SUPPLEMENT ARTICLE

Lactobacillus acidophilus CL1285, *Lactobacillus casei* LBC80R, and *Lactobacillus rhamnosus* CLR2 (Bio-K+): Characterization, Manufacture, Mechanisms of Action, and Quality Control of a Specific Probiotic Combination for Primary Prevention of *Clostridium difficile* Infection

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A specific probiotic formulation composed of *Lactobacillus acidophilus* CL1285, *Lactobacillus casei* LBC80R, and *Lactobacillus rhamnosus* CLR2 (Bio-K+) has been marketed in North America since 1996. The strains and the commercial products have been evaluated for safety, identity, gastrointestinal survival, and stability throughout shelf life. The capacity of both the fermented beverages and the capsules to reduce incidences of antibiotic-associated diarrhea and *Clostridium difficile* infection (CDI) has been demonstrated in human clinical trials. Individual strains and the finished products have shown antimicrobial activity against *C. difficile* and toxin A/B neutralization capacity in vitro. The use of this specific probiotic formulation as part of a bundle of preventive measures to control CDI in healthcare settings is discussed.

Keywords. Bio-K+; *Lactobacillus*; probiotic; *Clostridium difficile*; prevention.

Over the past 15 years, enormous effort and financial resources have been invested to better understand the role of the human microbiome, particularly the gut microbiome, in health and disease. Headed by the National Institutes of Health–funded Human Microbiome Project, scientists around the world have pooled their expertise to decipher structure, function, and diversity of a healthy human microbiome [1] and to establish differences between healthy individuals and diseased ones [2]. One of the main roles of the indigenous intestinal microbiota is to provide colonization resistance to the overgrowth of low-level resident bacteria or opportunistic pathogenic microorganisms [3–5]. Numerous studies have shown that antibiotic administration can hinder the microbial diversity and the richness of the gut's ecosystem, resulting in reduced resistance to colonization by intestinal pathogens such as *C. difficile* [6–8]. It is now known that antibiotic exposure and duration, along with advancing age, are the most important risk factors for *C. difficile* infection (CDI) [9, 10]. A series of clinical practices and recommendations to prevent healthcare-associated CDI were outlined by the European Centre for Disease Control and Prevention in 2008 [11] and by the Society for Healthcare Epidemiology of America (SHEA)/Infectious Diseases Society of America (IDSA) in 2010 [12]. Although antibiotic stewardship has proven effective in reducing incidences of healthcare-associated CDI [13], mixed results have been observed in the aftermath of publication of these guidelines, possibly due to poor compliance in healthcare settings [14, 15]. Moreover, CDI has been increasingly reported among young, healthy individuals in the community. In fact, 20%–28% of all CDI cases could

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have been acquired in the community [16]. Among these community-acquired CDI cases, 35.9% were not exposed to an antibiotic [16], meaning that antibiotic stewardship would not have been useful in preventing these cases.

As agreed upon by a group of experts mandated by the Food and Agriculture Organization of the World Health Organization in 2001 [17] and later by a panel convened in October 2013 under the auspices of the International Scientific Association for Probiotics and Prebiotics [18], the definition of a probiotic is "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host." These microorganisms are thought to interact with the gut ecosystem and protect from bacterial dysbiosis occurring after antibiotic exposure, thus improving resistance to colonization [19–21].

Although SHEA/IDSA and other infection prevention groups do not recommend probiotic usage for adjunctive therapy with antibiotics, recent meta-analyses concluded that probiotics can help reduce incidences of antibiotic-associated diarrhea (AAD) or CDI [22–24]. Moreover, Health Canada delivered specific health claims that a specific probiotic formulation, namely, Bio-K+, "help[s] to reduce the incidence of *C. difficile*–associated diarrhea in hospitalized patients." The probiotic is composed of 3 bacterial strains—*Lactobacillus acidophilus* CL1285, *Lactobacillus casei* LBC80R, and *Lactobacillus rhamnosus* CLR2—and has gained acceptance in North America among government officials, healthcare professionals, and the general public. Randomized clinical trials have demonstrated the efficacy of this probiotic formulation in reducing incidences of AAD [25, 26] and CDI in hospitalized patients also undergoing antibiotic therapy [27]. A quasi-experimental observational study conducted in a community hospital on 31 832 inpatients demonstrated the safety of Bio-K+ in healthcare settings [28].

Bio-K+ strains are available in a variety of vehicles, either in the form of freeze-dried powder in enteric coated capsules or as fermented beverages. In the latter, the strains are used to ferment milk, soy, or sprouted brown rice substrates. The resulting commercial probiotic products have been manufactured and marketed by Bio-K Plus International Inc in Canada since 1996 and in the United States since 2000, under the trade name Bio-K+ or Bio-K+ CL1285. The strain compositions and ratio have not been modified since the early developmental stages of the original fermented milk. It is important to note that even if *L. rhamnosus* CLR2 was not labelled before 2014, this strain has always been used in all the probiotic products. In Canada, probiotics can be sold in pharmaceutical dosage forms (eg, tablets, capsules, or powder), as natural health products, or as food (eg, fermented milk, yogurt, cheese).

Health Canada has developed a Probiotics Monograph to help industry stakeholders obtain licences to sell probiotics as natural health products. The Monograph includes information on acceptable health claims pertaining to doses as well as mandatory risk disclosures [29]. The Bio-K+ capsules comply with the Monograph. As for the fermented beverages, they are regulated as food products containing probiotic microorganisms under the guidance document published by the Food Directorate, Health Products and Food Branch of Health Canada [30]. In the United States, capsules are marketed as dietary supplements and fermented beverages as food. The US Food and Drug Administration regulates dietary supplements under a set of directives (Dietary Supplement Health and Education Act of 1994) separate from regulations governing regular food products.

In the 1970s, Dr Francois-Marie Luquet isolated numerous bacterial strains in stools from a newborn infant. He chose 3 strains in particular, based on their strong antimicrobial capacity to fight various pathogenic bacteria. Over ensuing years, Dr Luquet invested considerable time and effort to determine the ideal culture conditions to maximize the gastrointestinal (GI) survival of the strains, and allow them to optimize biological/probiotic functions in humans (François-Marie Luquet, personal communication).

The 3 strains were deposited at the Collection Nationale de Cultures de Microorganismes (CNCM; Institut Pasteur, Paris, France). The CNCM I-4099 strain (CL1285) was identified as *L. acidophilus* by comparing the sequence of the *rrs* gene coding for 16S ribosomal RNA (rRNA) to homologous bacterial sequences using BLAST (www.ncbi.nlm.nih.gov/BLAST). This identification was confirmed by the Analytical Index evaluation of carbohydrate utilization (API 50 CHL) and by hybridization with a species-specific primer (IDL04F 5′-AGGGTGAAGTCGTAACAAGTAGCC-3′ and IDL22R 5′-AACTATCGCTTACGCTACCACTTTGC-3′) [31], providing further evidence that CL1285 belongs to *L. acidophilus* (Bio-K Plus International Inc, internal report).

The CNCM I-3989 (LBC80R) strain belongs to the *L. casei* group by 16S rRNA comparison using BLAST and confirmed to be a member of *Lactobacillus paracasei* by API 50 CHL. Hybridization with a species-specific primer (Y2 5′-CCCACTGCTGCCTCCCGTAGGAGT-3′ and para 5′-CACCGAGATTCAACATGG-3′) [32], provided further evidence that LBC80R belongs to the *L. casei* group, but to the *L. paracasei* species. Repetitive sequence-based polymerase chain reaction (rep-PCR) profiles have been established to differentiate LBC80R from other *L. casei* group isolates (Bio-K Plus International Inc, internal report).

The CNCM I-3990 strain (CLR2) isolate belongs to the *L. casei* group by comparison of the 16S rRNA to homologous bacterial sequences using BLAST and was confirmed to be a member of *L. rhamnosus* by API 50 CHL. Hybridization with a species-specific primer (Y2 5′-CCCACTGCTGCCTCCCGTAGGAGT-3′ and rhamno 5′-TGCATCTTGATTTAATTTTG-3′) [32] provided further evidence that CLR2 belongs to the *L. casei* group, but to the *L. rhamnosus* species. Rep-PCR profiles have been established to differentiate CLR2 from other *L. rhamnosus* isolates (Bio-K Plus International Inc, internal report).

MANUFACTURING PROCESSES OF A PROBIOTIC PRODUCT

The probiotic market has greatly expanded during the last 15 to 20 years, and the number of products available has quite simply exploded due to the increase in consumer demand. Some of the products offer specific therapeutic results supported by compelling scientific evidence [24, 27, 33]. It is widely acknowledged that the functionality and clinical efficacy of probiotics is strain-specific and is affected by different steps in the manufacturing processes [34–36]. Factors such as the fermentation environment (temperature, substrates, pH control, etc), the method used to concentrate and stabilize the probiotic culture (freeze-drying, spray-drying), the final form of the probiotic product, and even storage conditions can all affect the concentration of viable microorganisms [37], as well as gene expression and transcription, thus modulating clinical efficacy [35, 38]. Because probiotics are available in a variety of delivery formats, including fermented beverages, sachets, powder, capsules, tablets, ice cream, cookies, juices, or spreads (reviewed in [38]), it is essential that the matrix have the capability to sustain efficacious dosage right up to the very end of shelf life [18].

The integrity of probiotic bacteria in products on the market is very important. The manufacturing process can stress bacteria, thereby comprising viability. Microorganisms can react differently if lyophilized or placed in other formulations such as milk or yogurt [39]. Also, storage conditions are crucial for stability. In fact, high temperature and water activity are known detriments to long-time stability of probiotic bacteria [40]. A number of studies have examined the stability of probiotic products in the marketplace [41–43]. Recently, Goldstein et al [44] evaluated the stability of 5 commercial probiotic products (including Bio-K+) by measuring the bacterial content and by comparing bacterial quantity to the label information. They concluded that most products are correctly labeled.

Also, standardized manufacturing processes that comply with Good Manufacturing Practice (GMP) guidelines are critical, as any deviation could possibly affect gene expression and efficacy. Strict quality controls including probiotic strain identity and enumeration, purity of raw material, absence of contaminants, and GI longevity are essential to ensuring product quality.

QUALITY CONTROL OF BIO-K+ PRODUCTS

As mentioned above, in Canada, probiotic products are regulated under the Probiotic Monograph as natural health products. As such, they are subjected to stringent and specific requirements established to guarantee quality [29]. Among these specific requirements, Health Canada demands the phenotypic and genotypic identification of all the microorganisms in a product's composition. Moreover, the manufacturer must certify that at least 80% of the microorganism quantity indicated on the product label remains viable until the expiry date of the product. Also, the finished product must comply with all microbial and chemical contamination requirements. Bio-K Plus International Inc manufactures specific probiotic formulation following GMP guidelines and implemented a strict quality control program to ensure consistently efficacious, high-quality products.

The following sections are a description of the procedures recently developed and implemented in the quality control laboratory of Bio-K Plus.

STRAIN STORAGE

The original cultures are stored at the CNCM, whereas the mother strains are kept frozen at −80°C at the manufacturing facility of Bio-K Plus International Inc (Laval, Quebec, Canada). Working cultures are prepared from the mother strains and kept frozen at −80°C until utilization. Identity, purity, and total absence of contaminants are routinely verified on each lot of a working culture.

IDENTIFICATION OF PROBIOTIC STRAINS

Because of the strain-dependent nature of a probiotic, the methods used for strain identification are critically important. Multiple methods exist to identify lactobacilli, based either on their phenotype or their genotype (see reviews in [45, 46]). Phenotypic identification is based on morphology, Gram staining, and biochemical tests such as carbohydrate fermentation profiling. Even if they do not provide identification at the strain level, phenotypic characterization allows confirmation of the purity of probiotic cultures before manufacturing and can help to detect variations of the normal phenotype. On the other hand, molecular-based approaches appear to be robust and rapid tools to confirm phenotypic results. Dazzling advances in molecular biology over the past years have led to the development of sensitive, specific, and affordable tools for microbial identification [45].

Each batch of working culture is subjected to a panel of tests combining phenotypic and genotypic methods to confirm the identity of the strains *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 before industrial production begins. In a first step, working cultures are grown on solid media to confirm the morphology and the purity of the bacterial strains. Phenotypic characterization, based on fermentation profile by API 50 CHL, is then used to identify the strains at the species level. DNA from working cultures is extracted before proceeding to the molecular identification of each probiotic strain. The entire gene coding for the 16S rRNA of each strain is PCR amplified and sequenced with primers designed by Edwards et al [47]. The 16S rRNA sequences are compared to the sequences of

the National Center for Biotechnology Information database. They are then aligned against the 16S rRNA reference sequences of strains *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 deduced from genome sequencing (Bio-K Plus International Inc, internal report). Simultaneously, rep-PCR [48] combining the parallel amplification of the repetitive extragenic palindromic elements and interspersed repetitive sequences $(GTG)_5$ is applied to confirm their identity at the strain level. Fingerprints obtained from working cultures are compared to the *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 reference fingerprints. This rep-PCR method is coupled with bioinformatics analyses using GelCompar II software (version 6.5 created by Applied Maths NV. Available from http://www.applied-maths.com) capable of distinguishing these strains from other strains in the same species. Any anomalies in anticipated results from both phenotypic and genotypic methods result in the rejection of the working cultures.

In addition to the identification of *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 strains, antibiograms are also done routinely to ensure that these strains have not acquired antibiotic resistance during transfer.

ENUMERATION OF TOTAL LACTOBACILLI AND SELECTIVE MEDIA

A probiotic product should contain the number of viable cells needed to deliver the claimed effect throughout the product's entire life span [18]. For example, the Canadian Natural Health Products Directorate (NHPD) recommends a minimum of 10^9 CFU per portion to claim non-strain-specific health effects [18]. Moreover, as mentioned above, a minimum of 80% of the labeled viable microorganism concentration must be present at the end of shelf life [29]. The current industry practice is to enumerate viable bacteria per portion, using the traditional nutritive solid medium for lactic acid bacteria (LAB), that is, de Man, Rogosa and Sharpe (MRS) agar. Bio-K Plus International Inc certifies a minimum of viable probiotic bacteria throughout the shelf life of its products. This quantity is ensured by total bacterial counts on MRS media throughout the manufacturing processes and storage period, until the expiry date using International Dairy Federation method 117:2003 with a modified sample treatment. In addition, selective media, based on the intrinsic antibiotic resistance of strains *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2, have been developed and are in the validation process. These tools could be implemented in the quality control laboratory to perform bacterial counts of individual strains.

ABSENCE OF CONTAMINANTS

Finished products must comply with NHPD quality requirements, in accordance with the Quality of Natural Health Products Guide [49]. That means every batch must be subjected to rigorous evaluation of microbial and chemical contamination in accordance with internationally recognized methods. Retention samples are stored for reanalysis if necessary. The quarantine is maintained for as long as evaluation results do not conform and only released once everything meets the proprietary specifications.

SURVIVAL UNDER GASTROINTESTINAL CONDITIONS

Survival of probiotic bacteria is critical to efficacy. Probiotic bacteria in commercial products must survive passage through the GI tract and reach the gut sufficiently intact [50]. The bacteria must overcome the presence of digestive enzymes, low pH, and bile salt activity. For example, some LAB possess a natural resistance to GI tract conditions [51, 52]. Evaluating the GI resistance is very important, and it can be done using different in vitro analysis: by static [53] or dynamic [54] simulated GI models. Several studies have demonstrated that some products available on the market can well survive passage through the stomach passage and colonize the gut [54, 55]. However, other products, even if they have the same probiotic bacteria, are not able to reach the gut in sufficient number [56]. Depending on the manufacturing process, probiotic bacteria may or may not be able to withstand the stomach environment. Millette et al [57] compared the GI survival of bacteria from 29 different commercial probiotic products using a static simulated GI model. Regarding Bio-K+ probiotic product, it was demonstrated that capsules and fermented milk protect *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 strains during GI transit, whereas most of the other commercial products were not protective. It is important to mention that encapsulated probiotics covered with an adequate enteric coating were resistant to GI passage.

SOME PROPOSED MECHANISMS OF ACTION OF BIO-K+

Growth Inhibition

Clostridium difficile is the major infectious cause of AAD. *Lactobacillus* species, a normal inhabitant of the intestinal microbiota, is thought to play an important role in protection from CDI. Furthermore, it is known that disruption in the composition of intestinal microbiota is a prerequisite for *C. difficile* colonization. The direct inhibitory effect of lactobacilli against various pathogenic bacteria is well known. It may be due to the production of organic acids such as lactic, acetic, or citric acid, or to the production of hydrogen peroxide and bacteriocins [58]. The probiotic formulation *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 has demonstrated a 99% growth inhibition of nosocomial methicillin-resistant *Staphylococcus aureus* strains [59]. Also, Millette et al [60] demonstrated

Table 1. Inhibition Zone Produced by Lactobacilli Strains and Fermented Beverages Against *Clostridium difficile*

Probiotic Strain and Product	*Clostridium difficile* Strain			
	BAA-1803	NAP2	L75–05	S75–01
Lactobacillus casei LBC80R	14	24 ± 1.7	24.7 ± 1.5	24.3 ± 1.2
Lactobacillus rhamnosus CLR2	13.3 ± 0.6	20.3 ± 2.5	23.7 ± 0.6	23.7 ± 0.6
Lactobacillus acidophilus CL1285	0	0	0	0
Fermented milk (Original)	22 ± 2	21.3 ± 1.2	27.7 ± 2.5	28.7 ± 1.5
Fermented milk (Strawberry)	19.7 ± 1.2	25.7 ± 2.5	24.7 ± 0.6	17.7 ± 1.2
Fermented soy	14	16.3 ± 1.5	25.7 ± 0.6	26.3 ± 0.6
Fermented brown rice	16.3 ± 1.5	26.3 ± 0.6	27 ± 1.7	23.3 ± 1.2

Data are presented as mean ± SD of inhibition zone, mm, n = 6.
Abbreviation: SD, standard deviation.

that supernatant from Bio-K+ fermented milk inhibits the growth of other pathogenic bacteria such as *Escherichia coli*, *Listeria monocytogenes*, and *Enterococcus faecium,* and cited the implication of both organic acids and bacteriocin-like inhibitory substances in the antimicrobial activity.

Recently, we conducted a study to demonstrate the in vitro inhibitory effects of *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 against on nosocomial isolates of *C. difficile*. The inhibitory potential was evaluated with an agar spot test using commercial fermented beverages or pure strains [61]. In a second step, a well diffusion assay (WDA) [62] against strains of *C. difficile* was performed to determine the inhibitory effect of the supernatants harvested from the fermented beverages or pure cultures (see Supplementary Data for complete methodology of these experiments).

The results (Table 1) demonstrate that lactobacilli mixed cultures in fermented beverages have a strong inhibition capacity against toxin A/B–producing *C. difficile*. Similar inhibition capacity, although to a lesser extent, was observed in *L. casei* and *L. rhamnosus* pure cultures. *Lactobacillus acidophilus* revealed no antimicrobial activity. In WDA, both pure strains and fermented products showed little or no effect on the *C. difficile* isolates evaluated (Table 2). Pure strains seem to have less effect compared with a fermented product. Original product has the better overall anti–*C. difficile* effect for all strains/products evaluated. However, further analysis should be done to determine the exact nature of the molecules secreted during fermentation by these probiotic strains.

Toxin Neutralization

In addition to direct growth inhibition resulting from the production of antimicrobial molecules, some studies suggest that *Saccharomyces boulardii* or other probiotic bacteria could affect the virulence of *C. difficile* via proteolytic cleavage of the toxins [63]. *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R, *L. rhamnosus* CLR2, and fermented beverage supernatants have been tested against *C. difficile*–mediated cytotoxicity on human enterocyte–like Caco-2 and HT-29 cell models and compared with other LAB. Cell-free supernatant harvested from pure strains of lactobacilli (CFS-LAC) or from the commercial fermented beverages were submitted to different treatments (temperature and pH gradients, enzymatic digestion, protein precipitation, and concentration) to verify their efficacy to protect enterocytes from cytotoxic effect of CFS harvested from *C. difficile* strains (CFS-CD) (see Supplementary Data for complete methodology of these experiments).

Figure 1*A* shows the normal aspect of Caco-2 cells (untreated control), whereas incubation with CFS-CD from *C. difficile* ATCC 9689 caused a cytopathic effect (rounding and cell detachment) on Caco-2 cells (Figure 1*B*). Caco-2 cells are protected from cytotoxicity induced by CFS-CD when it was previously treated with CFS-LAC of *L. casei* LBC80R (Figure 1*C*). However, not all lactobacilli are able to produce CFS-LAC with anticytotoxicity effect in the conditions evaluated. For example,

Table 2. Inhibition Zone Produced by Cell-Free Supernatants Harvested From Lactobacilli Strains or Fermented Beverages Against *Clostridium difficile*

Probiotic Strain and Product	*Clostridium difficile* Strains			
	BAA-1803	NAP2	L75–05	S75–01
Lactobacillus casei LBC80R	0	2	3 ± 1.5	6.7 ± 3.9
Lactobacillus rhamnosus CLR2	0	2	2.5 ± 0.5	3.7 ± 2.4
Lactobacillus acidophilus CL1285	0	0	2 ± 1.3	3.3 ± 2.5
Fermented milk (Original)	0	9	2	6.3 ± 0.5
Fermented milk (Strawberry)	1	3	2 ± 1.5	3.7 ± 2.4
Fermented soy	1	2	1.7 ± 0.5	7.2 ± 1.3
Fermented brown rice	1	3	3.5 ± 0.5	6.3 ± 2.6

Data are presented as mean ± SD of inhibition zone, mm, n = 6.
Abbreviation: SD, standard deviation.

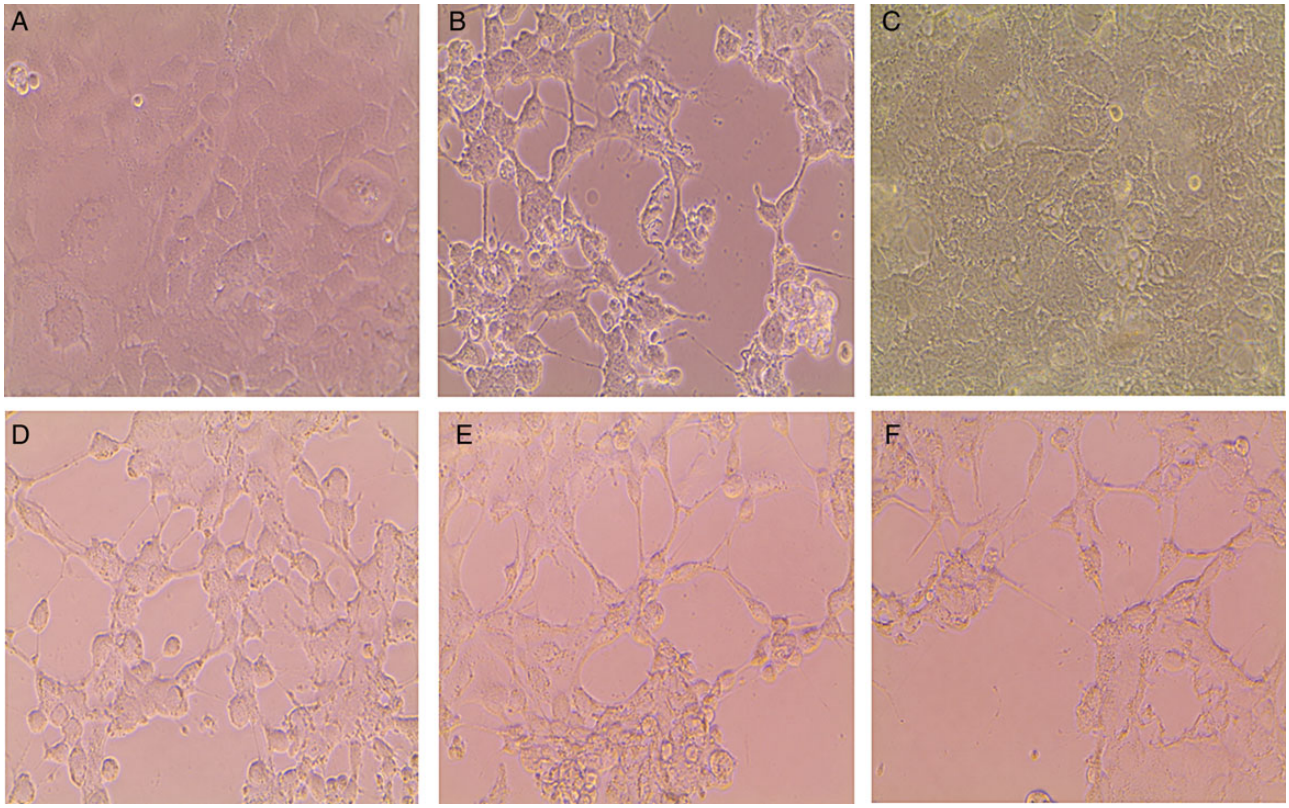

Figure 1. Images of Caco-2 cells incubated at 37°C for 24 hours in 5% CO_2 and 95% humidity in the presence of lactobacilli cell-free supernatant (CFS-LAC) and supernatant of *Clostridium difficile* ATCC 9689 (CFS-CD). *A*, Negative control: Caco-2 cells alone. *B*, Cytotoxicity control: Caco-2 cells with CFS-CD. *C*, Caco-2 cells with CFS-LAC from *Lactobacillus casei* LBC80R and CFS-CD. *D*, Caco-2 cells with CFS-LAC from *Lactobacillus acidophilus* ATCC 832 and CFS-CD. *E*, Caco-2 cells with CFS-LAC from *L. casei* ATCC 4007 and CFS-CD. *F*, Caco-2 cells with CFS-LAC from *L. acidophilus* ATCC 4796 and CFS-CD.

L. acidophilus ATCC 832 or ATCC 4007 and *L. casei* ATCC 4796 were not able to protect Caco-2 cells from damage induced by CFS-CD (Figure 1*D*–*F*, respectively). A similar anticytotoxicity pattern was observed when replacing Caco-2 by HT-29 cells (data not shown). The lack of protection showed by some LAB brings evidence that not all lactobacilli can prevent *C. difficile*–induced cytotoxicity.

Table 3 shows results of the toxin neutralization assay of various LAB. Of the 13 LAB evaluated, 9 strains seemed to exert an anticytotoxic effect including *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2. The Bio-K+ fermented milk was also effective in neutralizing *C. difficile* toxins.

To determine the type of component secreted in CFS-LAC, we submitted CFS-LAC to various treatments (see Supplementary Data for complete methodology of these experiments). Results indicate that heat treatment of CFS-LAC, even at 99°C for 1 hour, did not reduce anticytotoxic activity (result not shown). Then, we purified and concentrated the protein moieties of CFS-LAC and verified its resulting anticytotoxic activity. However, we observed no improvement of anticytotoxicity capacity of CFS-LAC, indicating that the inhibitory activity may be due to another mechanism rather than a protein (results not shown). Moreover, based on previous studies indicating that *S. boulardii* secretes a protease with the ability to degrade *C. difficile* toxins [63], we treated CFS-LAC with trypsin and proteinase K. Results show that supernatant produced from culture of *S. boulardii* and treated with both proteases lost its anticytotoxic activity, whereas CFS-LAB from lactobacilli were not affected by the proteolytic treatment (results not shown). Finally, to verify the role of the acidity of CFS-LAC, the pH of CFS-LAC was neutralized with NaOH (1 N). We observed a complete loss of anticytotoxic activity at pH >5 (results not shown). Then, to further confirm the role of organic acids in that activity, we evaluated the anticytotoxic activity of CFS prepared from sterile dairy substrate enriched with increasing concentrations of lactic acid (CFS-AM). Results indicated that *C. difficile*–mediated cytotoxicity was impaired by CFS-AM containing ≥1% of lactic acid. At these levels, the pH value was <4.2 (Table 4). In our experiments using CFS-LAC, toxin neutralization occurred at pH <4.8 (Table 3). Only *Bifidobacterium longum* ATCC 15708 had a low pH value (4.17) while

Table 3. Cytotoxicity Assay of Different Cell-Free Supernatant Against *Clostridium difficile* Cell-Free Supernatant on Caco-2 Cells

Cell-Free Supernatant	pH	*Clostridium difficile* Strains					
		CHUM	L75–05	S75–01	BAA-1803	NAP2	ATCC 9689
Bifidobacterium longum ATCC 15708	4.17	2	2	2	2	2	2
Lactobacillus acidophilus ATCC 832	4.93	3	3	3	3	3	3
L. acidophilus ATCC 53544	4.34	1	1	1	1	1	1
L. acidophilus ATCC 43121	3.81	1	1	1	1	1	1
L. acidophilus ATCC 4355	4.71	2	2	2	2	2	3
L. acidophilus ATCC 53671	4.94	3	3	3	3	3	3
L. acidophilus CL1285	4.31	1	1	1	1	1	1
Lactobacillus casei ATCC 393	3.97	1	1	1	1	1	1
L. casei ATCC 4007	4.76	3	3	3	3	3	3
L. casei ATCC 944	4.07	2	2	2	2	2	2
L. casei LBC80R	3.74	1	1	1	1	1	1
Lactobacillus rhamnosus ATCC 4796	4.86	3	3	3	3	3	3
L. rhamnosus CLR2	3.98	1	1	1	1	1	1
Bio-K+ fermented milk	4.01	1	1	1	1	1	1
CFS-CD	. . .	3	3	3	3	3	3

Cell morphology data are presented as follows: 1, cells morphologically healthy; 2, cells morphologically different but healthy; 3, dead, rounded, and detached cells.
Abbreviation: CFS-CD, cell-free supernatant prepared from a 24-hour culture of *Clostridium difficile* ATCC 9689 (negative control).

showing a weak toxin neutralization capacity. Qa'Dan et al [64] suggest that toxin binding to the receptor can be compromised by an acidic environment, thus reducing *C. difficile*–mediated cytotoxicity. Our experiments brought evidence that CFS produced from pure strains or Bio-K+ finished products reduced *C. difficile*–mediated enterocytotoxicity in cell culture models. This nhibition may be due by the acidic environment created by LAB. However, the antagonism could also be caused by a diminution of synthesis or secretion of both toxins. Determination of extracellular toxin concentration in *C. difficile*–spent culture supernatant or a study of the impact by LAB on virulence gene should be conducted to determine other plausible mechanisms of action.

When combined, these results demonstrate that CDI prevention could be due in part to secretion of antimicrobial and toxin neutralization molecules by lactobacilli strains. Obviously, the impact of the probiotic on the immune system or the intestinal microbiota could also explain the mechanisms of action.

Table 4. Effect of Milk Substrates Acidified With Increasing Concentrations of Lactic Acid on the *Clostridium difficile*–Mediated Cytotoxicity on Caco-2 Cells

Concentration of Lactic Acid, %	pH	*Clostridium difficile* Strain			
		L75-05	S75-01	BAA-1803	CHUM
0.5	4.97	3	3	3	3
1	4.18	2	2	2	2
2	3.58	1	1	1	1
3	3.04	1	1	1	1
4	2.64	1	1	1	1
Control (CSF-CD)	. . .	3	3	3	3

Cell morphology data are presented as follows: 1, cells morphologically healthy; 2, cells morphologically different but healthy; 3, dead, rounded, and detached cells.
Abbreviation: CFS-CD, cell-free supernatant prepared from a 24-hour culture of *Clostridium difficile* ATCC 9689 (negative control).

CONCLUSIONS

Taken as a co-therapy with antibiotics, a specific probiotic combination composed of *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 can reduce side effects such as diarrhea, bloating, and cramps. Also, clinical trials conducted with this specific probiotic formulation demonstrated safety and efficacy in reducing the incidences of AAD and CDI in hospitalized patients. Appropriate and standardized manufacturing processes respecting GMP guidelines and rigorous quality control are crucial to guaranteeing bacterial identity, viability, and GI survival throughout shelf life. Also, the results presented in this study should contribute to a better comprehension of the mechanisms of action of specific probiotic products or their isolated active culture against *C. difficile* growth and toxicity. However, more studies are needed to understand the impact of *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 on the immune system or the intestinal microbiome. Also, colonization, persistence,

and host–microbe interactions of these lactobacilli should be investigated carefully to complete the mechanisms of action of this probiotic formulation.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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