Bifidobacterium bifidum MIMBb75 colonizes the mouse intestinal tract impacting the resident microbial populations in a region-specific and time-dependent manner

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Keywords: *Bifidobacterium bifidum*, gut microbiota, quantitative PCR, mouse

ABSTRACT

Bifidobacterium bifidum MIMBb75 is a recently identified probiotic. However, its biogeographical distribution and impact on host intestinal microbiota are unknown. Herein, we established a quantitative real-time PCR assay targeting the *B. bifidum*-specific *BopA* gene for the quantification of *B. bifidum* in feces and we used it to investigate the ability of *B. bifidum* MIMBb75 to colonize the murine intestine. We also analyzed the consequential impact on endogenous microbial cohorts. C57BL/6J mice were daily gavaged with 0.2 mL of either sterile PBS or PBS containing 10^8 CFU of *B. bifidum* MIMBb75 for two weeks, after which intestinal contents and fecal samples were analyzed for microbial compositional changes. *B. bifidum* MIMBb75 was able to transiently colonize the murine intestine with the predominant niche being the caeco-promixal colonic region. Region-specific effects on host microbiota were observed including decreased levels of *C. coccoides* in the caecum, increased levels of bifidobacteria in the proximal and distal colon, total bacteria and *C. leptum* in the proximal colon, and of *C. coccoides* in the feces. These findings suggest that probiotic properties of *B. bifidum* MIMBb75 may partially depend on its ability to colonize the intestine and impact the endogenous microbial communities at various intestinal loci.

INTRODUCTION

Bifidobacterium bifidum MIMBb75 is a strain isolated from human feces (Guglielmetti et al. 2008) and whose probiotic properties have been recently demonstrated in a double-blind randomized clinical trial enrolling patients with irritable bowel syndrome (Guglielmetti et al. 2011). In this trial, *B. bifidum* MIMBb75 administration resulted in reduced global IBS symptoms and improved overall quality of life. *B. bifidum* is also the first *Bifidobacterium* for which a surface protein, named Bifidobacterial outer protein A (BopA), has been identified as a mean of adhering to intestinal epithelial cells *in vitro* (Guglielmetti et al. 2008). This study also showed that BopA is a discriminant of the *B. bifidum* species within the *Bifidobacterium* genus. The ability to adhere to intestinal cells has been considered a possible proxy for the capacity to colonize *in vivo* due to the potential for avoidance of peristaltic elimination and possibly the means by which these prokaryotes facilitate inter kingdom cross-talk with the host, particularly by impinging on the microenvironment via induction of signaling pathways and/or modulation of gene expression (Lebeer et al. 2010). It is intriguing to hypothesize that adhesion is essential for this strain to exert its probiotic properties (Guglielmetti et al. 2009); though, spatio-temporal patterns of its intestinal colonization and the consequential impact on the host, have not yet been determined.

Bifidobacteria are gram-positive, nonmotile, nonsporulating, anaerobic, autochthonous inhabitants of the human colon, and comprise 1-4.8% of the fecal microbial population in adults (Harmsen et al. 2002; Satokari et al. 2003; Zoetendal et al. 2006). Colonization studies assessing the niche environment of these indigenous residents reveal a region-

specific pattern subject to temporal modifications. More specifically, localization studies depict caeco-colonic regions as the preferential niche of bifidobacterial populations (Simon and Gorbach 1984; Cronin et al. 2008) with elevated levels of *B. bifidum* in fecal samples versus mucosal biopsies when taking into consideration both sigmoid and rectal mucosal–adherent bacteria and those shed in the feces (Turroni et al. 2009). Bifidobacteria, and in particular *B. bifidum*, are among the first gut colonizers and persist in the intestine throughout adulthood. As a whole, decreased levels of bifidobacteria accompany senility (Gueimonde et al. 2007). Several investigations have previously shown that the presence of bifidobacteria can infringe upon the endogenous microbial composition through modulation of the host intestinal milieu. In a double-blind randomized placebo-controlled cross-over trial, Ishizuka et al. demonstrated a capacity for *Bifidobacterium animalis* subsp. *lactis* to proliferate *in vivo* and increase total bifidobacteria numbers while compositionally maintaining the resident bifidobacterial cohorts and improving bowel regularity (Ishizuka et al. 2012). As a synbiotic preparation, both *B. bifidum*, *B. longum* and the prebiotic oligofructose have been used to enhance both the quantity and type of bifidobacteria found in elderly populations that often experience deleterious changes in their microbiological compositions (Bartosch et al. 2005). Moreover, both *in vitro* and *in vivo* assays have demonstrated a capacity for *B. bifidum* CECT 7366 to inhibit *Helicobacter pylori* (Chenoll et al. 2011).

However, studies assessing the distribution along the cephalo-caudal gut axis and implications of *B. bifidum* on host microbial composition are lacking. Although, historically culture-based techniques were employed for characterization of various bacterial cohorts the advent of molecular tools allowed for more efficient and accurate

quantification, particularly in terms of affording a greater discriminatory power between species of the same genus. Quantitative real-time polymerase chain reaction (qPCR) is now routinely employed in fecal microbiology including the assessment of bifidobacteria (Matsuki et al. 1998; Requena et al. 2002; Malinen et al. 2003; Haarman and Knol 2005; Penders et al. 2005; Gueimonde et al. 2007; Palmer et al. 2007; Junick and Blaut 2012). Moreover, to differentiate species within the *Bifidobacterium* genus assays targeting the *16S rRNA* gene (Matsuki et al. 1998) and, more recently, the housekeeping genes *groEL* (Junick and Blaut 2012) and *tal* (Requena et al. 2002) have been used. With respect to *B. bifidum* as of late, cell surface adhesion markers have been characterized including the extracellular transaldolase (Tal), which *in vitro* acts as a potential colonization mediator of certain strains of *B. bifidum* to intestinal HT-29 cells (Gonzalez-Rodriguez et al. 2012) and BopA outer cell surface lipoprotein (Guglielmetti et al. 2008). As suggested previously, the transaldolase gene has been utilized for bifidobacterial species determination though, the ability to employ real-time PCR for the enumeration of *B. bifidum* via the *tal* gene is limited (Requena et al. 2002). Considering the specificity afforded by targeting species specific constituents, in this study we developed a novel 5' nuclease based assay targeting the *BopA* gene, which encodes for an adhesion molecule, for enumerating *B. bifidum* in fecal and intestinal samples. Hence, making use of this assay, we sought to delineate the environmental niche of *B. bifidum* MIMBb75 via determining its geographical distribution within the intestine, interactions with indigenous microbial residents at diverse intestinal loci, and retention *in vivo* using a murine model.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

All strains used in this study are listed in Table 1. *Bifidobacterium bifidum* MIMBb75, *Bifidobacterium bifidum* DSM 20456^T, *Bifidobacterium longum* NCC2705, *Bifidobacterium adolescentis* DSM 20083^T, and *Bifidobacterium breve* DSM 20213^T were routinely grown in anaerobic conditions at 37°C in MRS broth (Difco, Detroit, MI, USA) supplemented with 0.05% (wt/vol) L-cysteine hydrochloride (cMRS). For gavage, *B. bifidum* MIMBb75 was grown overnight in cMRS, then cells were washed and re-suspended in PBS at a concentration of $1x10^8$ colony forming units (CFU)/0.2 mL. *Bacteroides thetaiotaomicron* ATCC 29148^T and *Clostridium coccoides* DSM 753^T were cultivated in PRAS cooked meat glucose medium in Hungate tubes (Remel, Lenexa, KS, USA) at 37°C. *Clostridium leptum* DSM 753^T was grown in Reinforced Clostridial Medium (Oxoid, Nepean, ON, Canada) supplemented with 10 g of maltose/L under anaerobic conditions at 37°C. *Escherichia coli* JM101 and *Bacillus subtilis* DSM 10^T were grown aerobically at 37°C in LB Broth (BD, Franklin Lakes, NJ, USA). *Lactobacillus helveticus* MIMLh5 and *Enterococcus faecium* ATCC 19434^T were grown anaerobically at 37°C in MRS and M17 (Difco) media, respectively.

Animals.

All animal study designs and procedures were approved by the local animal ethics committee at the University of Toronto and handled in accordance with the Regulations of Animals for Research Act in Ontario and the Guidelines of the Canadian Council on

Animal Care. Twenty-eight male C57BL/6J mice, 7 weeks of age, were obtained from Jackson's Laboratory (Sacramento, CA, USA) and housed at The Division of Comparative Medicine, University of Toronto. Upon arrival to the animal facility, mice were acclimatized for a period of one week prior to being randomly assigned into their respective treatment groups based on baseline body weights. All animals were provided *ad libitum* access to a standard chow diet (2018 Teklad, Harlan, Mississauga, ON, Canada) and water, and housed under the conventional 12h:12h light-dark cycle until the date of sacrifice. Mice were daily gavaged for two weeks with 0.2 mL of sterile PBS containing $1x10^8$CFU of *B. bifidum* MIMBb75 cells (treatment group) or with an equal volume of sterile PBS alone (control group). Subsets of seven mice per group were then sacrificed while the remaining fourteen mice (7/group) were maintained for an additional period of one week (day 21) without gavage (washout) before being sacrificed. Fecal pellets were collected at baseline day 0 (one week post-acclimatization) and subsequently after 7, 14 and 21 days. Intestinal retention of gavaged *B. bifidum* MIMBb75 was assessed in the feces for a period of 24 hours with fecal pellets collected at 3, 6, 9, 12, 18, 21 and 24 hours following the seventh and fourteenth day of gavage. Mice were sacrificed by CO_2 inhalation followed by cervical dislocation. Upon sacrifice, the caecum, proximal colon and distal colon were immediately excised and contents were collected separately for each region. Tissues were further cleaned with sterile 0.9% NaCl, divided into two halves longitudinally and alongside contents were snap-frozen in liquid nitrogen and stored at -80°C and -20°C, respectively, until further processing.

Gut microbiota composition analysis.

Fecal and intestinal contents microbial DNA was extracted with the Omega E.Z.N.A.TM Stool DNA Isolation Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions modified to include a lysozyme digestion step at 37˚C for 30 minutes. Both quantity and quality of DNA were assessed via UV absorbance (OD260/280/230), using Thermoscientific's Nanodrop 1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Extracted DNA samples were stored at -20°C until they were used for quantification of individual bacterial groups by TaqMan real-time PCR using the TaqMan Gene Expression Master Mix and specific custom made TaqMan assays (all reagents were from Applied Biosystems). Reactions (10µl) were run in triplicates using Applied Biosystems 7900 HT Real-Time PCR machine equipped with a 384 wells block. Bacterial numbers were calculated using pre-constructed standard curves obtained from serially diluted DNA extracted from known numbers of cells of each of the bacterial species of interest and expressed versus wet fecal weight or wet intestinal contents weight. The following bacteria were targeted (primers were from references in parentheses): total bacteria (Suzuki et al. 2000), Bacteroidetes (Furet et al. 2009), bifidobacteria (Furet et al. 2009), *C. coccoides* group (Furet et al. 2009), *C. leptum* group (Furet et al. 2009), and *B. bifidum* (this study, see below).

Establishment of a 5' nuclease assay for the quantification of *B. bifidum* in fecal samples.

Custom-made TaqMan MGB (Minor Groove Binding) primers and probe were designed to target the *oppD* region upstream of the *BopA* gene (GenBank: AM710395.1), specific to *B. bifidum* species (Guglielmetti et al. 2008), using the Primer Express v3.0 software

(Applied Biosystems, CA, USA) and were obtained from Applied Biosystems. Sequences of the primers and probe are as follows: Forward: 5'-ACCGAATTCGCCTGTCACTT-3', Reverse: 5'-ACGGCGCGGATTCGT-3', Probe: 5'-CCGCTGGATGTGAAC-3'. Specificity of the primers and probe sequences was confirmed *in silico* using BLAST alignment (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and experimentally using quantitative real-time PCR and DNA extracted from both target and non-target species (Table 1). Sensitivity was assessed through a standard curve constructed from 10-fold serial dilutions of DNA extracted from a known number of *B. bifidum* MIMBb75 cells, as determined in triplicate using a Neubauer Bright-Line Hemacytometer (Hausser Scientific, Horsham, PA, USA), after adjusting its concentration to 100 ng/µL.

Statistical analyses.

Microbiota inter-regional or time-course dependent compositional analysis in the feces and the intestinal contents were assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) followed by the Dunn's Multiple Comparison post-hoc test. Within a given region, differences in the microbial composition were determined with the Mann-Whitney test. Differences were considered significant at $p<0.05$. Outliers were detected using the Grubb's Outlier Test (extreme studentized deviate). All data analysis was performed using GraphPad Prism 5 Software (La Jolla, CA, USA).

RESULTS

Establishment of a 5' nuclease assay for the enumeration of *B. bifidum* in fecal and

intestinal contents samples.

The specificity of the new assay was first assessed *in silico* showing perfect match with the *oppD* region upstream of the *BopA* gene of *B. bifidum* but no other significant match with other bacterial sequences. Next, the assay was employed in real-time PCR with genomic DNA extracted from target and non-target microbial species, including dominant and sub-dominant representatives of the gut endogenous microbial community and *Bifidobacterium* species other than *B. bifidum*. Cross-reactions of the primers and probe were not observed for any of the non-target microbes tested, based on a lack of amplification, as listed in Table 1. Quantitative real-time PCR was used to assess the sensitivity of the novel *BopA* TaqMan assay. Ten-fold serial dilutions of DNA extracted from quantified *B. bifidum* MIMBb75 cells were subjected to qPCR. The average of triplicate amplifications as measured by cycle threshold (Ct) values for each corresponding dilution was plotted against $\log_{10}$ values of *B. bifidum* MIMBb75 cell counts per PCR reaction (Figure 1). The standard curve generated depicts a linearity range (R^2>0.999) between 6.8 and $6.8x10^6$ CFU/PCR reaction, suggestive of the fact that *B. bifidum* is quantifiable over a broad range of concentrations (PCR efficiency was 98%, as calculated from the slope of the standard curve). A similar plot was obtained with DNA isolated from a different strain of *B. bifidum* (*B. bifidum* DSM 20456^T) (data not shown).

Retention of gavaged *B. bifidum* MIMBb75 in the murine intestinal tract.

B. bifidum was not detected in mice from either of the groups (treatment/control) at day 0, as assessed by absolute quantitative real-time PCR (Figure 2), suggestive of the fact

that members of this species are not natural residents of the murine gastro-intestinal (GI) tract and thereby lending to the assumption that the bacteria detected with the *BopA*-specific TaqMan assay throughout our study stem from the inoculum, specifically *B. bifidum* MIMBb75.

In order to assess retention of *B. bifidum* MIMBb75 within the host following gavage, freshly passed fecal pellets were collected consecutively at intervals of 3, 6, 9, 12, 18, 21 and 24 hours following both 7 and 14 days of gavage, and analyzed for the presence of *B. bifidum* via qPCR (n=6-7/group/time point). During the 24 hour period following day 14 of gavage, *B. bifidum* cells were not detected in the control group (data not shown), however feces from mice in the treatment group contained between 6.8 – 9.2 log cell counts/g feces depending on the hour post-inoculation with a peak cell clearance of (9.2 +/- 0.2 log cell counts/g feces) at 6 hours (Figure 3). Levels of *B. bifidum* were significantly reduced at 18 hours (7.1 +/- 0.3 log cell counts/g feces) and were retained at 7.0 +/- 0.3 log cell counts/g feces from the 18th onwards to the 21st hour with a significant decrease from the peak levels shed in the feces once again at 24 hours (6.8 +/- 0.3 log cell counts/g feces) (Figure 3). A similar pattern was observed at day 7 (data not shown).

Temporal colonization pattern of *B. bifidum* MIMBb75.

Fecal shedding of *B. bifidum* cells at various time points before and throughout treatment including at day 0, 7, 14, and 21 (one week wash-out) were used as comparators of one another. At 7 and 14 days of treatment 6.1 ± 0.6 log cell counts/g feces and 6.8 ± 0.3 log cell counts/g feces, were recovered in the treatment group, respectively, with a complete

absence in control counterparts (n=6-7/group) (Figure 4). There was no difference in the number of fecal *B. bifidum* cells between 7 and 14 days of treatment (log cell counts/g; $p>0.05$). After a one-week wash-out period (day 21), *B. bifidum* cells declined significantly in the treatment group ($p<0.05$) but remained detectable at 4.4 ± 0.5 log cell counts/g feces (Figure 4) indicating that the administered bacterium had not completely cleared.

Geographical distribution of *B. bifidum* MIMBb75 within the large intestine and its impact on host microbial composition.

Localization of *B. bifidum* along the longitudinal gut axis was assessed at day 14 via quantification of *B. bifidum* in the luminal contents of caecum, proximal and distal colonic regions as well as the feces (Figure 5). For all regions considered, there were no *B. bifidum* cells detected in the control group (data not shown). In the treatment group, the proximal colon contained the highest number of *B. bifidum* cells with 8.2 ± 0.3 log cell counts/g intestinal contents which significantly outnumbered the levels in both the distal colon and fecal samples (6.9 ± 0.2 log cell counts/g intestinal contents and 6.8 ± 0.3 log cell counts/g feces, respectively). There was no significant difference between the levels of *B. bifidum* in the proximal colon and caecum. These results depict a uniform colonization pattern of *B. bifidum* throughout the caeco-proximal colonic regions, with levels decreasing within distal colonic regions.

To establish the implications of *B. bifidum* colonization on the host indigenous microbial composition, levels of Bacteroidetes (*Bacteroides*/*Prevotella*), bifidobacteria, *C. coccoides*, *C. leptum* and total bacteria were assessed in contents of the caecum, large

intestinal regions and feces and compared between control and *B. bifidum* MIMBb75-administered mice. There were no statistically significant differences in absolute cell counts between control and treatment groups in any of the bacterial groups of interest in the feces at baseline (Figure 2). Moreover, bacteria from the genus cluster *Bacteroides*/*Prevotella* and from the species *B. bifidum* were not detectable in the feces of all mice considered (Figure 2).

After 2 weeks of gavage, in the caecum, bifidobacteria could be quantified in all animals in the probiotic supplemented group (7.5 ± 0.7 log cell counts/g intestinal contents), however only two of control counterparts had detectable levels (6.8 ± 0.0 log cell counts/g intestinal contents), the increase in the number of bifidobacteria was accompanied by decreased levels of *C. coccoides* in treatment versus control group (6.4 ± 0.5 versus 7.4 ± 0.1 log cell counts/g intestinal contents; P=0.0022) (Figure 6A). Conversely, no significant differences in the levels of both total bacterial counts and *C. leptum* were observed (Figure 6A). In the proximal colon, not only was there an observed increase in bifidobacteria in treatment versus control group (8.3 ± 0.7 versus 5.0 ± 0.3 log cell counts/g of intestinal contents p=0.0043), but also, the number of total bacteria (10.5 ± 0.1 versus 10.1 ± 0.2 log cell counts/g intestinal contents, p=0.0095) and *C. leptum* levels (9.1 ± 0.4 versus 8.4 ± 0.36 log cell counts/g intestinal contents p=0.0317) increased in the probiotic administered mice (Figure 6B). *C. coccoides* levels remained unaffected. In consonance with the caecum and proximal colon, bifidobacteria increased in the distal colon in the treatment versus control group (7.6 ± 0.5 versus 5.4 ± 0.3 log cell counts/g intestinal contents, p=0.0022) (Figure 6C). Though, other bacterial groups were unaffected by treatment. Fecal shedding of bacteria following 14 days of gavage

was also assessed for composition; no significant differences were observed between treatment and control groups for bifidobacteria levels (7.9 ± 0.7 versus 6.9 ± 2.0 log cell counts/g feces), *C. leptum* (8.7 ± 0.5 versus 8.8 ± 0.5 log cell counts/g feces) or total bacteria cell counts (10.6 ± 0.4 versus 10.7 ± 0.1 log cell counts/g feces) (Figure 6D). Conversely, significantly elevated levels of *C. coccoides* were found in the feces of the treatment group compared to control (7.8 ± 0.6 versus 6.7 ± 0.7 log cell counts/g feces) (Figure 6D). These results show that *B. bifidum* MIMBb75 impacts intestinal microbial composition in a region-specific manner.

DISCUSSION

B. bifidum MIMBb75 is a human intestinal strain, which has been recently employed as probiotic (Guglielmetti et al., 2011). Though, its intestinal colonization pattern and the effects of its consumption on the endogenous intestinal microbiota are not known. To assess colonization, we first established a 5' nuclease assay able to specifically detect *B. bifidum*. The novelty of this assay is such that it enables for species specific quantification of *B. bifidum* through targeting of a single gene encoding for a cell surface constituent implicated in mediating attachment to Caco-2 cells and inherent to *B. bifidum* species of bifidobacteria (Guglielmetti et al. 2008). Previously, bifidobacterial species-specific primers targeting the *16S rRNA* gene were developed (Matsuki et al. 1998). Though, currently research suggests the use of the *16S rRNA* gene is limited, particularly for species-specific identification in the *Bifidobacterium* genus, due to the high resolution power needed to discriminate between closely related bifidobacteria species (Junick and

310 Blaut 2012). As a result, the use of housekeeping genes has been implemented as
311 demonstrated by the recent examination of *groEL* (Junick and Blaut 2012) and *tal*
312 (Requena et al. 2002). Here, we designed an assay to target the *BopA* gene, due to its
313 specificity to *B. bifidum* species (Guglielmetti et al. 2008). Interestingly, both *tal* and
314 *BopA* code for proteins that are involved in *B. bifidum* adhesion to intestinal cells
315 (Guglielmetti et al. 2008; Gonzalez-Rodriguez et al. 2012). Nevertheless, for *B. bifidum*
316 quantification, *tal*-based qPCR was not as powerful as for other bifidobacteria (Requena
317 et al. 2002).
318 With the newly developed *BopA* assay, negative cross-reactions for species other than *B.*
319 *bifidum* were observed when employing real-time qPCR enabling us to examine the
320 intestinal colonization pattern of *B. bifidum* in mice. At baseline, in all mice considered,
321 *B. bifidum* was undetectable and therefore, lends to the assumption that detection of *B.*
322 *bifidum* stems from the gavage of *B. bifidum* MIMBb75 cells. Surprisingly, Bacteroidetes
323 was also not detectable in any of C57BL/6J mice; the primers that were used in this study
324 were originally designed to quantify Bacteroidetes in human and farm animal fecal
325 samples (Furet et al. 2009) and target a *16S rRNA* gene sequence which has also been
326 used for rodents by fluorescence *in situ* hybridization (Salzman et al. 2002; Cani et al.
327 2007); though, it is possible that the strain of mice employed for this study carries a
328 diverse subset of *Bacteroides* that are not detected by these primers as a group of mouse-
329 specific Bacteroidetes in C57BL/6J mice has been described elsewhere (Salzman et al.
330 2002). One of the current underlying criteria for probiotic usage entails survival of
331 passage through the GI tract with viability at the site of action as an important secondary.
332 Although viability tests were not conducted, fecal samples were collected for a 24 hour

period following day 14 of gavage particularly at the 3, 6, 9, 12, 18, 21 and 24 hour mark in order to assess *B. bifidum* MIMBb75 clearance time. We were able to show that following a two week intervention period, *B. bifidum* was recovered in the feces of treatment mice with cell counts reaching $10^{9.2}$ bacterial cells at the six hour mark which is quantitatively greater than the gavaged amount of 1×10^8 bacterial cells suggesting the ability to multiply *in vivo*. Previous studies have demonstrated a physiological transit time of 5.8-10 hours in mice (Schwarz et al. 2002). Considering the presence of *B. bifidum* past the 10 hour excretion mark particularly in the order of $1 \times 10^{6.8}$ bacterial cells at 24 hours post inoculation it further depicts the ability to survive passage through the GI tract and remain alive at the active site. Mechanistically, we postulate that the survivability of *B. bifidum* through the gastric environment may be via its pH induced auto-aggregation properties which enable the bacteria to conceal cell surface constituents and once within the distal colonic regions disaggregate due to the increased pH allowing for exposure of adhesin proteins and colonization (Guglielmetti et al. 2009). Future studies should incorporate cell viability assays and/or the traditional culturing of fecal samples with selective agents followed by discrimination of bacterial subtypes through classical PCR procedures to illustrate viability of passage through the GI tract.

In order to assess the temporal colonization pattern of *B. bifidum,* fecal samples were collected at day 7, 14 and one week post treatment cessation day 21. There was no significant difference in the quantity of *B. bifidum* MIMBb75 found in the feces at both day 7 and 14 of gavage. Despite literature support for a two week intervention period for probiotic bacterium, assessment of time-points prior to may be relevant considering a similar colonization pattern found in our study. Following one week treatment cessation

significantly lower levels of *B. bifidum* MIMBb75 were found in the feces albeit not negligible. This is indicative of the transient membership of *B. bifidum* MIMBb75 once feeding is stopped.

To date, the colonization strategy of *B. bifidum* MIMBb75 has not been elucidated. In order to determine the preferential niche of *B. bifidum* MIMBb75 contents from the caecum, proximal and distal colonic regions were collected. This study provides evidence for a discriminate colonization pattern of *B. bifidum* with the highest quantities in the caeco-proximal colonic region with levels tapering distally and lowest quantities found in the feces. These findings strongly coincide with *in vitro* analysis of environmental factors affecting *B. bifidum* MIMBb75 including low pH, presence of bile salts, and specific sugars (Guglielmetti et al. 2009). The authors of this study concluded the adhesion of *B.bifidum* MIMBb75 may be restricted to distal sites of the alimentary canal. What is more, although the large intestine has been implicated as the niche habitat of bifidobacteria (Simon and Gorbach 1984), Mundy et al. found bifidobacteria in the caecal fluid at levels of 10^6 CFU/ml in humans (Mundy et al. 2003) with Cronin et al. identifying the caecum as the primary habitat for *B. breve* in mice (Cronin et al. 2008), a finding shared with *B. bifidum* MIMBb75.

Considering a discriminate colonization strategy of *B. bifidum* MIMBb75 along the alimentary canal we sought to determine region-specific effects on host microbial cohorts. Apart from the caecum and feces, a significant increase in the proportion of bifidobacteria was observed in the treatment group in all regions considered, a likely result of inoculation with *B. bifidum* MIMBb75. The lack of increase of bifidobacteria in

fecal samples may be due the adhesive properties of *B. bifidum* MIMBb75 lending to its establishment within the colon and lack of fecal shedding.

Within the context of the proximal colon there was an increase in total bacteria not observed elsewhere. The increase in total bacteria may have been directly related to a significant increase in the *C. leptum* and bifidobacteria in the proximal colonic region. Administration of *B. bifidum* MIMBb75 was able to selectively modulate the murine proximal colonic microbial composition favoring an increase in *C. leptum*, a butyrate producing bacterium of the Firmicutes phylum. Considering that *C. leptum* levels are under-represented in elderly populations compared to healthy middle-aged counterparts (Mariat et al. 2009) and that there are lower levels in the feces of active IBD and infectious colitis patients compared to healthy individuals (Sokol et al. 2009), it is straightforward to remark that probiotic interventions aimed at stimulating their increase could prove to be clinically relevant.

With respect to the feces, there was a significant increase in *C. coccoides* levels which were correspondent with a significant decrease in their levels within the caecum. Mariat et al. demonstrated an age-associated change in the proportions of bifidobacteria which correlate with *C. coccoides* (Mariat et al 2009). In other words, high proportions of bifidobacteria in infancy were shown to be associated with lower counts of *C. coccoides*, but with age (adulthood) bifidobacteria numbers significantly declined with simultaneous increases in *C. coccoides* proportions (Mariat et al. 2009). Moreover, in a study which fed healthy elderly volunteers a bifidogenic prebiotic, an increase in bifidobacteria was associated with a concomitant increase in *C. coccoides* in the feces (Vulevic et al. 2008).

In summary, we found that *B. bifidum* MIMBb75 behaves as an allochthonous inhabitant of the intestinal microbial community, which is capable of transiently colonizing the GI tract of mice. Its transient membership within the intestinal ecosystem is also reiterated by the fact that seven days post treatment cessation there is a significant decrease in the levels of *B. bifidum*. Furthermore, *B. bifidum* MIMBb75 is able to predominately establish in caeco-proximal colonic regions while impacting the resident microbial population in a region-specific and time-course dependent manner. Therefore, we concur with previous investigators that the fecal microbiota cannot be considered a proxy for bacterial communities along the GI tract (Sarma-Rupavtarm et al. 2004). Taken together, these data suggest that *B. bifidum* MIMBb75 probiotic properties may at least partially depend on its ability to colonize the intestinal tract and impact the endogenous microbial community.

Acknowledgments

This work was funded by grants from Natural Sciences and Engineering Research Council (NSERC) and Dairy Farmers of Canada. NS was the recipient of the NSERC Alexander Graham Bell Canada Graduate Scholarship award. AW and YSS were the recipients of a NSERC Undergraduate Research Opportunity Award. The authors would like to thank Wen Su and Aifeng Lin for technical help.

FIGURE LEGENDS:

Figure 1. Standard curve for the *BopA* assay. The curve was obtained by qPCR analysis of serially diluted *B. bifidum* MIMBb75 DNA corresponding to 1 to $1x10^7$ cells/PCR reaction.

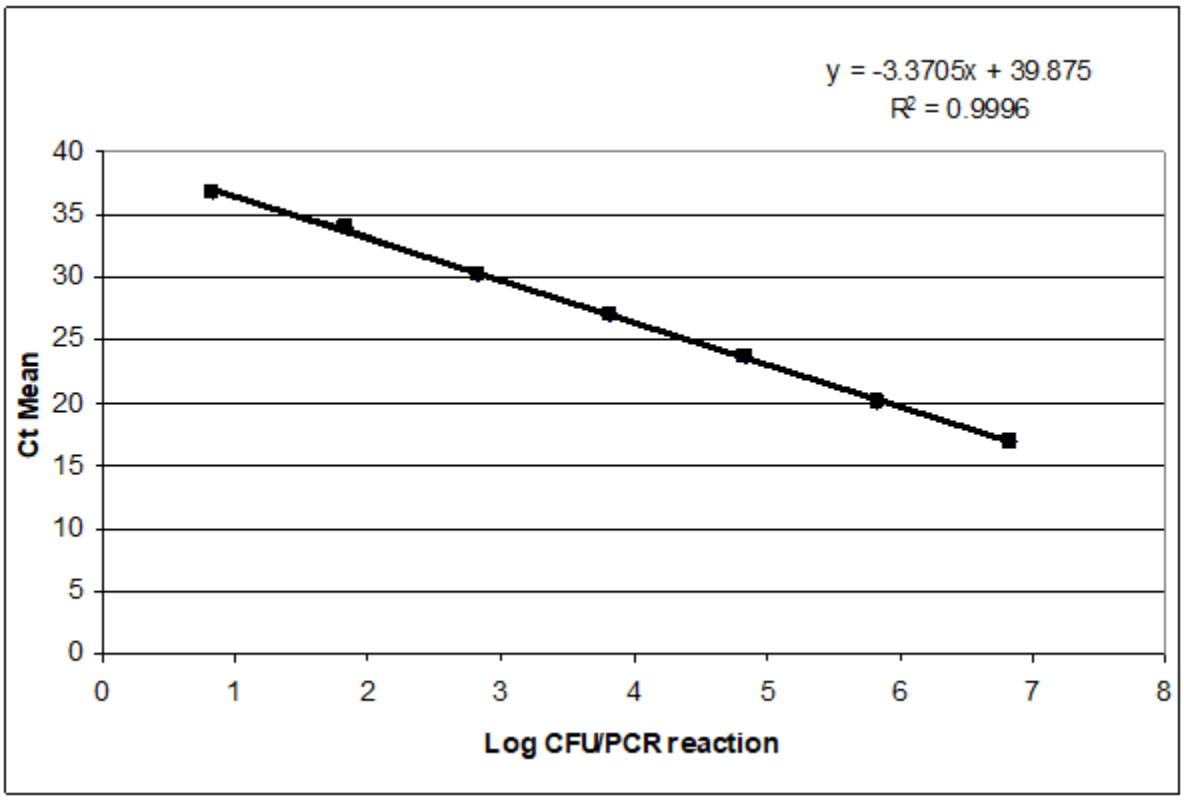

Figure 2. Fecal microbial composition analysis at day zero.

Absolute qPCR was used to assess the fecal microbial composition at baseline (n=7/group). Results were expressed as the mean log cell counts/g feces. The non-parametric Mann-Whitney test was performed to determine statistical significance with outliers removed based on Grubb's Test. N.D., not detectable.

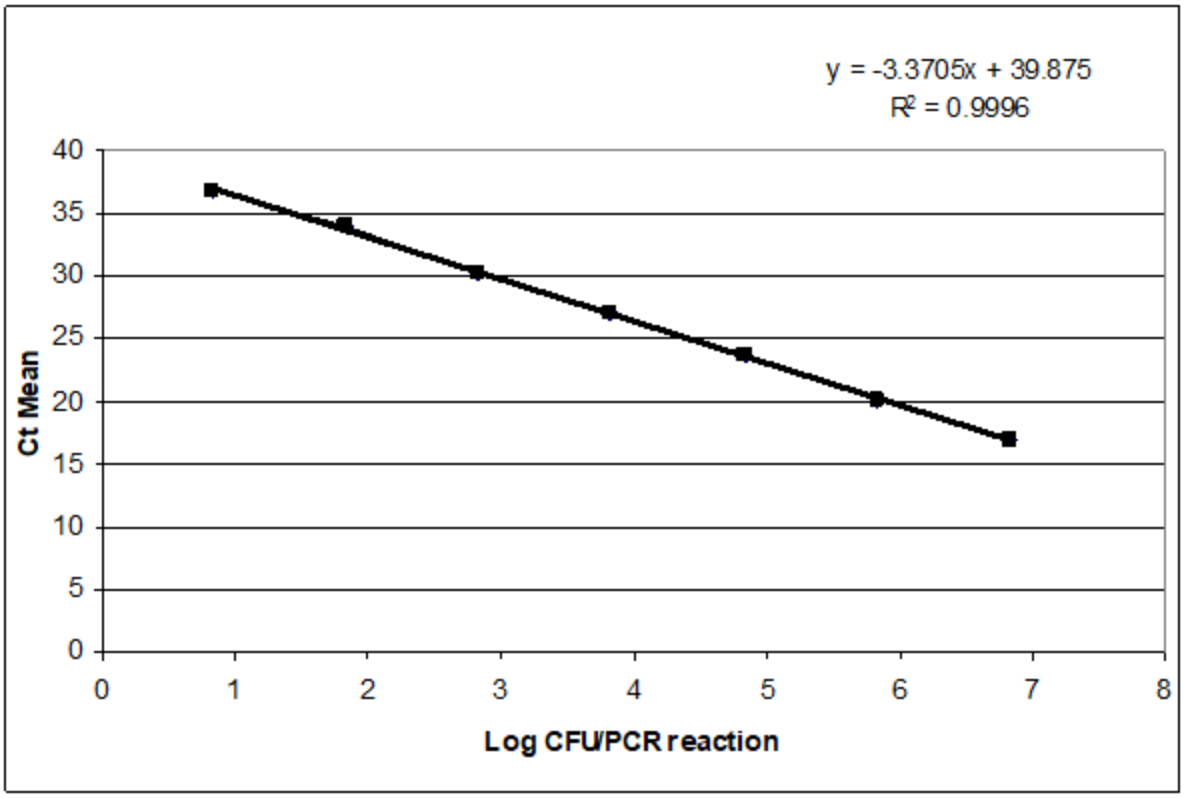

Figure 3. Intestinal transit of *B. bifidum* during 24 hours after gavage at day 14.

To assess the ability of *B. bifidum* MIMBb75 to persist within the gut, bacterial cells were enumerated by absolute quantitative real-time PCR in the feces of mice fed or not (n=6-7/group) *B. bifidum* MIMBb75 at 3, 6, 9, 12, 18, 21, and 24 hours after gavage on day 14. *B. bifidum* cells were not detected in the control group at any time point (not shown). One-way ANOVA using the Kruskal-Wallis test followed by the Dunn's Multiple Comparison Test was employed to determine quantitative differences at diverse time-points. Outliers were determined using Grubb's Test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

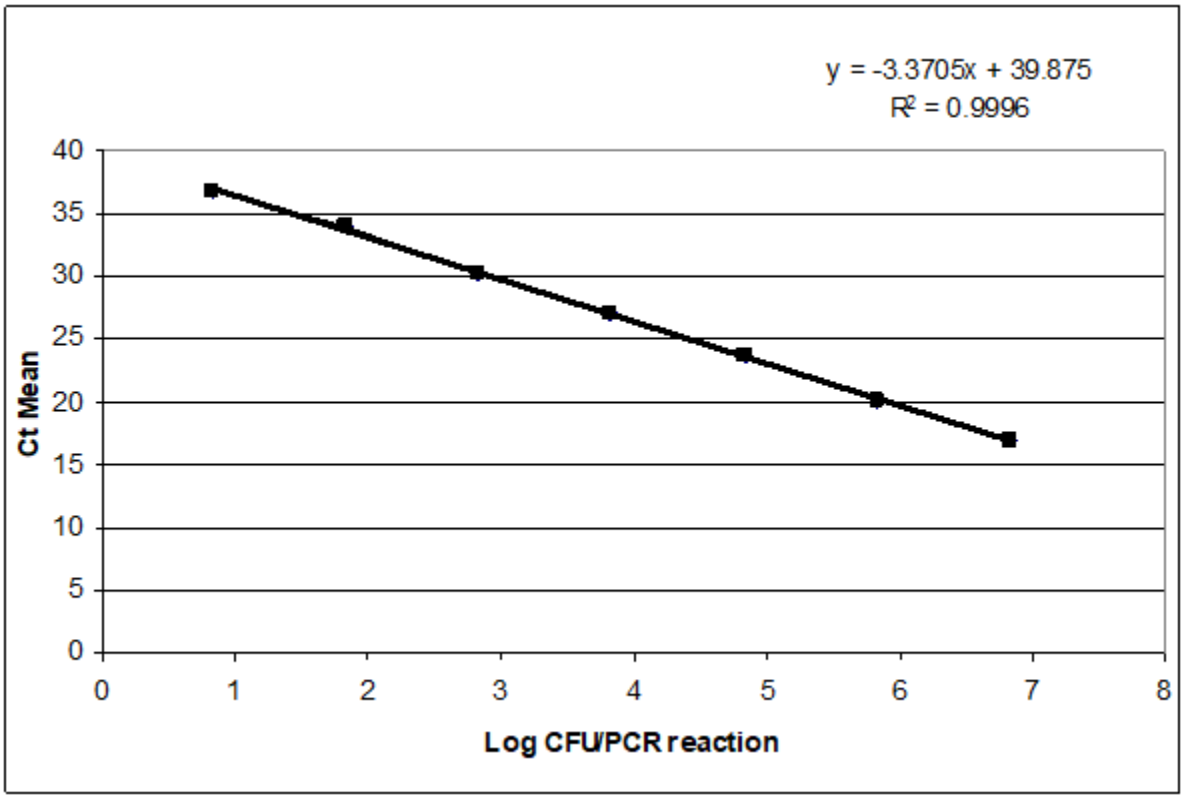

Figure 4. Temporal quantification of *B. bifidum* MIMBb75 in feces.

QPCR quantification of *B. bifidum* MIMBb75 cells in the feces of animals gavaged with PBS or *B. bifidum* MIMBb75 at 24 hours on day 0, 7, 14, and a washout period of one week (day 21) (n=6-7/group/timepoint). Data are presented as mean (indicated by horizontal bars) of absolute cell counts (log cell counts/g feces). Significance is based on one way ANOVA using the Kruskal-Wallis test followed by the Dunn's Multiple Comparison Test. Outliers were determined using Grubb's Test. N.D., not detectable; ***p<0.001.

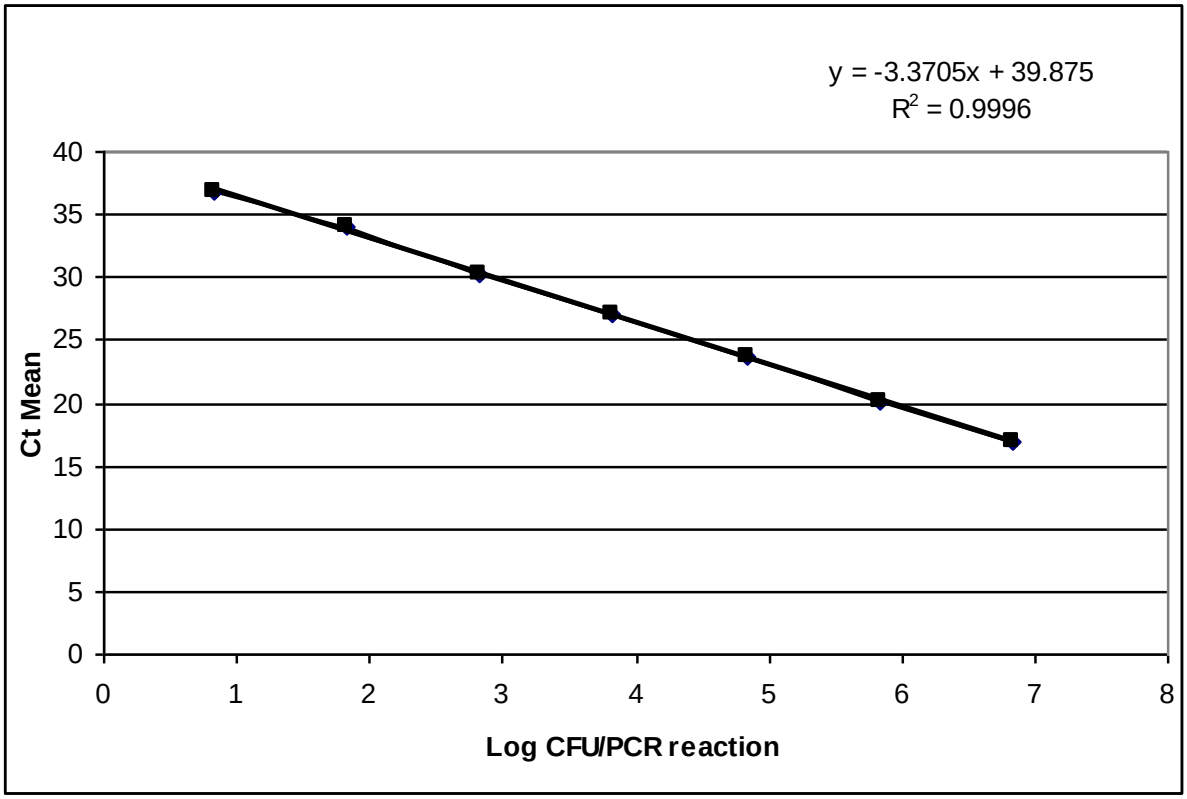

Figure 5. *B. bifidum* MIMBb75 intestinal colonization. To assess the colonization strategy of *B. bifidum* MIMBb75 along the longitudinal gut axis, luminal intestinal contents from the caecum, proximal colon, distal colon, and feces were collected at two weeks post-gavage and analyzed using absolute qPCR. There were no *B. bifidum* detectable in the control group in any region considered (data not shown). Data are represented as mean (horizontal bars) of absolute cell counts (log cell counts/g feces or intestinal contents) (n=6-7/group). One-way ANOVA using the Kruskal-Wallis test, followed by the Dunn's Multiple Comparison Test was used to determine significance between diverse regions. Outliers were removed based on Grubb's Test. * $p<0.05$, ** $p<0.01$.

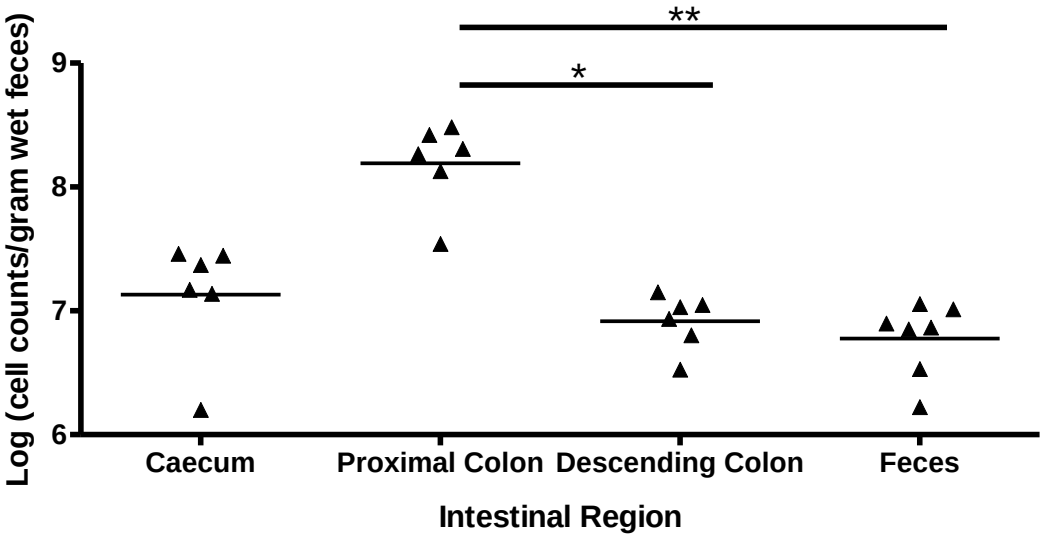

Figure 6. Intestinal microbial composition analysis at day 14.

Absolute qPCR quantification of dominant and subdominant bacteria in the caecum (A), proximal colon (B), distal colon (C) and feces (D) of mice gavaged or not with a suspension of *B. bifidum* MIMBb75 cells for 14 days (n=6-7/group). Data are presented as mean (horizontal bars) of absolute cell counts (log cell counts/gram intestinal contents or feces). Significance is based on non-parametric Mann-Whitney test with outliers removed as per Grubb's Test. N.D., not detectable; * $p<0.05$, ** $p<0.01$.

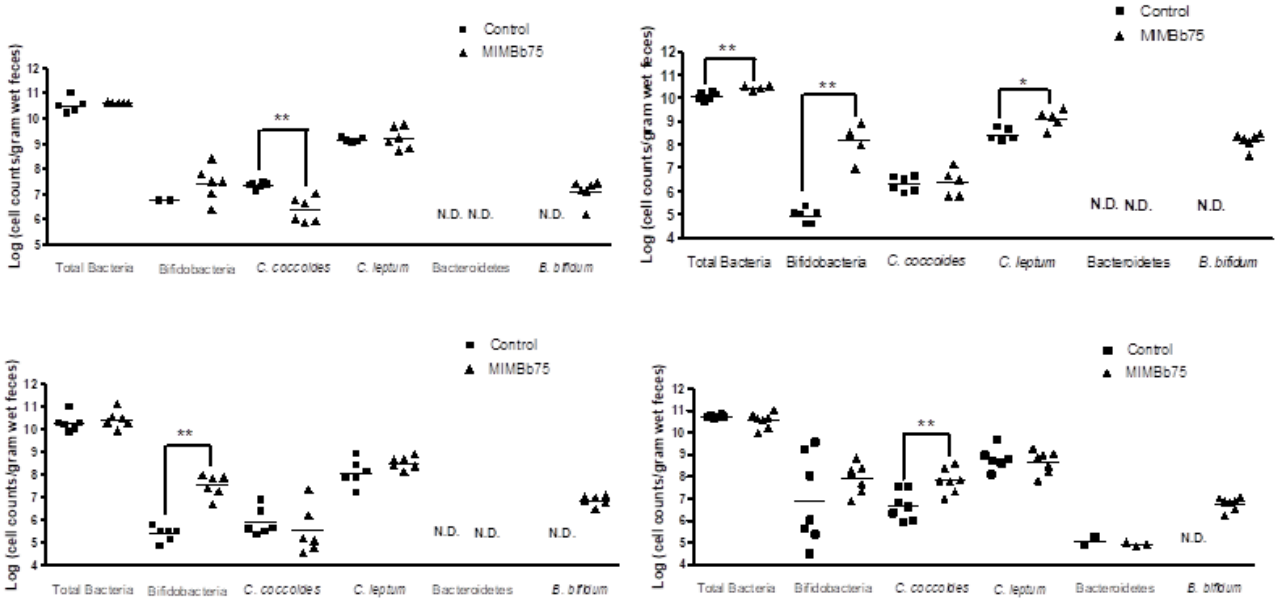

Table 1. Bacterial strains included in this study and experimental validation of the novel *B. bifidum* assay specificity.

Species	Specificity
Bacillus subtilis DSM 10^{T} [a]	-
Bacteroides thetaiotaomicron ATCC 29148^{T} [b]	-
Bifidobacterium bifidum MIMBb75 [a]	+
Bifidobacterium bifidum DSM 20456^{T} [a]	+
Bifidobacterium longum NCC2705 [a]	-
Bifidobacterium adolescentis DSM 20083^{T} [b]	-
Bifidobacterium breve DSM 20213^{T} [b]	-
Clostridium coccoides ATCC 29236^{T} [c]	-
Clostridium leptum DSM 753^{T} [b]	-
Enterococcus faecium ATCC 19434^{T} [a]	-
Escherichia coli JM101 [a]	-
Lactobacillus helveticus MIMLh5 [a]	-
Methanobrevibacter smithii DSM 861 [b]	-

a From the culture collection, DeFENS, University of Milan, Milan, Italy (Gugliemetti et al, 2010).
b From German Collection of Microorganisms and Cell Cultures, DSMZ, Braunschweig, Germany.
c From ATCC, Manassas, VA, USA.
"+" implies cross-reactivity while "-" a lack thereof

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