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RESEARCH ARTICLE

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Comparative genomics of *Bifidobacterium* species isolated from marmosets and humans

Celeste J. Brown[1,2,3] | Dorah Mtui[1] | Benjamin P. Oswald[1,3] | James T. Van Leuven[2] | Eric J. Vallender[4*] | Nancy Schultz-Darken[5] | Corinna N. Ross[6,7] | Suzette D. Tardif[6] | Steven N. Austad[8†] | Larry J. Forney[1,3]

[1]Department of Biological Science, University of Idaho, Moscow, Idaho

[2]Center for Modeling Complex Interactions, University of Idaho, Moscow, Idaho

[3]Institute for Bioinformatics and Evolutionary Studies, University of Idaho, Moscow, Idaho

[4]New England Primate Research Center, Harvard Medical School, Southborough, Massachusetts

[5]Wisconsin National Primate Research Center, University of Wisconsin, Madison, Wisconsin

[6]Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, Texas

[7]Department of Science and Mathematics, Texas A&M University, San Antonio, Texas

[8]Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, Texas

Correspondence
Celeste J. Brown, Department of Biological Sciences, University of Idaho, 875 Perimeter Drive MS3051, Moscow, Idaho.
Email: celesteb@uidaho.edu

Present address
*Eric J Vallender, University of Mississippi Medical Center, Jackson, MS 39216; Tulane National Primate Research Center, Covington, LA.
†Steven N Austad, Department of Biology, University of Alabama at Birmingham, Birmingham, AL

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Abstract

The genus *Bifidobacterium* is purported to have beneficial consequences for human health and is a major component of many gastrointestinal probiotics. Although species of *Bifidobacterium* are generally at low relative frequency in the adult human gastrointestinal tract, they can constitute high proportions of the gastrointestinal communities of adult marmosets. To identify genes that might be important for the maintenance of *Bifidobacterium* in adult marmosets, ten strains of *Bifidobacterium* were isolated from the feces of seven adult marmosets, and their genomes were sequenced. There were six *B. reuteri* strains, two *B. callitrichos* strains, one *B. myosotis* sp. nov. and one *B. tissieri* sp. nov. among our isolates. Phylogenetic analysis showed that three of the four species we isolated were most closely related to *B. bifidum*, *B. breve* and *B. longum*, which are species found in high abundance in human infants. There were 1357 genes that were shared by at least one strain of *B. reuteri*, *B. callitrichos*, *B. breve*, and *B. longum*, and 987 genes that were found in all strains of the four species. There were 106 genes found in *B. reuteri* and *B. callitrichos* but not in human bifidobacteria, and several of these genes were involved in nutrient uptake. These pathways for nutrient uptake appeared to be specific to *Bifidobacterium* from New World monkeys. Additionally, the distribution of *Bifidobacterium* in fecal samples from captive adult marmosets constituted as much as 80% of the gut microbiome, although this was variable between individuals and colonies. We suggest that nutrient transporters may be important for the maintenance of *Bifidobacterium* during adulthood in marmosets.

KEYWORDS
Bifidobacterium, genomics, humans, marmosets

1 | INTRODUCTION

Members of the genus *Bifidobacterium* are purported to have beneficial consequences for human health (Backhed, Ley, Sonnenburg, Peterson, & Gordon, 2005; Guarner & Malagelada, 2003; Nowak, Paliwoda, & Blasiak, 2018; Tojo et al., 2014). Currently, GenBank Taxonomy recognizes 71 species of *Bifidobacterium* that have been isolated from the gastrointestinal tracts of mammals, birds and social insects as well as human-impacted environments (Bottacini, Ventura, van Sinderen, & O'Connell Motherway, 2014; Killer et al., 2010; Michelini, Modesto et al., 2016; Milani et al., 2017). Bifidobacteria are noted for their ability to degrade ingested polysaccharides and proteins, and to synthesize vitamins and other nutrients that are beneficial to their hosts (Coakley et al., 2006; Kiyohara et al., 2012; Lugli et al., 2018; Milani et al., 2016; Milani, Lugli et al., 2015; O'Connell Motherway, Kinsella, Fitzgerald, & van Sinderen, 2013; Pompei et al., 2007). Species that are often found in the human gut microbiome have been intensively studied and are transmitted from mothers to infants where they occur at relatively high frequency until weaning. The bifidobacteria of the infant gut are proposed to play crucial roles in protecting infants against pathogenic bacteria, contributing to priming the mucosal immune system, and consequently protecting against susceptibility to diverse diseases later in life (Eckburg et al., 2005; Fukuda et al., 2011; Lee & O'Sullivan, 2010; Marco, Pavan, & Kleerebezem, 2006; O'Hara & Shanahan, 2007; Schell et al., 2002). After weaning, the frequencies of different species of bifidobacteria change over an individual's life span (Bäckhed et al., 2015; Stewart et al., 2018; Turroni et al., 2012) and while their absolute abundance remains constant, their relative abundance decreases to around 4% due to colonization by other bacteria (Bäckhed et al., 2015; Milani, Mancabelli et al., 2015; Stewart et al., 2018; Tanaka & Nakayama, 2017; Turroni et al., 2012; Turroni et al., 2018).

Comparative genomics studies of members of the genus *Bifidobacterium* have provided valuable information regarding the characteristics that are unique to, shared between, or distinguish *Bifidobacterium* species from other gut microbiota. These characteristics likely evolved as a consequence of interspecies interactions among bacteria and adaptation to the host environment (Bottacini et al., 2018; Bottacini, O'Connell Motherway et al., 2014; Lee & O'Sullivan, 2010; Lugli et al., 2018; Schell et al., 2002; Sela et al., 2008). In view of what is known about the role of *Bifidobacterium* species in animal health, comparisons between species with different host specificity can provide information relevant to understanding adaptation to the host environment.

Species of *Bifidobacterium* have been isolated from the feces of adult and infant common marmosets (*Callithrix jacchus*; Endo, Futagawa-Endo, Schumann, Pukall, & Dicks, 2012; Lugli et al., 2017; Michelini et al., 2015; Michelini, Oki et al., 2016; Modesto et al., 2014; Toh et al., 2015). As found in humans (Stewart et al., 2018; Yatsunenko et al., 2012), the relative abundance of bifidobacteria is highly variable between individuals and populations. However, unlike humans the abundance of *Bifidobacterium* in marmosets can remain quite high well into adulthood (Ross et al., 2017). Genomic comparisons between *Bifidobacterium* isolated from marmosets and those isolated from humans may provide information that could be exploited in the development of probiotic strains of bifidobacteria that persist in high numbers in adult humans.

In this study, we tested the hypothesis that bifidobacteria from adult marmosets have genes that are not found in closely related bifidobacteria from humans. To do so, we isolated bifidobacteria from adult animals and determined their genome sequences. We proceeded to identify protein coding sequences from the genomes of these isolates that were not found in bifidobacteria that reside in humans, several of which were components of nutrient transport systems.

2 | METHODS

2.1 | Biological sources

The *Bifidobacterium* strains characterized in this study were originally isolated from fecal samples of adult common marmosets (*Callithrix jacchus*) raised in either barrier conditions at the University of Texas Health Science Center San Antonio (UTHSCSA) or a conventional colony at Southwest National Primate Research Center (SNPRC; Ross et al., 2017; Table 1). To determine whether the high abundance of *Bifidobacterium* in adult marmosets was a common phenomenon, additional fecal samples from marmosets over 1 year of age were provided by the SNPRC ($n = 34$), the Wisconsin National Primate Research Center (WNPRC; $n = 45$) and the New England Primate Research Center (NEPRC; $n = 43$). Animals were housed in family groups and underwent routine health and prevention procedures. They had free access to water. The UTHSCSA barrier colony of marmosets receives irradiated Teklad purified marmoset diet with Harlan irradiated primate enrichment mix and no other dietary enrichment items. SNPRC provides a mix of Teklad Purified and Mazuri Regular diets to their conventional colony. NEPRC provides a mix of Teklad NWM and ZuPreem diets, and WNPRC provides the Mazuri High Fiber diet. Each of the conventional colonies provide different combinations of protein sources (eggs, peanuts, garbanzo beans, yogurt or cottage cheese), fruits, vegetables, and sweets ad libitum.

2.2 | Analysis of microbial communities by 16S rRNA gene sequencing

The first step toward obtaining *Bifidobacterium* isolates for genomic sequencing was to identify fecal samples that had high proportions of bifidobacteria. The composition of bacterial communities in all fecal samples were analyzed by sequencing the V1-V3 region of 16S rRNA genes that had been amplified from each sample as follows. Fecal samples were taken from marmosets by inserting a mini e-swab

TABLE 1 Descriptions of marmosets sampled, *Bifidobacterium* species, and genome sequences

ID	Sex	Colony[a]	Isolate	Coverage[b]	Putative species	# of Contigs	Genome size (Mb)	CDS[c]	rRNA[d]	tRNAs
A	Female	Barrier	UTBIF-56	336	*B. reuterii*	63	2.75	2063	4	51
B	Female	Barrier	UTBIF-68	264	*B. reuterii*	71	2.86	2191	3	55
C	Male	Barrier	UTBIF-78	319	*B. myosotis*	87	2.81	1984	9	53
D	Male	Conven	UTCIF-1	46	*B. reuterii*	51	2.7	2004	2[e]	50
D	Male	Conven	UTCIF-3	201	*B. reuterii*	51	2.7	2009	4	54
E	Female	Conven	UTCIF-24	149	*B. reuterii*	62	2.76	2060	4	52
F	Male	Conven	UTCIF-36	253	*B. reuterii*	69	2.72	2022	3	51
F	Male	Conven	UTCIF-37	179	*B. callitrichos*	50	2.78	2119	3	58
G	Male	Conven	UTCIF-38	222	*B. callitrichos*	48	2.77	2116	3	57
G	Male	Conven	UTCIF-39	113	*B. tissieri*	46	2.74	2031	4	58

[a]Barrier = UTHSCSA barrier colony; Conven = SNPRC conventional colony.
[b]Coverage = average number of unique reads that include a given nucleotide in a reconstructed sequence.
[c]CDS = number of protein coding sequences.
[d]Number of 5S, 16S, or 23S rRNA genes.
[e]Missing 5S sequences.

(Copan Diagnostics, Murrieta, CA) into the rectum. E-swabs were placed in Amies transport medium and immediately frozen at −80°C. Samples were mailed to the University of Idaho on dry ice and stored at −80°C. Genomic DNA was extracted from fecal samples, and the V1-V3 region of the 16S rRNA gene was amplified and sequenced by 454 pyrosequencing using our standard protocols (Hickey et al., 2015), while the 16S rRNA genes from the WNPRC were sequenced using an Illumina MiSeq platform (Shen et al., 2016).

For the bacterial communities from which *Bifidobacterium* isolates were purified, raw sequence reads were demultiplexed and processed sequences according to our standard protocols (Hickey et al., 2015). The RDP Bayesian classifier was used to assign sequences to genus or higher (RDP 2.5). An alternative strategy was needed to identify *Bifidobacterium* sequences to species level, and the following protocol was used for sequences from the three colonies. Raw reads were demultiplexed with QIIME (Caporaso et al., 2010), adapter trimmed using Cutadapt (Martin, 2011), and quality filtered using DADA2 (truncQ = 2, maxN = 0, maxEE = 2, truncLen = 450; Callahan et al., 2016). Unique sequence variants were quantified using DADA2 and classified using DADA2 with the silva_species_assignment_v128 database or SPINGO (Allard, Ryan, Jeffery, & Claesson, 2015) with RDP_11.2_species database. Before taxonomic assignment, we manually added the 16S rRNA gene sequences of our four *Bifidobacterium* species (*B. reuteri*, *B. myosotis*, *B. callitrichos*, and *B. tisseri*). Classifications from DADA2 and SPINGO were merged in phyloseq (McMurdie & Holmes, 2013) so that the lowest classification was retained. In some instances the classification of sequences differed between DADA2 and SPINGO at the genus level. In these instances, we trimmed back the classification to the family level, where the methods never disagreed. Amplicon sequence variants (ASV) with 100% identity to the 16S rRNA gene sequences for our four bifidobacteria were combined for each species. All other ASVs that were designated as *Bifidobacterium* were combined into a single count for the *Bifidobacterium* genus.

2.3 | Isolation of *Bifidobacterium* strains and growth conditions

The second step in identifying *Bifidobacterium* isolates for genomic sequencing was to isolate putative *Bifidobacterium* from the fecal samples for which *Bifidobacterium* phylotypes represented more than 50% of the sequence reads. This was done by streaking fecal material on modified DB agar plates (g/L; Columbia agar base 42.5, glucose 2.5, lactulose 2.5, L-cysteine 0.5, riboflavin 0.01, propionic acid 5.0 ml, pH 5.5+/−0.2). The plates were incubated anaerobically at 37°C for 72 hr (Anaerobe Systems Model: AS-580, (www.aanerobesystems.com) supplied with 90% N_2, ~5% CO_2, and ~5% H_2. Colonies with typical bifid, gram-positive rods were restreaked onto modified DB agar plates and well-isolated colonies were used to inoculate de Man-Rogosa-Sharpe broth supplemented with 500 mg/L L-cysteine (MRS medium) then incubated at 37°C for 24 hr under anaerobic condition. Aliquots of these cultures were stored at −80°C.

2.4 | Genomic DNA extraction and amplification of 16S rRNA genes for *Bifidobacterium* isolates

The third step in identifying isolates for genomic sequencing was to extract genomic DNA from each isolate and identify different strains of *Bifidobacterium* by sequencing their 16S rRNA genes. To do this, colonies grown on modified DB agar plates were used to inoculate MRS medium and the cultures were incubated at 37°C for 24 hr under anaerobic conditions. Bacterial genomic DNA was isolated from each culture using the QIAamp DNA mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Briefly, 500 µl of each bacterial cell suspension was incubated with 100 µl of lytic enzymes (50 µl lysozyme (10 mg/ml; Sigma-Aldrich, St Louis, MO), 6 µl mutanolysin (25,000 U/ml; Sigma-Aldrich), 3 µl lysostaphin (4,000 U/ml in sodium acetate; Sigma-Aldrich) and 41 µl TE buffer (10 mmol/l Tris-HCl; 50 mmol/l EDTA; pH 8.0) at 37°C for 1 hr. Bacterial cells were then mechanically disrupted by adding

~750 mg of 0.1-mm zirconium silica beads (BioSpec, Bartleseville, OK) to the mixture and using in a mini-bead-beater machine (BioSpec) set at 36 oscillations per second at 2,100 rpm. After cell disruption, the suspensions were centrifuged at 2,100 rpm for 1 min. To digest proteins, 50 µl proteinase K (20 mg/ml) and 500 µl AL buffer (Qiagen) were added to the crude cell lysates, which were then incubated for 30 min at 56°C. Afterwards, 500 µl of ethanol and 50 µl sodium acetate was added to crude lysate and DNA was bound to QIAmp spin columns that were then washed with AW1 and AW2 buffers. DNA was eluted from the spin columns using 100 µl AE buffer. Genomic DNA concentrations were measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) and quality was assessed using gel electrophoresis.

The procedure for sequencing 16S rRNA genes from bacterial isolates was different from that used for bacterial communities. The V1-V3 region of the 16S rRNA genes was amplified from genomic DNA using primers 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 926r (5′-CCGTCAATTCCTTTRAGTTT-3′). The reaction mixture for PCR consisted of 5.0 µl 10× PCR buffer II (Applied Biosystems, Foster City, CA), 6.0 µl $MgCl_2$ (25 mM; Applied Biosystems), 2.5 µl Triton X-100 (1%), 0.4 µl deoxyribonucleoside triphosphates (25 mM), 0. 5 µl each of primer 8F and 926r (20 pmol/µl each), 0.2 µL AmpliTaq DNA polymerase (5 U/µl; Applied Biosystems), and 5 ng of template DNA in a total reaction volume of 50 µl. Samples were initially denatured at 95°C for 5 min, then amplified by using 30 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 90 s. A final extension of 7 min at 72°C was added at the end of the program to ensure complete amplification of the target region.

Amplicons were cleaned using Qiagen QIAamp mini spin columns (Qiagen Inc., Valencia, CA). Amplicons were sent to Elim Biopharmaceuticals (Hayward, CA) for Sanger sequencing. The 16S rRNA gene sequences were aligned to those of other *Bifidobacterium* sp. to verify that they were indeed species of *Bifidobacterium and* free of contamination. Using the 16S rRNA gene sequence data, different species and strains were chosen for genome sequencing.

2.5 | Genome sequencing and genomic analysis of *Bifidobacterium* isolates

The genome sequences of 10 strains were determined using the Illumina MiSeq platform in the IBEST Genome Resources Core at the University of Idaho. Reference-guided genome assembly was performed using our in-house program, ARC (https://ibest.github.io/ARC/). Genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/). The genome sequence data for these strains were deposited at GenBank under the accession numbers JFAG0000000–JFAO00000000, JEOG00000000.

Annotated sequences were analyzed using OrthoMCL (Li, Stoeckert, & Roos, 2003) to infer homologous sequences among the 10 strains plus the 33 *Bifidobacterium* strains listed in Table S1. The criteria for homology were 50% for the coverage cutoff and 50% for the percentage of identical amino acid residues and 1.1 for the MCL setting. The R statistical package was used to extract core, shared and species-specific genes from the OrthoMCL database (https://www.r-project.org/).

2.6 | Phylogenetic analysis of 76 *Bifidobacterium* strains

To place the bifidobacteria from marmosets (*C. jacchus*, *C. pygmaea*) within an evolutionary context of bifidobacteria from humans and other primates (*Eulemur macaco*, *Lemur catta*, *Macaca mulatta*, *Saguinus imperator*, *S. midas*, *S. oedipus*), a phylogenetic analysis was performed using the Type I polyketide synthase amino acid sequence, which is over 3200 amino acids long. Sequences for this protein were extracted from the genomic sequences for our strains and for the genomes listed in Table S1. Additionally, the protein sequences listed in Table S2 were downloaded from GenBank (https://www.ncbi.nlm.nih.gov). These protein sequences were used to infer the phylogenetic relationships among 76 bifidobacteria. The sequences were aligned using MUSCLE (Edgar, 2004), and the phylogeny was inferred using the maximum likelihood algorithm and the WAG scoring matrix as implemented in RAxML (Stamatakis, 2014).

2.7 | Ethics statement

This study was carried out in adherence with the guidelines put forth by the American Society of Primatologists' Principles for the Ethical Treatment of Primates. All procedures were reviewed and approved by the respective Institutional Animal Care and Use Committees at UTHSCSA, SNPRC, NEPRC, and WNPRC.

3 | RESULTS

3.1 | Isolation and genome sequences of *Bifidobacterium* strains from marmoset fecal samples

The purpose of our study was to determine whether *Bifidobacterium* from marmosets carry genes that are not found in *Bifidobacterium* from humans. The first step in answering this question was to isolate and sequence the genomes of *Bifidobacterium* strains from marmoset fecal samples so that gene content could be compared to human-derived bifidobacteria. Eleven adult marmosets were identified as having greater than 50% bifidobacteria in their bacterial communities. Multiple isolates were purified from the fecal samples of these individuals. Isolates were identified as *Bifidobacterium* based on their 16S rRNA gene sequences, and ten isolates from seven individuals were chosen for genome sequencing based upon having different 16S rRNA gene sequences (Table 1).

All ten genomes were greater than 2.7 Mbp and the gene content varied from 1984 to 2191 inferred coding sequences (Table 1). All but one of the genomes had three or more copies of ribosomal RNA genes, and all genomes had >50 tRNA genes. Although the sequence coverage was >100× for nine of the ten isolates, it was not possible to assemble scaffolds into closed genome sequences.

Analysis of 16S rRNA gene sequences showed that we had isolated six *B. reuteri* strains, two *B. callitrichos* strains, one *B. myosotis* sp. nov. and one *B. tissieri* sp. nov. (Endo et al., 2012; Michelini et al., 2015; Michelini, Oki et al., 2016; Table 1). Two of the six *B. reuteri*

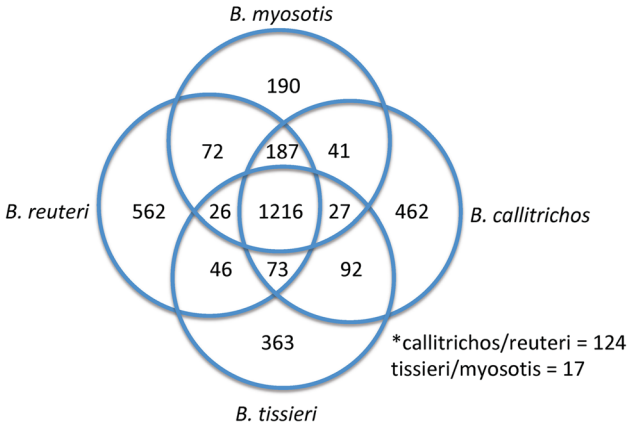

FIGURE 1 Venn diagram comparing four species of *Bifidobacterium* isolated from marmoset feces. Numbers indicate orthologous genes shared between at least one strain from each species as determined by OrthoMCL (50% protein sequence identity with at least 50% coverage; MCL inflation value = 1.1). *counts not depicted in diagram

isolates came from the same animal and were very similar in gene content and nucleotide sequence (UTCIF-1, UTCIF-3). Two other marmoset fecal samples provided two isolates each, and these were different species.

There was little variation in the gene content of strains belonging to the same species. The genomes of *B. reuteri* and *B. callitrichos* type strains had been previously sequenced (Table S1). The gene content of our isolates of these species were similar to those of their type strains, but differences were also evident. In general, the type strains had larger genomes and more coding sequences. Five of our *B. reuteri* strains had only one to seven unique coding sequences, Although samples came from separately housed colonies, the barrier colony was started with animals from the conventional colony 2 to 5 years before sampling for this study (Ross et al., 2017). The genome size and gene content of *B. reuteri* strain UTCIF-68 was greater than that of other isolates. Of the 118 unique genes, some were putative prophage sequences. Our two *B. callitrichos* isolates were also quite similar, sharing more than 98% of their genes.

The gene content of all four species was fairly conserved (Figure 1). For the criteria used here (50% identity, 50% coverage), the four species shared 1216 genes, and another 313 genes were shared among three of the four species. Between 128 and 258 genes were unique to each species. Finally, between 62 and 376 genes were found in only one marmoset *Bifidobacterium* species, but these genes were shared with other *Bifidobacterium* species of human origin (see below).

3.2 | Phylogenetic relationships of *Bifidobacterium* from marmosets and humans

Meaningful comparisons between bacterial genomes require a clear understanding of the evolutionary relationships among the bacteria. To place the bifidobacteria from marmosets within an evolutionary context of bifidobacteria from humans and other primates, their Type I polyketide synthase amino acid sequences were used to infer the phylogenetic relationships of these species (Figure 2). Our phylogenetic analysis shows that *B. myosotis* shared a common ancestor with *B. reuteri*, *B. breve*, and *B. longum*, whereas the most recent common ancestor of *B. tissieri* shares a more distant common ancestor with these four, *B. bifidum* and *B. callitrichos* (Figure 2). Note that *Bifidobacterium* species isolated from marmosets and tamarins, which are New World monkeys with overlapping species ranges, occurred intermingled within clades (Figure 2; green and blue labels). Also, the *Bifidobacterium* species from *Eulemur macaco* and *Lemur catta* were outgroups to the bifidobacteria from humans and New World monkeys, reflecting the phylogeny of the primate host.

3.3 | Comparison of *Bifidobacterium* genomes from marmosets and humans

The genomes of bifidobacteria isolated from marmosets were compared to the genomes of 47 *Bifidobacterium* strains from the species *B. adolescentis* (*n* = 3), *B. animalis* (*n* = 16), *B. bifidum* (*n* = 3), *B. breve* (*n* = 8), *B. callitrichos* (*n* = 1), *B. dentium* (*n* = 1), *B. longum* (*n* = 13), *B. reuteri* (*n* = 1), and *B. thermophilum* (*n* = 1) (Table S1; www.ncbi.nlm.nih.gov). The genomes of the human-derived *Bifidobacterium* species were generally smaller than those from marmosets and accordingly, had fewer genes (Table S1). There were 937 genes found in at least one strain of each species. Of these, 712 were found in every strain of every species. Genetic distance plays a large role in detecting evolutionary relationships; the faster a protein evolves, the less likely homologs will be found among distantly related organisms. Thus, the core genome of the *Bifidobacterium* genus has only 413 genes when all species and strains were considered (Milani et al., 2016).

We sought to determine if there were features of *Bifidobacterium* species in marmosets that allow them to persist in the adult gastrointestinal tract. To explore this we compared the gene content of strains of bifidobacteria isolated from humans, which do not persist at high relative frequency in adults, to those from marmosets that can be found at high relative frequency in adult animals. The genes found in marmoset bifidobacteria, but not in humans may allow these species to persist into adulthood. To minimize the probability of declaring a difference that is only due to genetic distance, we chose to compare the genomes of two marmoset-derived species with multiple strains, *B. reuteri* and *B. callitrichos*, to the genomes of the two most closely related human-derived species, *B. longum* and *B. breve* (Figure 3). Using our criteria, there were 1357 genes that were found in at least one strain from each of the four species, and 987 genes that were found in all strains of the four species. More important, there were 106 genes in *B. reuteri* and *B. callitrichos* that were not found in *B. breve* and *B. longum*. Forty five of the 106 genes encoded hypothetical proteins, the rest had sufficient similarity to known proteins that they were functionally annotated.

We investigated the 61 annotated genes in greater depth using BLAST to find homologous sequences in other bacteria. Twenty of

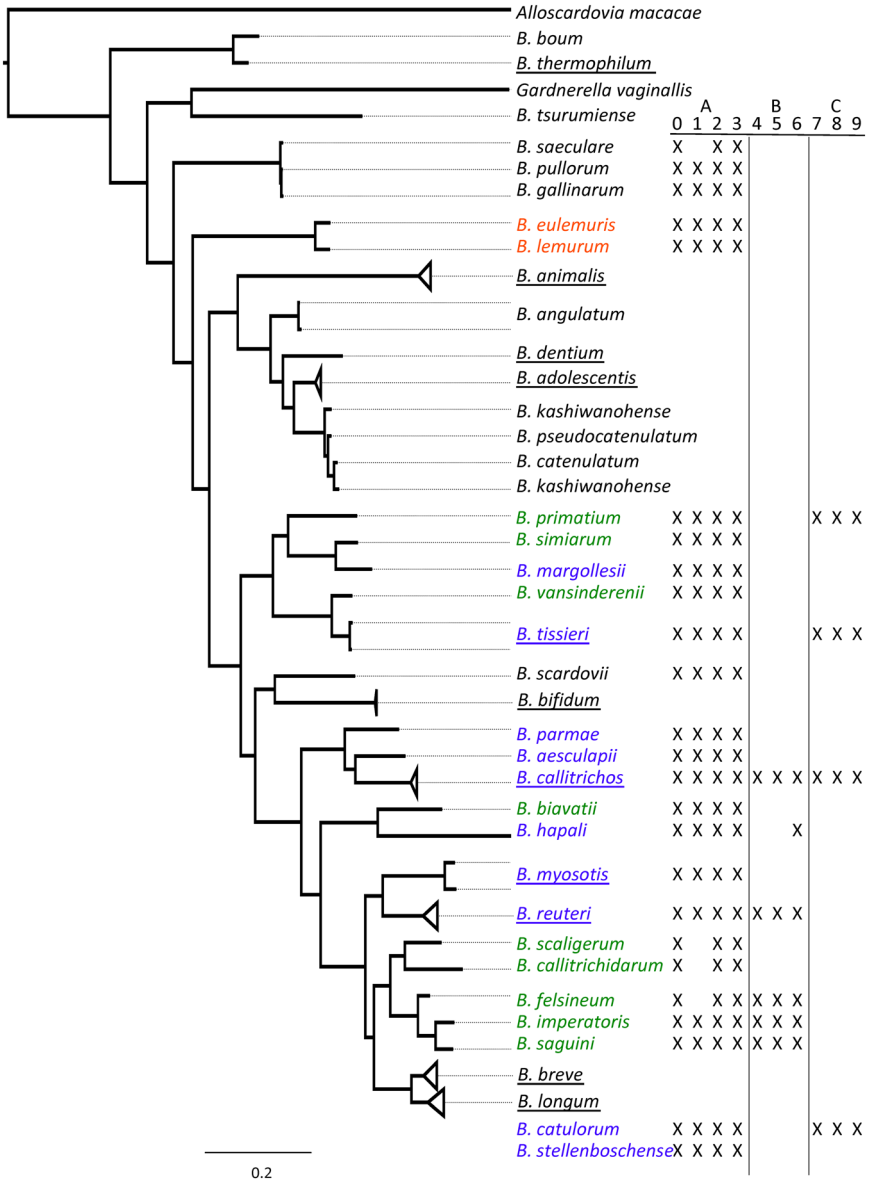

FIGURE 2 Maximum likelihood phylogeny of *Bifidobacterium* species based upon the type 1 polyketide synthase protein. *Alloscardovia macacae* was used to root the phylogeny; *Gardnerella vaginalis* has recently been recognized as a member of the genus *Bifidobacterium*. Species used in the comparative genome analysis are underlined. Names of *Bifidobacterium* isolated from nonhuman primates are colored: blue=marmosets, green=tamarins, orange=lemurs. Branches with more than two strains for a single species are collapsed. The error bar indicates the mean number of nucleotide substitutions per site. The table shows the distribution of genes associated with three ABC transport systems: A) 2-aminoethylphosphonate ABC transporter (0. ATP binding protein, 1. periplasmic 2-aminoethylphosphonate-binding and permease protein, 2. 2-aminoethyl-phosphonate:pyruvate aminotransferase, 3. phosphono-acetaldehyde hydrolase); B) peptide ABC transport (4. peptide-binding protein, 5. two permease proteins, 6. two ATP binding proteins); C) aliphatic sulfonate ABC transporter (7. alphatic sulfonate binding protein, 8. permease protein, 9. ATP binding protein). Complete type 1 polyketide synthase sequences were not available for *B. catulorum* and *B. stellenboschense*, but their ABC transporter contents are shown at the bottom of the phylogeny

the 61 genes were found in human-derived bifidobacteria other than *B. breve* and *B. longum*, and we excluded them from further consideration. The remaining 41 genes encoded proteins with various functions, including transcriptional regulation, membrane proteins and a range of enzymes (Table S3). Seven proteins were listed as components of ABC transporters, suggesting that differences in nutrient uptake might distinguish marmoset bifidobacteria from human bifidobacteria.

3.4 | ATP-binding cassette transport systems

We looked more closely at the genes encoding ATP-binding cassette (ABC) proteins that appeared to be specific for *Bifidobacterium* species isolated from marmosets. ABC importers are complexes of two or three proteins, with the following functions: 1) an ATPase that provides energy for transport across the cell membrane, 2) a permease that mediates passage of the nutrient into the cell and 3) an extracellular substrate binding protein that recognizes the

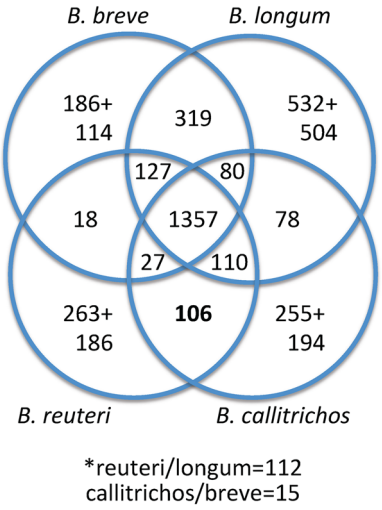

FIGURE 3 Venn diagram comparing marmoset-derived versus human-derived bifidobacteria. Numbers indicate orthologous genes shared between at least one strain from each species as determined by OrthoMCL (50% protein sequence identity with at least 50% coverage; MCL inflation value = 1.1). Genes unique to each species are indicated as # shared with other human *Bifidobacterium* species + # unique to all *Bifidobacterium* strains. *counts not depicted in diagram

nutrient and brings it to the permease (Eitinger, Rodionov, Grote, & Schneider, 2011). Sometimes the permease and substrate binding protein are encoded by a single gene.

The genomes of *B. callitrichos* and *B. reuteri* have genes encoding a putative 2-aminoethylphosphonate ABC transporter, but *B. breve* and *B. longum* do not. These proteins include both a permease domain and a substrate binding domain, and an ABC transport ATPase domain. Immediately downstream of these genes were ones encoding a putative phosphonoacetaldehyde hydrolase (EC:3.11.1.1) and 2-aminoethylphosphonate:pyruvate aminotransferase (EC:2.6.1.37). We found the distribution of these four genes to be limited within bifidobacteria (Figure 2), occurring in ten species derived from marmoset feces (Figure 2; blue labels), nine species from tamarin feces (Figure 2; green labels), one species from human blood (*B. scardovii*), two from lemur feces (Figure 2; orange labels), one from rabbit feces and two from chicken feces. Genes for these four proteins were not found in any of the human fecal isolates of bifidobacteria even though by our phylogenetic analysis, these human-derived bifidobacteria share common ancestors with those bifidobacteria isolated from marmosets and tamarins. On the other hand, these four genes are found in a wide range of other species including Firmicutes, β-proteobacteria, and γ-proteobacteria, especially those that are known to inhabit the human gut, such as *Clostridium*, *Salmonella*, and *Klebsiella*.

The two metabolic genes are involved in the cleavage of C–P bonds in organophosphate compounds, releasing the P as a nutrient source (Kamat & Raushel, 2013). The wide-spread but sporadic distribution of this metabolic pathway has been noted previously, and its presence in diverse genomes suggests that lateral gene transfer might be important in its distribution across phyla (Huang, Su, & Xu, 2005). The distribution of this pathway within the genus *Bifidobacterium* suggests that it was present in the ancestral genome but was subsequently lost in all species associated with the human gut microbiome, as well as with many other environments (Figure 2).

Two other clusters of ABC importer genes were found that have an even more restricted range (Figure 2). One cluster encoded a peptide ABC substrate binding protein, two peptide ABC permeases and two ABC ATPases. Within the genus *Bifidobacterium*, the first three genes were found only in *B. callitrichos*, *B. reuteri* and three closely related species isolated from tamarins. The ATPase was also found in *B. hapali*, a species of bifidobacteria that had been isolated from the common marmoset. One ATPase was also found in a *Bifidobacterium* isolated from human blood. The proteins encoded by this cluster of genes had greater than 60% sequence identity to homologs in the genus *Paenibacillus* and were widespread in other *Firmicutes*. The second cluster was similar to an aliphatic sulfonate ABC transporter. The three proteins that comprised this importer were found only in *B. tissieri*, *B. callitrichos*, *B. catulorum*, and the tamarin-derived *B. primatium*. Proteins from clostridia had the closest sequence similarity to these transport proteins. The sporadic distribution of these two transport systems suggests that lateral gene transfer has played an important role in their distribution. The other three ABC transport components that were not found in human-derived bifidobacteria were ABC exporters that are putatively involved in multidrug export.

3.5 | Phosphotransferase systems

Phosphotransferase systems (PTS) are used by bacteria to actively import carbohydrates at the expense of ATP and concomitantly catalyze their phosphorylation (Kotrba, Inui, & Yukawa, 2001). PTS are found in many bacterial species, however, there are also many species that lack PTS homologs. Differences in their distributions sometimes exist even among strains of the same species (Barabote & Saier, 2005). Because this system seems to evolve rapidly, possibly in response to nutrient availability, we categorized the types of PTS that were found in all of the genomes that we studied (Tables 1 and S1).

The strains of *B. animalis* that we studied did not have any PTS genes, whereas strains of the other species had all three PTS genes necessary for both β-glucoside and glucose transport. *B. bifidum* and *B. callitrichos* had PTS genes for sugar and cellobiose-specific transfer. In contrast, *B. bifidum*, *B. breve*, and *B. callitrichos* had PTS genes for *N*-acetylglucocosamine and ascorbate, whereas *B. breve* also had PTS genes for fructose. The distribution of the PTS genes in both marmoset- and human-derived bifidobacteria suggests that this is not a system associated with the persistence of bifidobacteria into adulthood.

3.6 | Distribution of *Bifidobacterium* species in adult marmoset fecal samples

Our goal was to test the hypothesis that bifidobacteria from adult marmosets carry genes that allow them to persist at high levels, as

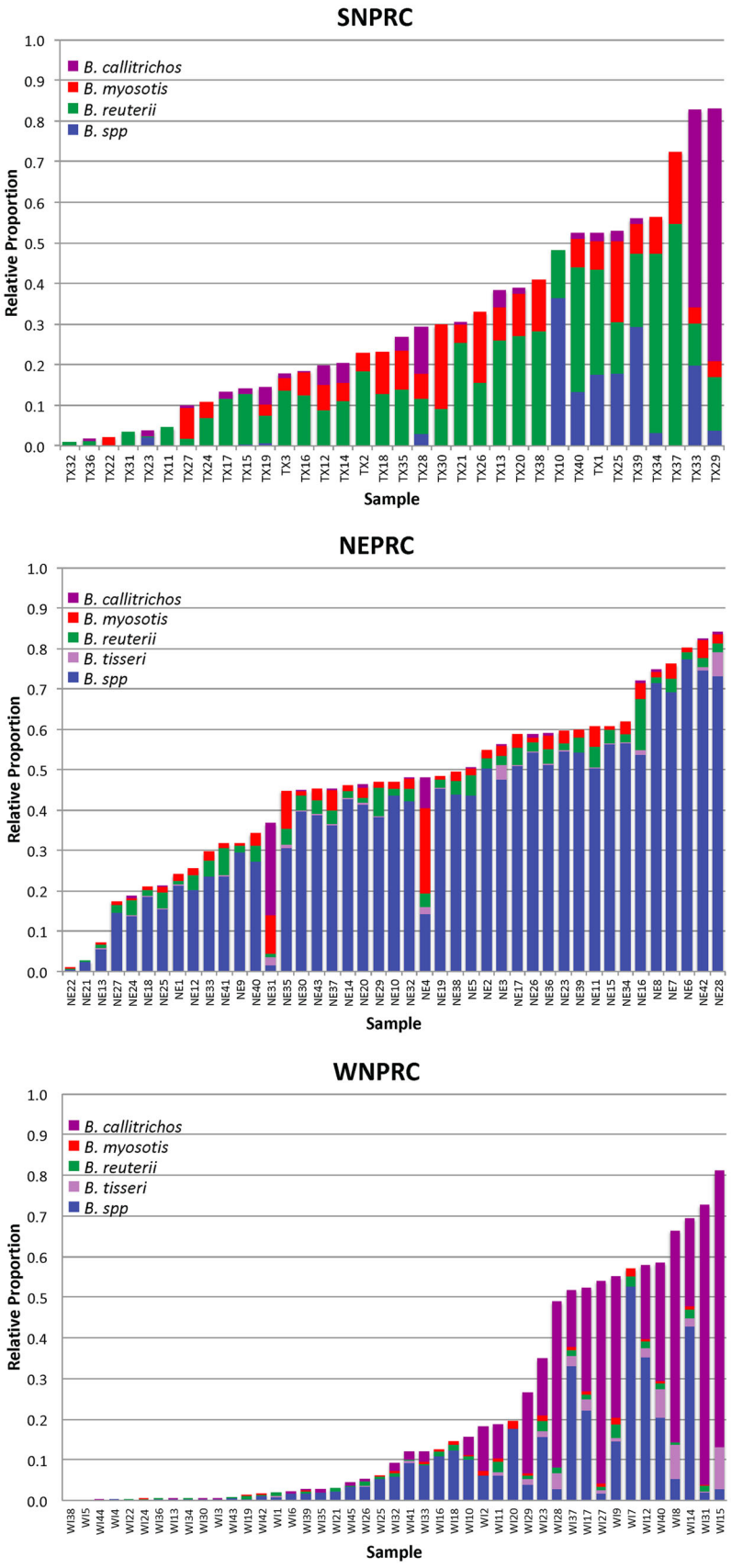

FIGURE 4 Relative abundance of *Bifidobacterium* species in marmoset fecal samples from three primate research centers. Each fecal sample was from a different individual, who was over one year old

compared to bifidobacteria in humans that have low abundances in adults. This hypothesis was based upon our previous results showing that the gastrointestinal tracts of adult marmosets often have high proportions of bifidobacteria (Ross et al., 2017). To confirm the high proportion of bifidobacteria in a larger sample, we determined the relative abundance of *Bifidobacterium* species in bacterial communities of 122 adult marmosets. This was done by sequencing and classifying amplicons of 16S rRNA genes in fecal samples. As seen previously, there was wide variation in the relative abundance of *Bifidobacterium* species ranging from zero to more than 80% (Figure 4). About 72% of our adult marmosets had a relative abundances of *Bifidobacterium* greater than 10%, which is more than three times what is commonly seen in adult humans (Yassour et al., 2016), and over half had abundances greater than 25%.

Additionally, we determined the relative abundance of different species of bifidobacteria. *B. reuteri* and *B. myosotis* were found in fairly high proportions in the SNPRC colonies, which is the center from which the isolates originated. *B. tissieri* was found at less than 10% relative frequency in samples from NEPRC and WNPRC. Oddly, it was not found at the SNPRC from which it was originally isolated. *B. callitrichos* predominates in samples from the WNPRC colony (Figure 4). Sequences from species other than our four species of *Bifidobacterium* predominated in samples from the NEPRC colony. The sequence with the highest frequency at NEPRC was 100% identical to GenBank accession EU459101, which was originally isolated from Geoffrey's marmoset (*C. geoffroyi*) feces (Ley et al., 2008).

4 | DISCUSSION

4.1 | Genome comparisons between *Bifidobacterium* species from marmosets and from humans

Our goal in this study was to compare *Bifidobacterium* from the common marmoset with those from humans with the intent of identifying mechanisms that allow bifidobacteria to persist at high levels in adult marmosets. There may be multiple reasons for the differential effect of age on the frequency of human-derived and marmoset-derived bifidobacteria. The reasons for differences are not known, but diet, host physiology, exposure to different microbes and genetic differences between marmosets and humans seem like reasonable possibilities. The results of our genomic comparison suggested that diet might be a major influence. There were only 106 proteins found in *B. reuteri* and *B. callitrichos* genomes that were not found in genomes of *B. breve* and *B. longum*. Forty one of these proteins had been assigned putative functions and were not found in any other human-derived bifidobacteria strain (Table S3). Because diet influences the composition of the gut microbiome and adult diets are different from infant diets, we focused on nutritional transporters in the marmoset-derived bifidobacteria that might explain the persistence of bifidobacteria in the gut microbiomes of adult marmosets. We found examples of ABC transporters, but not PTS transporters, that distinguished bifidobacteria from marmosets and humans.

An important component of nutritional transport systems are the substrate binding proteins that are secreted into the environment to capture nutrients and facilitate nutrient uptake. In a study that examined the secretomes of *Bifidobacterium*, *B. biavatii* and *B. aesculapii* isolated from New World monkeys were predicted to encode the largest arsenal of secreted proteins (Lugli et al., 2018). Indeed, with few exceptions, New World *Bifidobacterium* have the highest number of predicted extracellular proteins. These results suggest that there are multiple genomic differences between New World primate bifidobacteria and human bifidobacteria. An evolutionary comparison of *Bifidobacterium* from different primates may provide insights to the evolution of this interesting genus.

We found several examples of ABC transporters that were found in certain species of *Bifidobacterium* and not others. In our analysis, the bacteria outside of *Bifidobacterium* with protein sequences most similar to these transporters were *Firmicutes*, which are often found

TABLE 2 Differences among colonies in content of prepared diets

	NEPRC		SNPRC		WNPRC
Base diet(s)	Teklad NWM	ZuPreem	Teklad purified	Mazuri regular	Mazuri high fiber
Top five ingredients in order by weight	Ground corn	Cracked wheat	Dextrin	Glucose	Glucose
	Ground wheat	Soybean meal	Sucrose	Soybean meal	Ground corn
	Wheat middlings	Sugar	Lactalbumin or	Ground corn	Soybean meal
	Wheat germ	Dried egg product	Whey protein	Casein	Casein
	Soybean meal	Vegetable oil	Soybean oil	Wheat middlings	Corn gluten meal
Gelling agent	None	None	Agar or gelatin	Gelatin	Gelatin
				Xanthum gum	Xanthum gum
Crude DM protein %	20	25.5	14	20	20
Crude DM fat %	7.5	10	5.6	7.0	4.5
Kcal/kg DW	3,200	3,600	3,600	3,410	3,200

Abbreviations: NEPRC: New England Primate Research Center; SNPRC: Southwest National Primate Research Center; WNPRC: Wisconsin National Primate Research Center.

in the gut microbiomes of adult humans. We propose that these transporters allow the marmoset-derived *Bifidobacterium* to use resources in the marmoset gut that are also used by *Firmicutes* in the human gut, and this may permit the persistence of bifidobacteria at high relative abundance in the adult marmoset.

4.2 | Phylogenetic relationships of *Bifidobacterium* from marmosets and humans

Our analysis of the taxonomic distribution of ABC transporters led us to include species of *Bifidobacterium* obtained from other primates in our phylogenetic analysis. An interesting pattern was seen in the phylogeny inferred using the type 1 polyketide synthase protein. The clade that includes *B. bifidum*, *B. breve*, and *B. longum*, the first colonizers of the human infant gut, contains many species that are found in New World monkeys. Thus, it appears that New World monkeys share bifidobacteria with a common ancestor of the human bifidobacteria, but the genus has undergone evolutionary radiation and multiple species have emerged in the gut microbiomes of marmosets and tamarins. Although these species were isolated from specific primates, it would be interesting to determine whether the New World monkeys share these species or if they are specific to the host from which they were derived.

4.3 | Distribution of *Bifidobacterium* species in adult marmosets

Within each marmoset colony, the relative abundances of *Bifidobacterium* species vary widely from 0 to 80% (Figure 4). However, closer examination shows that the predominant species are different among the three marmoset colonies considered in this study (Figure 4). Studies that view microbiomes with similar distributions of phyla or genera as an "enterotype" may tend to overlook important variation within members of the enterotype (Arumugam et al., 2011; Koren et al., 2013; Liang et al., 2017; Wu et al., 2011). For instance, putative aliphatic sulfonate ABC transporter genes were found in *B. callitrichos* and not in *B. reuteri* (Figure 3), and there are >500 other proteins that are not shared between strains of these two species (Figure 2). Thus, simply identifying the frequency of *Bifidobacterium* in these colonies would not convey the underlying differences among them that could be important in defining their ecological roles.

Diet plays an important role in determining the species composition of the gut microbiome (David et al., 2014; Muegge et al., 2011; Wu et al., 2011). Our three marmoset colonies were fed different formulations of prepared diets that provided similar caloric and protein content, but from different sources (Table 2). All colonies were also provided supplemental foods, such as fruits, vegetables, and yogurt ad libitum, and these supplements also differed among colonies. These differences in diet may account for the distribution of the different *Bifidobacterium* species among the colonies. For instance, the NEPRC marmosets received far less simple carbohydrate by weight than the other two colonies (Table 2). On the other hand, the species composition of a particular colony may be maintained by the close proximity of marmosets in adjacent cages or by contact with their caregivers. Evaluating the effect of dietary differences on species composition in the gut is an important next step and the goal of our current research.

Although *B. myosotis* and *B. tissieri* were previously isolated from infant marmoset feces (Michelini et al., 2015; Michelini, Oki et al., 2016), our isolates of these two species came from fecal samples of adult animals. In addition, both species were found in multiple adults from different colonies (Figure 4). It is possible that—as with human infants (Stewart et al., 2018)—the frequency of *Bifidobacterium* species changes over time, and these two species may have greater prevalence in infant marmosets. Common marmosets are housed as family groups with multiple litters, thus adults are constantly exposed to the gut microbiomes of their offspring. Further work is needed to understand the development of the marmoset microbiome from infancy onwards.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ORCID

Celeste J. Brown http://orcid.org/0000-0002-1202-323X
Suzette D. Tardif http://orcid.org/0000-0002-3412-7742

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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