Bacterial populations and metabolites in the feces of free roaming and captive grizzly bears

Clarissa Schwab, Bogdan Cristescu, Mark S. Boyce, Gordon B. Stenhouse, and Michael Gänzle

Abstract: Gut physiology, host phylogeny, and diet determine the composition of the intestinal microbiota. Grizzly bears (*Ursus arctos horribilis*) belong to the Order Carnivora, yet feed on an omnivorous diet. The role of intestinal microflora in grizzly bear digestion has not been investigated. Microbiota and microbial activity were analysed from the feces of wild and captive grizzly bears. Bacterial composition was determined using culture-dependent and culture-independent methods. The feces of wild and captive grizzly bears contained log 9.1 ± 0.5 and log 9.2 ± 0.3 gene copies·g^{-1}, respectively. Facultative anaerobes Enterobacteriaceae and enterococci were dominant in wild bear feces. Among the strict anaerobes, the *Bacteroides–Prevotella–Porphyromonas* group was most prominent. Enterobacteriaceae were predominant in the feces of captive grizzly bears, at log 8.9 ± 0.5 gene copies·g^{-1}. Strict anaerobes of the *Bacteroides–Prevotella–Porphyromonas* group and the *Clostridium coccoides* cluster were present at log 6.7 ± 0.9 and log 6.8 ± 0.8 gene copies·g^{-1}, respectively. The presence of lactate and short-chain fatty acids (SCFAs) verified microbial activity. Total SCFA content and composition was affected by diet. SCFA composition in the feces of captive grizzly bears resembled the SCFA composition of prey-consuming wild animals. A consistent data set was obtained that associated fecal microbiota and metabolites with the distinctive gut physiology and diet of grizzly bears.

Key words: carnivore, *Ursus arctos horribilis*, SCFA, fecal microflora, gut physiology.

Résumé : La physiologie de l'intestin, la phylogénie de l'hôte et la diète déterminent la composition de la flore intestinale. L'ours grizzly (*Ursus arctos horribilis*) appartient à l'ordre des carnivores, même s'il a une diète omnivore. Le rôle de la flore microbienne intestinale dans la digestion chez le grizzly n'a pas été examiné. La flore intestinale et l'activité microbienne ont été analysées à partir des fèces d'ours grizzlys sauvages et d'ours gardés en captivité. La composition bactérienne a été déterminée à l'aide de méthodes dépendantes et indépendantes de la culture. Les fèces des grizzlys sauvages et gardés en captivité contenaient log 9,1 ± 0,5 et log 9,2 ± 0,3 copies de gènes·g^{-1}, respectivement. Les anaérobies Enterobacteriaceae et entérocoques étaient dominants dans les fèces des ours sauvages. Parmi les anaérobies stricts, le groupe *Bacteroides–Prevotella–Porphyromonas* était prédominant. Enterobacteriaceae était prédominant dans les fèces des ours grizzly captifs, avec log 8,9 ± 0,5 copies de gènes·g^{-1}. Les anaérobies stricts du groupe *Bacteroides–Prevotella–Porphyromonas* et de la grappe *Clostridium coccoides* étaient présents avec log 6,7 ± 0,9 et log 6,8 ± 0,8 copies de gènes·g^{-1} respectivement. La présence de lactate et d'acides gras à chaîne courte (AGCC) a permis d'évaluer l'activité microbienne. Le contenu total en AGCC et sa composition était affecté par la diète. La composition en AGCC des fèces des ours grizzly captifs ressemblait à celle des animaux sauvages nourris de proies. Les résultats cohérents obtenus ont permis d'associer la flore fécale et les métabolites à la physiologie caractéristique de l'intestin et à la diète des ours grizzlys.

Mots-clés : carnivore, *Ursus arctos horribilis*, AGCC, flore microbienne fécale, physiologie de l'intestin.

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Introduction

Grizzly bears (*Ursus arctos horribilis*) belong to the Order Carnivora. Yet, with the exception of the polar bear (*Ursus maritimus*), the diet of members of the bear family consists, to a large extent, of plant material (Christiansen 2008). The diet of grizzly bears can vary, depending on the availability of resources within different areas. Coastal bears mainly feed on salmon; interior bears consume new vegetation in the spring, acorns, nuts, and berries in the fall, and only occasionally catch prey (Mowat and Heard 2006; Munro et al. 2006). The presence of berries is crucial for increasing body mass before hibernation, as mass gain has been related to reproduction in females (Samson and Huot 1995; Welch et al. 1997). To date, feeding high quantities, extensive chewing, and rapid fecal excretion have been identified as the digestive processes that pandas and grizzly and black bears rely on to fulfill energy demands (Christiansen 2008; Pritchard and Robbins 1990). Little is known about the composition or role of the gastrointestinal (GI) mi-

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croflora in the digestion of grizzly bears and closely related species.

The GI tract of mammals is a complex ecosystem that stages a dynamic interplay between food, host, and intestinal microbiota. The composition of gut microflora varies among hosts (Ley et al. 2008*a*, 2008*b*). The GI tract of Ursidae consists of a long small intestine and a short bowel, and lacks a cecum (Stevens and Hume 1995). In contrast, omnivores, such as humans and pigs, possess a complex GI system composed of a small intestine, a cecum, and a large haustrated colon. Microbial counts and diversity differ within gut sections (Marteau et al. 2001; Mentula et al. 2005; Metzler et al. 2009; Stevens and Hume 1998). The small intestine of omnivores contains between 1×10^3 and 1×10^8 CFU·mL^{-1} aerobes, facultative anaerobes (lactobacilli, enterococci, staphylococci, and Enterobacteriaceae), and strict anaerobes (clostridia, bifidobacteria, and *Bacteroides*). The cecum is populated by approximately 1×10^8 CFU·mL^{-1}, 75% ananerobes, and 25% facultative anaerobes. The highest concentration of bacteria can be found within the large intestine (1×10^{10} to 1×10^{11} CFU·g wet mass^{-1}), where the strict anaerobes are dominant (Hao and Lee 2004; Leser et al. 2002; Marteau et al. 2001; Mentula et al. 2005; Metzler et al. 2009). The fecal microbiota is a reliable indicator of the microbiota present within the distal colon (Moore et al. 1978). Feces also contain microorganisms originating from food or the oral cavity, which do not persist in and only transit the GI tract (Dal Bello and Hertel 2006; Tannock et al. 2000).

Intestinal microbiota and the mammalian host live in a symbiotic partnership. The gut microflora acts as a stimulator of the intestinal immune system, provides protection against colonization by pathogens, and is involved in nutrient processing (Hooper et al. 2002; Neish 2009). The mammal provides substrates for use by the host microorganism. Formation of short-chain fatty acids (SCFAs) by the bacterial fermentation of carbohydrates or proteins not otherwise digestible by the host delivers between 10% and 35% of energy needed by humans, dogs, and pigs. The ruminal microbiota of herbivores supplies up to 70% of required energy through the breakdown of dietary polysaccharides (Hooper et al. 2002; Williams et al. 2001). Comparison of conventional and sterile mice or pigs showed that, in the presence of an intact flora, body mass can be increased even though food intake is reduced (Hooper et al. 2002).

Interactions between gut microflora and the host are complex. Environment-, diet-, or disease-triggered impacts on gut health will render nutrient uptake and processing, and will thus affect the animal as a whole (Cani and Delzenne 2007; Neish 2009; Williams et al. 2001). In domesticated animals, the relationship between intestinal microflora, welfare, and reproduction has been recognized and applied in practice to increase productivity (Flickinger et al. 2003; Williams et al. 2001). In contrast, the role of microflora in wild animals has gained little attention because of a lack of economic interest and difficulties in obtaining samples.

Nevertheless, the relation between the health of intestinal microflora and the animal host is of special importance in a declining species such as the grizzly bear. Numerous studies have investigated and defined habitat requirements for grizzly bears, as their persistence is dependent on the availability of high-quality habitat (Ciarniello et al. 2007; McLellan and Banci 1999; Nielsen et al. 2003, 2006; Weaver et al. 1996). Because "habitat" includes the availability of food resources, some studies focus on the nutrition of grizzly bears (Robbins et al. 2004). Future bear conservation and management decisions will have to take into consideration detailed information about the digestive processes and energy demands of grizzly bears (Munro et al. 2006). The little investigated distinctive combination of an omnivorous diet and carnivorous GI tract also raises interest from a research perspective, as recent metagenomic studies indicate that gut physiology, host phylogeny, and diet determine the composition of the intestinal microbiota (Ley et al. 2008*a*, 2008*b*).

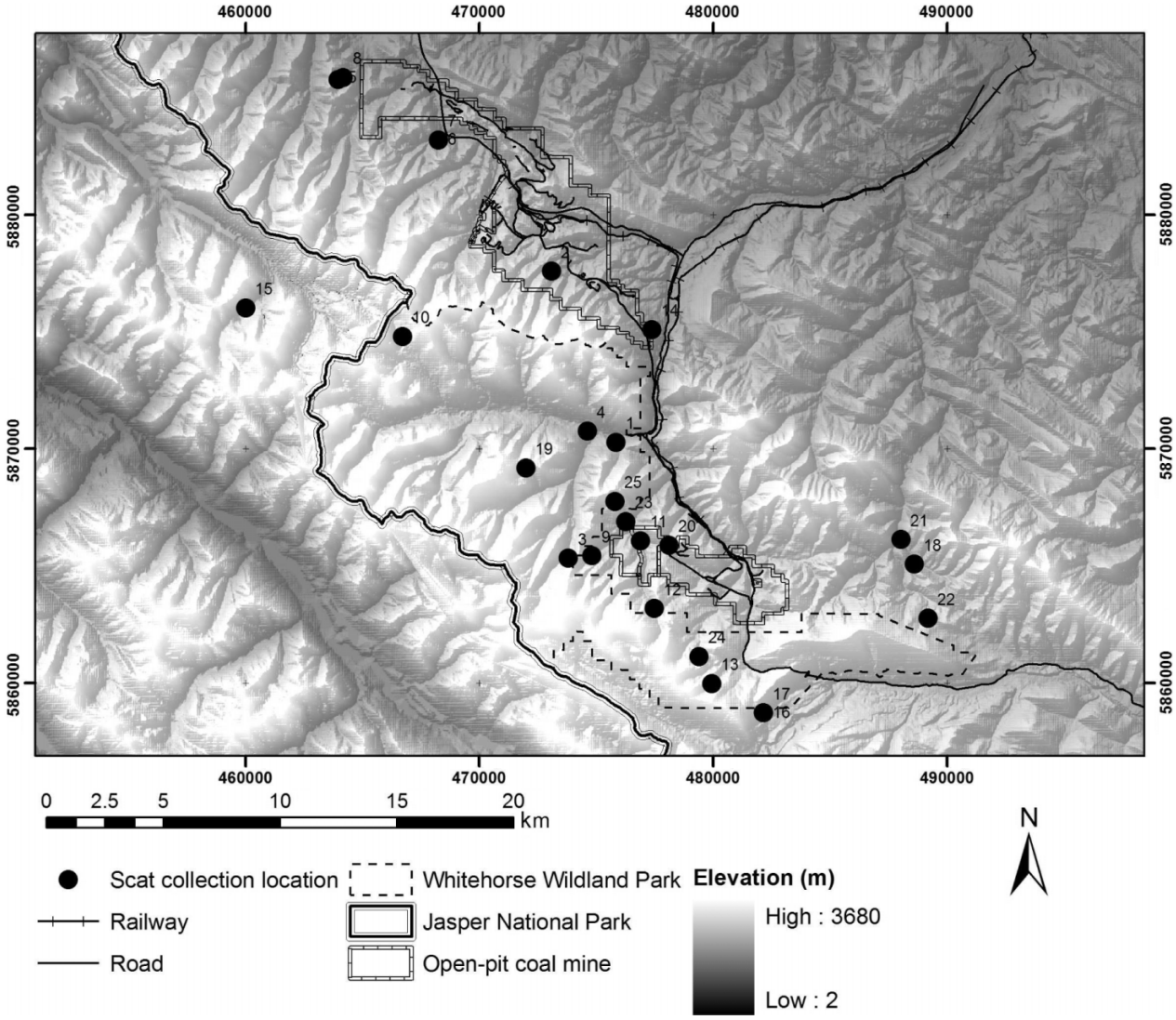
This work characterized the composition of the fecal microflora of an interior grizzly bear population from west-central Alberta in Canada. In this region, grizzly bear habitat is threatened by industrial development (Nielsen et al. 2004, 2006). Fecal samples from free roaming grizzly bears were collected throughout 2008. Samples of captive grizzly bears were obtained from the Calgary Zoo to validate our approach. Culture-dependent and culture-independent methods were employed. Group-specific PCR is a technique commonly used to investigate the composition of complex microflora, and relies on the stable presence of DNA in environmental samples (Lamendella et al. 2008; McCartney 2002; Rajendram et al. 2006; Renter et al. 2006). Lactate and SCFAs were investigated as a link between gut physiology, microbial community, and diet.

Methods

Sampling from wild grizzly bears

The study area was in west-central Alberta, Canada, on the eastern slopes of the Rocky Mountains (53°150′N, 118°300′W; Fig. 1). The vegetation consists of montane, conifer, and subalpine forests, and alpine meadows and shrubs. The highest elevation is 3680 m, and there are rocky peaks, steep mountain sides, and flatter narrow valleys (Achuff 1994). Landscape characteristics and available foraging resources favour the settlement of grizzly bears, whereas black bears are rarely reported in the area where our samples were collected (G.B. Stenhouse, unpublished data). This is not surprising, because spatial landscape partitioning exists between grizzly and black bears (Apps et al. 2006). During the June–November 2008 sampling period, 2 adult grizzly bears (1 male and 1 female) wearing global positioning system (GPS) radiocollars (Televilt, Lindesberg, Sweden) were monitored as part of a larger study on grizzly bear foraging and movement ecology in the region (University of Alberta Animal Care and Use Committee for Biosciences Protocol 552712). Fixed-wing aircraft and helicopter flights were carried out to acquire GPS data from the collars remotely, without the need for bear recapture. Sites used by the bears were visited by field teams after the animals had left the area, and 21 fecal samples (2 mL vials) were collected (Research and Collection Permits 8219, RC08WC002, and JNP-2008-1494). Four additional samples were collected on wildlife trails or at bear root digging sites; these could not be assigned to a specific grizzly bear. The location of each feces deposit (e.g., the bear bedding site)

Fig. 1. Collection sites for fecal samples obtained from free-ranging grizzly bears during June–November 2008 on the eastern slopes of the Rocky Mountains in Alberta. Grid projection is universal transverse mercator (UTM) zone 11.



was recorded in the field, and all sampling sites were marked with a hand-held GPS. Samples were recovered from the inside of the feces using sterile techniques. Fecal material was assessed visually for contents in the field and in the lab. Contents were grouped as prey related (hair, bones, and meat) or as vegetative matter (roots, stems, leaves, and berries).

Sampling from captive grizzly bears

Fecal samples from 1 male and 1 female grizzly bear housed at the Calgary Zoo were obtained with the cooperation of the Zoo (Biological Research Permit 2009-01). These samples were less than 24 h old. The animals were fed their regular diet, consisting of dog chow and fruit (24%–31% protein, 15%–18% fat, and 0.37% fibre).

Determination of lactate and SCFAs using high-performance liquid chromatography

Fecal samples were incubated with 7.5% perchloric acid at 4 °C overnight to remove proteins. Metabolites were separated using an Aminex 87H column (Bio-Rad, Mississauga, Ontario) at a temperature of 70 °C, and the solvent was 5% acetonitrile in 5 mmol·L^{-1} H_2SO_4, at a flow rate of 0.4 mL·min^{-1}. Metabolites were visualized using a UV detector at 210 nm, and identified using external standards. Lactate and SCFAs were analyzed in duplicate. For statistical analysis, Student's *t* test was used.

Qualitative determination of mono-, di-, and polysaccharide content in feces of wild grizzly bears

Sugars were water extracted from freeze-dried feces samples after incubation at 80 °C for 2 h. Mono- and di-saccharides were analysed directly from the extract. To determine pectin, amylose, cellulose, and chitin content, 40 µL of extract was mixed with 40 µL of the respective buffer and digested for 20 h with 2.4 units of pectinase (resuspended in 50 µL of 50 mmol·L^{-1} sodium acetate buffer, pH 4.1, 50 °C), amyloglucosidase (50 mmol·L^{-1} sodium acetate buffer, pH 4.8, 50 °C), cellulase (50 mmol·L^{-1} sodium acetate, pH 5.0, 37 °C), and chitinase (50 mmol·L^{-1} sodium phos-

phate, pH 6.0, 25 °C; all purchased from Sigma). The presence of glucose in the amylase and cellulase digested samples indicated the presence of amylose and cellulose, respectively. The presence of *N*-acetylglucosamine after chitinase treatment indicated the presence of chitin. Xylose, arabinose, galactose, and glucoronic acid indicated pectin content. Monosaccharides were analysed with a CarboPacPA20 column (Dionex, Oakville, Ontario), using water, 200 mmol·L^{-1} NAOH, and 1 mol·L^{-1} sodium acetate as solvents, at a flow rate of 0.25 mL·min^{-1}. Glucose and NAG were separated with the following gradient: 0 min at 6% NAOH and 20 min at 100% NAOH. Pectinase-treated samples were analysed according to the methods of Currie and Perry (2006) with modifications: 0 min at 3% NAOH and 2% sodium acetate, 10 min at 3% NAOH and 2% sodium acetate, and 30 min at 75% NAOH and 17% sodium acetate.

Culture-independent microflora analysis

DNA was isolated from feces with a Qiamp DNA stool mini kit (Qiagen, Mississauga, Ontario), which has been successfully employed before for DNA isolation from fecal microflora (Li et al. 2003). Successful isolation of DNA was verified on an agarose gel. General 16S primers (hda1 and hda2) were used for amplification to verify the absence of PCR inhibitors in the environmental sample (Tannock et al. 2000).

Microflora analysis by group-specific primers using PCR and qPCR

Fecal bacterial composition was analysed in all fecal samples of wild and captive grizzly bears using PCR (feces IDs are summarized in Table 1). Group-specific primers (summarized in Metzler et al. 2009) were used to detect the presence of lactobacilli (forward 5′-AGCAGTAGGGAATCTTCCA-3′, reverse 5′-CACCGCTACACATGGAG-3′), enterococci (forward 5′-CCCTTATTGTTAGTTGCCATCATT-3′, reverse 5′-ACTCGTTGTACTTCCCATTGT-3′), *Bifidobacterium* spp. (forward 5′-TCGCGTCYGGTGTGAAAG-3′, reverse 5′-CCACATCCAGCRTCCAC-3′), Enterobacteriaceae (forward 5′-GTTAATACCTTTGCTCATTGA-3′, reverse 5′- ACCAGGGTATCTAATCCTGTT-3′), *Bacteroides–Prevotella–Porphyromonas* group (forward 5′-GGTGTCGGCTTAAGTGCCAT-3′, reverse 5′-CGGAYGTAAGGGCCGTGC-3′), *Clostridium leptum – Fecalibacterium prausnitzii* subgroup (*Clostridium* genus cluster IV) (forward 5′-GCACAAGCAGTGGAGT-3′, reverse 5′-CTTCCTCCGTTTTGTCAA-3′), and *Clostridium coccoides – Eubacterium rectale* subgroup (*Clostridium* cluster XIVa and XIVb) (forward 5′-AAATGACGGTACCTGACTAA-3′, reverse 5′-CTTTGAGTTTCATTCTTGCGAA-3′). The primer pair Eu-forward 5′-CGGYCCAGACTCCTACGGG-3′ and Eu-reverse 5′-TTACCGCGGCTGCTGGCAC-3′ was used to determine gene copies of the total bacteria domain (Lee et al. 1996). PCR conditions were. 94 °C for 3 min for initial denaturation followed by 34 cycles of 94 °C for 30 s, 60 or 62 °C for 30 s, and 72 °C for 45 s. Bacterial counts in 6 fecal samples from wild grizzly bears (IDs 1, 3, 4, 10, 15, and 16) and in the feces of the 2 captive animals were also quantified with qPCR, using the same primers. A 7500 Fast Real-Time PCR unit (Applied Biosystems, Streetsville, Ontario) was used. The PCR cycle was set to 94 °C for 5 min for initial denaturation followed by 40 cycles at 94 °C for15 s, 62 °C for 15 s, and 72 °C for 30 s. Master mixes (25 μL) contained 12.5 μL Applied Biosystems Fast SYBRGreen master mix, 1 μL DNA, and 0.05 pmol·L^{-1} primer. Melting curve analysis and size determination of amplificates on agarose gels verified amplification of the target fragments. For statistical analysis, Student's *t* test was used.

Culture-dependent microflora analysis

Fecal samples (IDs 1, 3, 4, 10, 15, 16, and Zoo male) were thawed, resuspended (0.02–0.2 g) in 900 μL of peptone water, vortexed, and shaken at room temperature for 1 h. Large particles were removed by centrifugation at low speed, and the supernatant was serially diluted in peptone water. Samples were plated on PCA, BHI, APT, m-Enterococcus, and Lactobacillus-MRS agar plates (all BD Biosciences, Mississauga, Ontario). Plates were incubated aerobically and anaerobically at 37 °C for 48 h. Three morphologically different isolates were picked from plates preferably representing cell counts of 10^7 CFU·g^{-1} and higher, unless otherwise indicated. DNA was isolated using a DNeasy Blood & Tissue kit (Qiagen). Identical isolates were identified by RAPD-PCR employing primers Box2AR (5′-ACGTGGTTTGAAGAGATTTCG-3′) and GTG5 (5′-GTGGTGGTGGTGGTG-3′) (Koeuth et al. 1995; Versalovic et al. 1994). For sequencing, a partial sequence of the 16S rRNA gene was amplified using primers 616V (5′-AGAGTTTGATYMTGGCTC-3′) and 630R (5′-CAKAAAGGAGGTGATCC-3′) (Loy et al. 2002) or 616Vcont (3′-CGTGAGTGATGAAGGCTTTC-5′) and 630Rcont (3′-CGGTGTGTACAGGCCCG-5′). All PCR master mixes contained 5 μL of PCR buffer, 1.5 mmol·L^{-1} $MgCl_2$, 1.5 U of *Taq* DNA polymerase, 10 nmol·L^{-1} dNTPs (all Invitrogen, Burlington, Ontario), 0.5 pmol·L^{-1} primer, and 1 μL of DNA. Amplicons were sequenced by the DNAcore unit of the Bioscience Department (University of Alberta, Edmonton, Alberta) or by MacrogenUSA. For strain identification, sequences of at least 700 bp were blasted against the type strains of the Ribosomal Database Project (http://rdp.cme.msu.edu/).

Results

Sampling from wild grizzly bears

Feces were collected in areas where radiocollared bears had spent several hours to ensure that all feces collected came from grizzly, not black, bears. Sample locations are indicated in Fig. 1. Of 25 samples, 21 could be assigned to the male or female radiocollared animal. Sample characteristics are summarized in Table 1. Half of the fecal samples (n = 12) were collected from bear bedding sites, 8 samples were collected from root digging or grazing sites, and 2 were collected from sites where bears had been feeding on ungulate carcasses. Three samples were collected on trails on the way to identified microsites. With the exception of 2 sites, 1 sample was collected per site. The feces obtained from the same site (IDs 1 and 7, 11, and 12) differed in compositional matter or freshness. Fecal content was vegetative (stems, leaves, roots, and berries) or originated from prey (hair, bones, and meat). Thirteen samples contained stems,

Table 1. Deposition month, bear sex, site, elevation, visible fecal content, qualitative analysis of fibre content, and bacterial groups present in wild grizzly bear feces collected in 2008 on the eastern slopes of the Rocky Mountains in Alberta.

						Fibre content[b]			Bacterial groups present within scat samples[c]						
ID	Deposition month[a]	Sex	Site	Elevation (m)	Visible fecal content	Cellulose	Amylose	Pectin	Enterococci	Lactobacilli	Enterobacteriaceae	Bifidobacteria	BPP	CL	CC
Zoo	March	M	Housing	–	–	–	–	–/+	+	–	+	+	+	+	+
Zoo	March	F	Housing	–	–	–	–	–/+	+	–	+	–	+	–	+
1	June	F	Root dig	1676	S, L	–	–	–	+	+	+	–	+	–	–
2	June	F	Ungulate carcass	1771	R, m, h	–	+	–	+	–	–	–	–	–	–
3	July	M	Bed	1922	S, L	+	+	–	+	–	–	–	–	–	–
4	July	M	Bed	1686	S, L	+	–	–	+	–	–	–	+	–	–
5	July	F	Bed	1702	S, L	+	+	–	+	+	+	–	+	–	–
6	July	F	Root dig, grazing	1556	R, S, L	+	+	–	+	–	+	+	+	–	+
7	July	F	Root dig grazing	1556	R, S, L	+	+	+	+	–	+	+	+	–	+
8	July	F	Bed	1628	S, h	+	+	–	–	–	+	+	+	–	+
9	July	F	Grazing	1905	S, L	+	–	+	+	+	+	–	+	–	–
10	July	?	Wildlife trail	2053	h, b, m	+	+	–	+	+	+	–	+	–	+
11	July	?	Wildlife trail	1785	R, S	–	+	–	–	–	–	–	+	–	–
12	August	M	Root dig	1962	R	+	+	+	+	–	+	–	+	–	–
13	August	M	Bed	2069	S, L	+	+	+	+	–	–	–	+	–	–
14	August	F	Wildlife trail	1616	L, h	+	+	–	+	+	+	–	+	–	–
15	August	?	Root dig	1880	R, S	–	+	+	+	+	–	–	+	+	+
16	September	M	Bed	1944	R, S, B	+	+	+	+	+	+	–	–	–	–
17	September	M	Bed	1944	R, S, B, h	+	–	+	+	+	+	–	–	–	+
18	September	M	Bed	1682	R, L, B	+	+	+	+	–	–	–	–	–	–
19	September	F	Bed	2140	L, B, h	+	+	+	+	–	–	–	+	–	+
20	September	F	Root dig	1907	L, B	+	+	+	+	+	+	–	–	–	–
21	October	M	Bed	1671	R, h	+	+	–	+	+	+	–	–	–	+
22	October	M	Ungulate carcass	1814	m, h	+	+	–	+	+	–	–	–	–	–
23	October	F	Bed	1786	R, B	+	+	+	+	–	–	–	–	–	–
24	October	?	Root dig	2028	R	+	+	+	+	–	–	–	–	–	–
25	November	F	Bed	2012	R, S, L	+	+	+	+	–	–	–	–	–	–

Note: +, present; –, absent; BPP, *Bacteroides–Prevotella–Porphyrmonas* group; CL, *Clostridium leptum* cluster; CC, *Clostridium coccoides* cluster; S, stems; L, leaves; R, roots; B, berries; m, meat; h, hair; b, bones.

[a]Known from tracking of radiocollared grizzly bears. For feces collected along wildlife trails or at root digging sites, from unknown bears, the deposition date was estimated from visual inspection of the scat condition (i.e., dryness, colour, smell).

[b]Determined by monosaccharide composition after digestion with cellulase, amylase, and pectinase. No chitin was detected in any scat sample.

[c]Visible amplification products on agarose gels after 34 PCR cycles.

leaves, and roots, and 6 samples consisted of meat, hair, and bones, in addition to vegetative matter. Four samples contained roots, leaves, and berries. Berries, vegetative matter, and prey digest were present in 2 samples.

Metabolite content in fecal samples of wild and captive grizzly bears

Butyrate was detectable in all samples obtained from wild grizzly bears, and isobutyrate was present in 23 of 25 feces samples. Lactate, acetate, and isovaleric acid were detected in 88% of the samples. Butyrate (average 50.5 mmol·kg^{-1}, median 31.2 mmol·kg^{-1}) was the most abundant SCFA, as indicated in Fig. 2, followed by acetate (average 42.7 mmol·kg^{-1}). Lactate was present at an average of 70.0 mmol·kg^{-1} and a median of 16.7 mmol·kg^{-1}. Average contents of butyrate in fecal samples containing hair and meat, vegetative matter, or berries were not significantly different (48.4, 57.6, and 46.9 mmol·kg^{-1}, respectively). The amount of lactate in feces samples containing berries was significantly higher than in samples with vegetative matter (281.3 versus 36.0 mmol·kg^{-1}, $p < 0.005$) or prey (281.3 versus 25.6 mmol·kg^{-1}, $p < 0.05$). Feces with hair and meat contained significantly ($p < 0.05$) higher amounts of valerate (67.7 mmol·kg^{-1}) than feces with vegetative matter (2.6 mmol·kg^{-1}). Two feces samples (IDs 17 and 19) were omitted from statistical analysis of various feed groups because they contained prey compounds and berries, in addition to vegetative matter. Acetate (average 50.5 mmol·kg^{-1}) was the most abundant SCFA in feces of captive grizzly bears followed by butyrate (average 44.0 mmol·kg^{-1}) and propionate (average 40.2 mmol·kg^{-1}).

Qualitative determination of mono-, di-, and polysaccharides present in feces of wild grizzly bears

Mono- and di-saccharides were not detectable in 22 of 25 fecal samples obtained from free roaming grizzly bears (Table 1). Three samples contained traces of arabinose or xylose. The majority of samples contained cellulose and amylose. Pectin was detected in 7 of 17 samples. Pectin was first observed in samples obtained in July, and was present in all 5 samples collected in September. No chitin was detected. The feces of captive grizzly bears did not contain mono- or di-saccharides, amylose, cellulose, or chitin. Traces of xylose and galactose, but not glucoronic acid, were present after pectinase treatment.

Culture-independent microflora analysis

DNA was successfully isolated from each feces sample and amplified by PCR using group-specific primers. Amplicons verified the presence of enterococci, Enterobacteriaceae, bifidobacteria, the *Bacteroides–Prevotella–Porphyrmonas* group, and the *C. coccoides* and *C. leptum* clusters in the feces of wild and captive grizzly bears, as indicated in Table 1. Lactobacilli were only detected in the feces of wild grizzly bears. The highest occurrence within the fecal matter of wild grizzly bears was observed for enterococci, which were detectable in 23 of 25 samples. The presence of *Enterobacteriacae* and the *Bacteroides–Prevotella–Porphyrmonas* group could be confirmed in 13 and 14 samples, respectively. The *C. coccoides* cluster was identified in 8 samples, and bifidobacteria and the *C. leptum* cluster was identified in 3 and 1 sample, respectively. Enterobacteriaceae, enterococci, the *Bacteroides–Prevotella–Porphyrmonas* group, and the *C. coccoides* cluster were detected in the feces of female and male grizzly bears; bifidobacteria and the *C. leptum* cluster were only present in the male fecal samples.

Quantitative analysis of fecal microflora

The microflora of feces IDs 3, 4, and 16 (wild male), 1 (wild female), and 10 and 15 (unidentified animals) and of captive animals were analysed with qPCR to determine the quantitative composition of the fecal microbiota of at least 4 different animals (results are shown in Table 2). Average total gene copies were log 9.1 ± 0.5 gene copies·g^{-1} and log 9.2 ± 0.3 gene copies·g^{-1} for wild and captive animals, respectively. Among the strict anaerobic microflora, the *Bacteroides–Prevotella–Porphyrmonas* group was the most dominant (log 7.6 ± 0.8 gene copies·g^{-1}) in the feces of wild bears. Gene copies of the *C. coccoides* cluster, the *C. leptum* cluster, and the bifidobacteria were significantly lower ($p < 0.001$) at log 5.4 ± 1.3 gene copies·g^{-1}, log 5.4 ± 1.2 gene copies·g^{-1}, and log 5.9 ± 1.2 gene copies·g^{-1}, respectively. Enterococci and Enterobacteriaceae were the most prominent of the facultative anaerobes in the feces of wild bears at log 8.4 ± 0.8 and log 8.2 ± 1.2 gene copies·g^{-1}, respectively, and counts of lactobacilli were significantly lower at log 5.6 ± 1.0 gene copies·g^{-1} ($p < 0.001$). Enterococci were present in significantly higher counts than the *Bacteroides–Prevotella–Porphyrmonas* group ($p < 0.05$). The Enterobacteriaceae were the most prominent group present in the feces of the captive male and female grizzly bears and represented 29% and 73% of the fecal bacterial population, respectively. The *Bacteroides–Prevotella–Porphyrmonas* group and the *C. coccoides* cluster were the most prominent among the strict anaerobes; bifidobacteria were not detected.

Culture-dependent microflora analysis

Facultative anaerobes present in feces IDs 3, 4, and 16 (wild male wild), 1 (wild female), and 10 and 15 (unidentified animals) and the captive male were also identified by cultivation to determine the presence of bacterial groups not targeted with the primers used in qPCR. Isolated strains are shown in Table 3. Cell counts obtained from the feces of wild animals with the different media were log 8.4 ± 0.8 CFU·g^{-1} for aerobically incubated PCA, log 8.7 ± 2.0 CFU·g^{-1} for anaerobically incubated PCA, log 8.0 ± 2.5 CFU·g^{-1} for m-Enterococcus, log 7.6 ± 1.4 CFU·g^{-1} for LacMRS, and log 7.2 ± 1.1 CFU·g^{-1} for APT. CFUs in the feces of captive animals were log 8.5 CFU·g^{-1} for anaerobically incubated PCA, log 7.4 CFU·g^{-1} for m-Enterococcus, and log 8.7 CFU·g^{-1} for LacMRS. Colonies on BHI and Nutrient agar were difficult to enumerate because of slimy growth. The media used were not specific. m-Enterococcus agar harboured enterococci, streptococci, and vagococci. Enterococci and vagococci were recovered from all media. Bacilli grew on Nutrient agar, BHI, and PCA. Enterobacteriaceae were recovered from Nutrient agar and staphylococci were recovered from anaerobically incubated APT, BHI, and LacMRS (log > 7 CFU·g^{-1}). In total, 151 colonies were purified, distinguished using RAPD PCR, and identified by sequencing, as summarized in Table 3. Enterococci were detected in the feces of wild and captive animals. Staphylo-

Fig. 2. Metabolite contents in the feces of wild and captive grizzly bears. Metabolite contents present in all fecal samples of wild grizzly bears (A), and in wild grizzly bear feces containing vegetative matter (stem, leaves, and roots) (B), vegetative matter and berries (C), or vegetative and animal matter (hair, meat, and bones) (D) are displayed as box plots. Shown are the 5th and 95th percentiles. Average values are indicated by diamonds. Circles represent outliers. Metabolite contents in the feces of captive grizzly bears are depicted by squares in Fig. 2A. Metabolite content was determined by high-performance liquid chromatography.

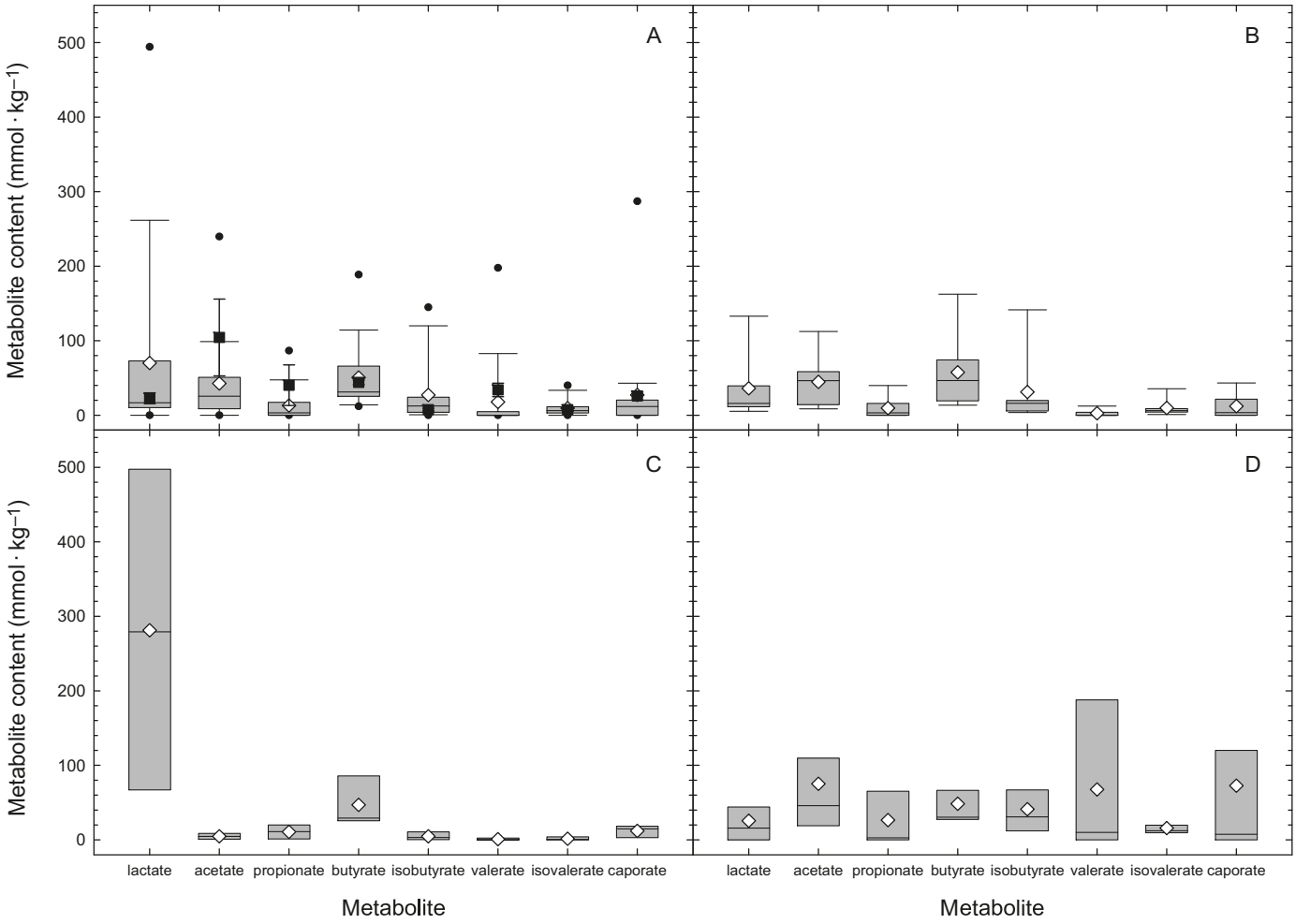


Table 2. Group-specific quantitative PCR (log gene copies·g^{-1}) of enterococci, lactobacilli, Enterobacteriaceae, the *Bacteroides–Prevotella–Porphyrmonas* group (BPP), bifidobacteria, the *Clostridium leptum* cluster (CL), and the *Clostridium coccoides* cluster (CC) present in fecal samples of wild (IDs 1, 3, 4, 10, 15, and 16) and captive grizzly bears (n = 2–4).

Sex (feces ID)	Total bacteria	Enterococci	Lactobacilli	Enterobacteriaceae	BPP	Bifidobacteria	CL	CC
Wild animals								
Male (3)	8.4±0.4	7.7±0.1	5.7±0.2	7.8±0	8.0±0.1	6.1±0.6	5.0±0.3	4.5±0.5
Male (4)	9.4±0	9.3±0	3.5±1.6	nd	7.0±0.1	3.5±0.1	nd	nd
Male (16)	8.8±0.1	7.3±0.2	5.6±0.4	9.5±0.3	7.8±0.4	7.1±0.7	4.9±0.2	3.6±0.2
Female (1)	9.6±0.1	9.4±0	6.5±0.1	9.0±0.7	7.9±0.1	7.0±0.2	3.6±0.6	4.9±1.0
Unknown (10)	8.7±0.2	8.4±0.2	6.1±0.2	6.8±0.7	6.5±0.2	6.3±0.8	5.9±0.3	6.7±0.8
Unknown (15)	9.7±0.1	8.5±0.2	5.8±0.1	7.7±0.3	8.5±0.4	5.1±0.5	7.0±0.3	6.2±1.0
Mean	9.1±0.5	8.4±0.8	5.6±1.0	8.2±1.2	7.6±0.8	5.4±1.2	5.4±1.3	5.9±1.2
Captive animals								
Male	8.9±0.1	7.7±0.5	nd	8.4±0.1	7.1±0.7	5.1±0.2	5.3±0.2	6.2±0.5
Female	9.5±0.1	7.4±0.2	nd	9.4±0.2	6.2±0.9	nd	5.1±0.1	7.4±0.5
Mean	9.2±0.3	7.6±0.3	nd	8.7±0.5	6.7±0.9	—	5.2±0.2	6.8±0.8

Note: nd, not detected.

Table 3. Facultative anaerobes isolated from wild (IDs 1, 3, 4, 10, 15, and 16) and captive grizzly bear feces.

Sex (ID)	Isolates	Enterococci/streptococci	Staphylococci	Bacilli	Enterobacteriaceae
Wild animals					
Male (3)	19	*Enterococcus hirae* (100%) *E. canintestini/dispar* (99%) *Enterococcus* spp. I	nd	*Bacillus muralis* (98.4%) *B. pumilis* (98.0%) *Bacillus* spp. IV	nd
Male (4)	23	*E. hirae* (100%) *E. canintestini/dispar* (100%) *Enterococcus* spp. II	nd	nd	*Escherichia albertii* (100%)
Male (16)	18	*E. faecium* (100%)	*Staphylococcus hominis* (98.3%)	*B. muralis* (98.7%) *Paenibacillus amylolyticus* (97.7%)	*E. albertii* (100%)
Female (1)	21	*E. caccae/silesiacus* (100%) *E. canintestini/dispar* (99.5%) *Vagococcus lutrae* (99.4%)	*S. warneri* (100%)	*Bacillus* spp. V	nd
Unknown (10)	26	*E. hirae* (99.1%) *E. faecium* (97.5%) *E. mundtii* (99.9%)	nd	*Bacillus* spp. VI	nd
Unknown (15)	18	*E. hirae* (100%) *E. avium* (100%) *Enterococcus* spp. III	nd	*Bacillus* spp. VI	nd
Captive animals					
Male	26	*E. hirae* (98.9%) *E. faecium* (100%) *Streptococcus lutetiensis/infantis* (98.9)	nd	*Viridbacillus arvi* (98.2%)	nd

Note: Percent homology to Ribosomal Database Project (http://rdp.cme.msu.edu/) type strains given in parentheses. I, *E. ratti* (96.4%); II, *E. termiti* (95.2%); III, *E. malodurans* (96.4%); IV, *B. niacini* (92.4%); V, *P. amylolyticus* (95.4%); VI, *B.muralis* (93.8%); VII, *B. benzoevorans* (95.3%). Clonal isolates were identified using RAPD PCR. The 16S rRNA gene was partially sequenced and sequences were blasted against the type strain collection of the Ribosomal Database Project. nd, not detected.

cocci and Enterobacteriaceae were recovered from 2 feces samples of wild animals. Bacilli, paenibacilli, brevibacilli, and viridibacilli were present in 6 of 7 analysed feces.

Discussion

This study is the first report on the composition of the fecal microflora of wild grizzly bears. An approach combining culture-dependent and culture-independent methods was employed to investigate the aerobic, facultative, and strict anaerobic fecal microbiota of grizzly bear fecal samples collected from June to November 2008 on the eastern slopes of the Rocky Mountains. Because fresh samples of a wild species like the grizzly bear are difficult to obtain without major disturbances to the animals, we relied on the stability of bacterial DNA in fecal samples, most of which were between 2 days and 3 weeks old. The feces of captive grizzly bears less than 24 h old were included in this study for comparison and method validation. The successful amplification of the 16S rRNA genes from all samples, the conformity of results using culture-dependent and culture-independent methods, and the stability of bacterial counts in fecal samples obtained at different points in time from different locations and from different animals support the validity of our approach. Samples contained no or only traces of fermentable sugars, minimizing the risk of external contamination.

Total bacterial counts in wild grizzly bear fecal material were log 9.1 ± 0.5 gene copy numbers·g^{-1}. Counts in samples of captive animals were comparable at log 9.2 ± 0.3 gene copy numbers·g^{-1}. Facultative anaerobes Enterobacteriaceae, enterococci, bacilli, and staphylococci outnumbered the strict anaerobes. The fecal microflora of wild and captive grizzly bears showed similarity to fecal microbiota of the giant panda (*Ailuropoda melanoleuca*), which contains approximately log·9 CFU·g^{-1}, and was dominated by facultative anaerobes Enterobacteriaceae, streptococci, and enterococci (Hirayama et al. 1989; Wei et al. 2007). Because of the predominance of facultative anaerobes and generally lower bacterial fecal counts, the fecal microflora of grizzly bears and pandas more closely resembles the ileal than the fecal microflora of pigs, dogs, and humans (Hao and Lee 2004; Mentula et al. 2005; Metzler et al. 2009). The apparent likeness of grizzly bear and giant panda fecal microflora to the small intestine microbiota of omnivores most likely reflects the distinctive carnivorous GI system, which consists of a long small intestine and a short undeveloped colon (Stevens and Hume 1995).

In this study, Enterobacteriaceae and enterococci were identified as the dominant microbial groups in grizzly bear feces by qPCR and cultivation of facultative anerobes. Enterococci are natural inhabitants of the GI tract of mammals and humans, and were first isolated from black bear feces in 1963 (Köhler 2007; Mundt 1963). Goatcher et al. (1987) characterized the culturable aerobic microflora of rectal, vaginal, and nasal grizzly bear swabs, and also identified Enterobacteriaceae (*Escherichia coli*, *Citrobacter*, *Enterobacter*, and *Proteus*) beside plant-associated bacteria (*Erwinia*, *Xanthomonas*, *Gluconobacter–Acetobacter*, *Rhizobium*, and *Agrobacterium*).

Five of the 6 cultured fecal samples of wild bears contained facultative anaerobic bacilli and paenibacilli. *Paenibacillus amylolyticus*, *Bacillus pumilis*, and the isolated *Bacillus* spp. are air or soil associated. *Bacillus muralis* was initially isolated from mural paintings and was recovered from the hindgut of insects (Cook et al. 2007; Felske 1999; Heyrman et al. 2005; Nagel and Andreesen 1991; Shida et al. 1997). The close connection of the identified bacilli and paenibacilli to soil raises questions about their origins. In this study, samples were always taken from the inside of the feces, where there was no contact between the sample and the soil. Bacilli and paenibacilli could be taken up with plant and root materials, and could survive passage through the grizzly bear GI tract. Ingested soil is frequently recovered from wildlife feces (Beyer et al. 1994). Mattson et al. (1999) observed the deliberate uptake of soil by grizzly bears (geophagy), and postulated that soil was consumed as an anti-diarrheal. Soil-related and undefined bacilli were also present in fresh feces of wild otter and chimpanzees, respectively (Oliveira et al. 2008; Uenishi et al. 2007). The presence of *Viridibacterium arvi* at more than log 8 CFU·g^{-1} in the fresh feces of a captive animal further indicates that the isolated bacilli originated from the GI tract. Lactobacilli were only detected in the feces of wild grizzly bears. In humans, most lactobacilli isolated from feces originate from fermented foods and the oral cavity, and only transit the gut. A possible correlation between food intake and the fecal presence of lactobacilli might account for the differences observed in wild and captive grizzly bears (Dal Bello and Hertel 2006; Tannock et al. 2000; Walter 2008).

The prevalence of Enterobacteriaceae and enterococci not capable of digesting complex carbohydrates indicates inefficient feed utilization. Grizzly bears feed on a diet consisting of animal protein, plants (roots, forbs, grasses, and berries), and plant concentrates, such as seeds, with changing priority from spring to fall (Gau et al. 2002; Munro et al. 2006). The presence of cellulose and amylose in a majority of the samples confirmed that dietary fibres constituted a large part of grizzly bear food intake (Munro et al. 2006). In contrast to ruminants or swine, grizzly bears and pandas do not harbour cellulolytic or hemicellulolytic bacterial species, and can only poorly digest cellulose and hemicellulose (Dierenfeld et al. 1982; Schwartz et al. 2003; Varel and Yen 1997). Within the dominant facultative anaerobic microbial community, polymer degradation has been only reported for bacilli and paenibacilli (Cook et al. 2007).

SCFA formation depends on gut physiology, bacterial microflora, and substrate availability. Cecum and the proximal colon, which are predominantly colonized by strict anaerobes, are the main sites of fermentation in omnivores (Bergman 1990). SCFAs are absorbed during the passage; the amount of SCFAs, and especially butyrate, in feces decreases with increasing colonic transit time (Topping and Clifton 2001; Wong et al. 2006). Reported transit times of 7 and 13 h for vegetative matter (clover) and meat, respectively, in the grizzly bear GI tract indicate that retention time is limited and therefore the opportunity for microbial fermentation is reduced (Pritchard and Robbins 1990). The presence of SCFAs in the feces of grizzly bears, nevertheless, verified intestinal microbial activity. Generally, acetate is the most abundant SCFA in mammal feces, and molar ratios between 75:15:10 and 40:40:20 for acetate–propionate–butyrate have been reported (Alvaro et al. 2007; Delgado et

al. 2006; Metzler et al. 2009; Meijer-Severs and van Santen 1989; Swanson et al. 2002; Wong et al. 2006). A ratio of 76:4:19 was observed in the feces of a brown bear fed an unspecified diet (McKay and Eastwood 1983). In this study, a ratio of 55:21:23 was detected in feces from captive animals fed a regular protein-rich diet. In contrast, the molar ratios of SCFAs differed after the intake of various feeds in the feces of wild animals. A molar ratio of 50:18:32 in wild bear feces containing prey resembled the ratios reported for other mammals more than feces composed of vegetative matter (40:9:51) or berries (7:17:75). Total SCFA contents of 82, 168, and 347 mmol·kg^{-1} in wild animal feces consisting of berries, plant material, and animal matter, respectively, and of 263 mmol·kg^{-1} in the feces of captive animals were comparable to total SCFA contents reported for various mammals (Von Engelhardt et al. 1989). Remarkably, lactate was routinely detected in grizzly bear feces. In omnivores and dogs, lactate is metabolized in the cecum, and only traces are detected in the feces (Alvaro et al. 2007; Banta et al. 1979; Clemens et al. 1975; Delgado et al. 2006; Meijer-Severs and van Santen 1989; Metzler et al. 2009; Swanson et al. 2002). During the passage through the GI tract of raccoons (*Procyon lotor*), however, which also possess the simple GI system of a carnivore, lack a cecum, and are fast digesters, no lactate metabolism occurs, and amounts remain stable throughout the GI tract (Clemens and Stevens 1979). A direct correlation between diet, lactate, and SCFA content was observed. Berry consumption increased fecal lactate content, whereas meat and hair in the feces correlated to increased amounts of valerate. Fruits contain easily digestible carbohydrates, which are preferably fermented to lactate by the facultative anaerobes.

In conclusion, the fecal bacterial populations characterized in this study were comparable to a species with largely similar gut physiology, the giant panda. Grizzly bears and giant pandas feed on different diets, but have a comparable fecal microflora dominated by facultative anaerobes, suggesting that gut physiology is the primary determinant of bear GI microbiota. This hypothesis is strengthened by results obtained in the metagenomic studies of Ley et al. (2008*a*, 2008*b*), which pointed out that members of the *Ursuid* family contain a similar "carnivorous microflora," independent of their feed source. The presence of SCFAs as the result of microbial fermentation suggests that the intestinal microbiota contribute to energy maintenance in grizzly bears. This study brings insights into the influence of intestinal microflora on the nutrition of wild and grizzly bears.

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