colostrum feeding of BUCY pigs may reduce cytotoxic liver injury, as indicated by lower circulating concentrations of transaminases and bilirubin. Collectively, we conclude that there is a potential to improve chemotherapy-induced toxicity by inclusion of BC into the enteral diet for such patients. More studies are required, however, to define the optimal timing and amount of colostrum in relation to different types of cytotoxic chemotherapies. In our studies on children subjected to chemotherapy, supplementary BC is provided during a limited time frame before and shortly after chemotherapy (clinicaltrials.gov: NCT01766804).

BC is the milk produced in the first 1–2 d after parturition. It contains a high amount of bioactive components, such as IgG, TGFβ, insulin-like growth factors, platelet-derived growth factor, fibroblast growth factor, and epidermal growth factor, all known to stimulate growth in mammalian cells in vitro and in vivo (11, 13). BC has shown potential in reducing the severity of NEC in preterm piglets (16), and it also preserves intestinal integrity and reduces bacterial translocation in rats treated with diclofenac (44) and inhibits NF-κB signaling in human colon cancer cell lines (45). In our study, colostrum feeding did not prevent a reduction in serum citrulline concentrations, relative to MR feeding. There were also lower concentrations of serum citrulline and a trend toward reduced intestinal mass in the Ctr-BC group vs. the Ctr-MR group. These findings suggest that feeding exclusively colostrum for up to 11 d may be less than ideal. The relatively long period of exclusive colostrum feeding in this study may also explain why we did not detect a positive effect on structural intestinal variables in the BUCY-BC group, but only on functional variables. We cannot exclude the possibility that the diet effects in this study could also in part result from a difference in the source of macronutrients between diets. However, it is unlikely that a single component, such as a cytokine, growth factor, or amino acid alone, can ameliorate gastrointestinal toxicity in an effective manner (46). Actually it might be the unique combination of growth factors, immune-regulating cytokines, antimicrobial peptides, and specific nutrients such as special proteins, amino acids, and milk fat molecules in BC that mediate the observed effects (47). For the MR formula used in this study, fats were mainly vegetable oils (not milk fats as in BC), proteins were whey proteins plus nonmilk proteins (not IgG, casein, and whey as in BC), and carbohydrates were mainly maltodextrins (not lactose as in BC). For these reasons, the BC intervention diet was compared to a formula diet containing the same overall energy amount as the BC diet, and with formula ingredients that reflect those often incorporated in enteral diets for tube-fed hospitalized patients suffering from oral and gastrointestinal mucositis.

Previous investigations have demonstrated significant chemotherapy-induced effects on the gut microbiota. These include decreased bacterial diversity (48) and increased density of potentially pathogenic bacteria, such as *Escherichia, Clostridia*, and *Enterococci* (17, 19, 48–51), and decreased density of potentially beneficial bacteria, such as *Lactobacilli* and *Bifidobacteria* (19, 48, 49). The results of the present study support these previous findings in that BUCY treatment reduced microbial diversity in the small intestine. BUCY pigs with oral mucositis tended to have more *Escherichia* in the small intestine than did BUCY pigs without mucositis and, in our study, BUCY pigs also had more *Lactobacilli*. The limited effects of diet on the intestinal microbiota suggest that the BC effects on the mucosa are primarily related to host responses via the trophic and immune-modulatory properties of BC (51). However, we cannot exclude effects on absolute bacterial abundance and we previously showed NEC to be more closely related to bacterial load than to bacterial composition (52). It is also possible that both diet and BUCY treatment affected the density and diversity of microbiota specifically along the mucosal lining. Finally, it is possible that compositional changes in the colonic microbiota depend on the time after start of treatment, because chemotherapy-induced dysbiosis may not be present until several days or even weeks after chemotherapy (53, 54).

In conclusion, BC may reduce gastrointestinal complications during myeloablative chemotherapy by preserving gastrointestinal function and reducing inflammation. Further animal studies should investigate the use of colostrum as a supplement to other diets and in relation to different chemotherapy regimens and feeding periods before and after treatment. As such, BC may become an important adjunct dietary therapy to reduce chemotherapy-induced mucositis.

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