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Immunomodulation of Antiretroviral Drug-Suppressed Chronic HIV-1 Infection in an Oral Probiotic Double-Blind Placebo-Controlled Trial

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Abstract

A putative source of inappropriate immune activation that drives human immunodeficiency virus (HIV)-1 immunopathogenesis is the gastrointestinal tract. Even with effective antiretroviral treatment, residual activation persists. We hypothesized that an oral probiotic could improve the residual immune activation in chronic treated HIV-1 infection, and tested a *Bacillus coagulans* GBI-30, 6086 capsule probiotic in HIV-1-infected persons with suppressed viremia on stable antiretroviral therapy in a 3-month double-blind placebo-controlled trial (10 probiotic, 7 placebo). The Gastrointestinal Symptom Rating Scale (GSRS) was administered monthly. Blood was tested at the start and end of placebo/probiotic administration for viremia, $CD4^+$ T cell percentage/concentration, soluble (s)CD14, soluble intestinal fatty acid binding protein, sCD163, D-dimer, C-reactive protein (CRP), interleukin-8, and tumor necrosis factor-α. All participants maintained viremia <40 RNA copies/ml. The probiotic was safe and well tolerated, and appeared to improve chronic gastrointestinal symptoms. Its administration was associated with a significant increase in the percentage of blood $CD4^+$ T cells compared to placebo ($+2.8\%$ versus -1.8%, $p=0.018$) although $CD4^+$ T cell concentrations were generally unchanged in both groups. None of the biomarkers showed significant changes on probiotic treatment or between-group differences in change (although significance was borderline for a greater sCD163 drop in the probiotic versus placebo group, $p=0.05$). Some biomarkers showed significant correlations to each other, particularly D-dimer with CRP and sCD14 with tumor necrosis factor (TNF)-α. These data demonstrate the safety and possible benefit of this probiotic for residual inflammation in treated HIV-1 infection, although further study will be required to determine the immune pathways involved.

Introduction

Untreated human immunodeficiency virus type 1 (HIV-1) infection generally leads to progressive loss of $CD4^+$ T cells, and growing evidence indicates a potential central role of gut-associated lymphoid tissue. This compartment, which contains the majority of total body lymphocytes,[1] is massively depleted by direct HIV-1 infection of $CD4^+$ T cells in early acute infection,[2] does not recover after acute infection, and poorly reconstitutes with combination antiretroviral treatment (cART) that restores blood $CD4^+$ T cell levels.[3] It has been suggested that this persisting defect leads to systemic translocation of gut bacteria causing the immune activation that drives chronic disease progression.[4] Alternatively, the depletion of key regulatory $CD4^+$ iNKT cells in the gut[5] or ongoing viral replication in this compartment[6] drives inappropriate immune activation.

As a major reservoir of immune cells that are constantly exposed to antigens from food, microbial flora, and pathogens, the gut is a major determinant of systemic immune processes. Gut cells interact with antigens through pattern recognition receptors such as toll-like receptors (TLRs), setting the net state of inflammation.[7] Many normal bacterial flora provide antiinflammatory signals, while pathogens can drive inflammatory danger signals. It is evident that the complex gut flora profoundly influences health and disease; for example, different profiles are associated with obesity and nonobesity in monozygotic twins,[8] and vary with diseases ranging from autoimmunity[9] to cancer.[10]

Administering probiotics to alter gut microbial flora has been considered for health and disease. In the simian immunodeficiency virus (SIV)-macaque model of AIDS, probiotic administration to antiretroviral-treated macaques increased reconstitution of colonic $CD4^+$ T cells, reducing inflammation-

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associated fibrosis and increasing antigen-presenting cell frequency and function.[11] For human HIV-1 infection probiotic data are very limited, and probiotics have not been tested in infected persons on effective cART who have significant residual immune activation. Here we study the safety and immune effects of the probiotic *Bacillus coagulans* GBI-30, 6086 (Ganedin**BC**^{30}), a gram-positive spore-forming bacterium with potential immunomodulatory effects.[12–15]

Materials and Methods

Study design

This was a randomized, double-blind, placebo-controlled study in persons with chronic HIV-1 infection who were receiving effective cART. Twenty-four subjects were randomized to placebo or probiotic and monitored for 90 days. The study was performed under a BioMed Central Institutional Review Board (San Diego, CA) approved protocol and registered with ClinicalTrials.gov (NCT01184456).

Study subjects

Adults with documented HIV seropositivity were recruited from greater metropolitan Los Angeles. Inclusion criteria included blood $CD4^+$ T cell counts ≥ 250 cells/mm^3 and plasma viremia measurements < 50 HIV RNA copies/ml for at least 6 months, high functioning Karnofsky score $\geq 60\%$, baseline (within 30 days of study entry) blood values of absolute neutrophils $> 1{,}000$ cells/mm^3, hemoglobin > 9 g/dl, platelets $> 75{,}000$/mm^3, creatinine $< 1.5\times$ the upper limit of normal, AST and ALT $< 3\times$ the upper limit of normal, and bilirubin < 2 mg/dl. Exclusion criteria included pregnancy, active infections, treatment with other probiotics, any antibiotic therapy within 30 days, immunosuppressive drug treatments, malabsorption syndrome, and liver disease. The first subject and last subjects were enrolled on August 9, 2010 and February 18, 2011, with the last follow-up on May 13, 2011.

Probiotic administration

For 24 participants 1:1 randomization was predetermined to receive placebo or probiotic (Ganedin**BC**^{30}). Subjects received a daily capsule containing either placebo or 2 billion colony-forming units of BC^{30} (*Bacillus coagulans* GBI-30, 6086), with instructions to swallow the capsule with water at the same time each day, regardless of meals. Adherence to the study protocol was determined by caplet counts. Both care providers and participants were blinded.

Clinical follow-up

Subjects received physical examinations at days 0, 30, 60, and 90. At each visit they were administered the Gastrointestinal Symptoms Rating Scale (GSRS) survey.[16] Blood was obtained at days 0 and 90 for assessment of $CD4^+$ T cell counts, viremia, and biomarker testing.

Proinflammatory blood biomarkers

Testing for serum C-reactive protein (CRP), interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α were performed by IBT Laboratories (Lenexa, KS). Serum D-dimer testing was performed by Saint Luke's Regional Laboratory (Kansas City, MO). Serum lipopolysaccharide (LPS) testing was performed by Viracor-IBT Laboratories (Lee's Summit, MO). Serum soluble CD14 (sCD14) and serum soluble CD163 (sCD163) levels were determined using the Human Quantikine ELISA assay (R&D Systems, Minneapolis, MN). Serum intestinal-type fatty acid binding protein (sI-FABP) was measured using the Human FABP-2 DuoSet ELISA (R&D Systems, Minneapolis, MN). Unfortunately, LPS, IL-1β, and IL-6 were not detected in most samples (< 0.05 EU/ml, < 7.1 pg/ml, and < 0.6 pg/ml respectively), and therefore not analyzed.

Statistics

Within-group changes from days 0 to 90 were evaluated using paired Student's *t*-test (two-tailed, nonequal distributions). Between-group comparisons were performed using Wilcoxon rank-sum test. Correlations between variables were evaluated using Spearman rank correlation. A p-value ≤ 0.05 was considered significant (unadjusted for multiple comparisons in this pilot study).

Results

Participant demographics and clinical symptoms

Twenty-four subjects were randomized 1:1 to receive probiotic or placebo for 90 days. Seven did not complete the study (all men), including two receiving probiotic (one withdrew due to lack of transportation and the other did not follow-up) and five receiving placebo (one withdrew because he felt no benefit and suspected he was receiving a placebo and the others did not follow-up); none of these subjects withdrew due to complaints related to probiotic or placebo.

The remaining 17 participants (10 probiotic and seven placebo) who completed the study had reasonably comparable ages, durations of cART including time on their most current cART regimen, nadir $CD4^+$ T cell counts and percentages, and baseline $CD4^+$ T cell counts and percentages (Table 1). No serious adverse events were reported. The GSRS was monitored at baseline (0) and 4, 8, and 12 weeks after the start of probiotic or placebo. Gastrointestinal symptoms were low for both groups at baseline and only mild symptoms were reported during the study. In the probiotic group 3/10 reported bloating. In the placebo group, 2/7 reported increased flatulence and 1/7 reported increased diarrhea. Summed GSRS scores for all categories (diarrhea, indigestion, constipation, abdominal pain, reflux) and the combined totals were examined (Fig. 1). For in-group comparisons of symptoms at 4, 8, or 12 weeks compared to baseline (paired Student's *t*-test), there were no significant differences in the placebo group. In the probiotic group, there were significant reductions in constipation scores at weeks 4 and 8 ($p = 0.037$ and $p = 0.005$, respectively), and total scores at weeks 4 and 8 ($p = 0.050$ and $p = 0.036$, respectively). Between groups, there were significant differences only at week 8 for the constipation and combined total categories (each $p = 0.049$). Overall, these findings suggest potential benefit in gastrointestinal symptoms due to probiotic administration.

Increased percentage of $CD4^+$ T cells was observed in the probiotic group

Blood $CD4^+$ T cells at baseline and 90 days were assessed. For both placebo and probiotic groups, median absolute $CD4^+$

Table 1. Demographics of the Participants

Group	ID	Age	Sex	Days on cART	Days current cART	Current cART	$CD4^+$ T cell nadir/mm^3	$\%CD4^+$ T cell nadir	Baseline $CD4^+$ T cells/mm^3	Ending $CD4^+$ T cells/mm^3	Baseline $\%CD4^+$ T cell	Ending $\%CD4^+$ T cell
Probiotic	GAN02	37	M	4,506	1,161	TDF/FTC/ATV/r	17	4.0%	335	343	23.9%	26.4%
	GAN04	42	M	1,679	449	TDF/FTC/DRV/r	77	N/A	452	494	21.5%	24.7%
	GAN07	59	M	5,186	771	RAL/FTC/NVP	139	19.8%	449	366	26.4%	30.5%
	GAN09	50	M	2,499	980	TDF/FTC/LPV/r	168	8.0%	377	377	14.5%	13.0%
	GAN11	40	M	1,542	251	ABC/DRV/r/TDF/FTC	18	3.5%	278	510	25.3%	30.0%
	GAN12	45	M	3,533	306	TDF/FTC/EFV	N/A	N/A	571	757	40.8%	44.5%
	GAN13	48	M	4,336	835	ABC/3TC/NVP	191	N/A	521	622	41.4%	44.4%
	GAN15	72	M	5,087	589	TDF/FTC/ATV/r	51	25.4%	572	505	28.6%	29.7%
	GAN17	59	M	3,029	1,128	ATV/r/RAL	180	18.0%	517	686	28.7%	31.2%
	GAN23	52	M	2,170	1,450	ABC/3TC/FPV/r/NVP	384	24.0%	818	709	37.2%	37.3%
	Median	49		3,281	803		139	13.0%	485	508	27.5%	30.3%
	SD	11		1,375	395		115	8.8%	152	150	8.6%	9.4%
Placebo	GAN01	53	M	262	356	TDF/FTC/EFV	374	17.8%	600	631	25.0%	28.7%
	GAN03	51	M	3,771	1,552	TDF/FTC/EFV	371	23.0%	644	580	46.0%	44.6%
	GAN10	37	M	1,066	658	TDF/FTC/RAL	252	19.4%	432	351	27.0%	19.5%
	GAN14	52	M	4,353	460	ABC/3TC/RTV	N/A	N/A	416	486	19.8%	18.0%
	GAN18	49	M	3,784	791	ABC/3TC/TDF/DRV	7	2.0%	303	325	27.5%	25.0%
	GAN21	46	M	3,957	692	ATV/r/RAL	194	19.4%	290	333	24.2%	23.8%
	GAN24	51	F	1,704	794	ABC/3TC/ATV	34	4.3%	649	963	29.5%	27.5%
	Median	51		3,771	692		223	18.6%	432	486	27.0%	25.0%
	SD	6		1,645	387		159	8.8%	155	229	8.3%	8.8%

TDF, tenofovir; FTC, emtricitabine; ATV, atazanavir; /r, low dose ritonavir; DRV, darunavir; RAL, raltegravir; LPV, lopinavir; ABC, abacavir; EFV, efavirenz; NVP, nevirapine; 3TC, lamivudine; FPV, fosamprenavir; RTV, ritonavir; NA, not available.

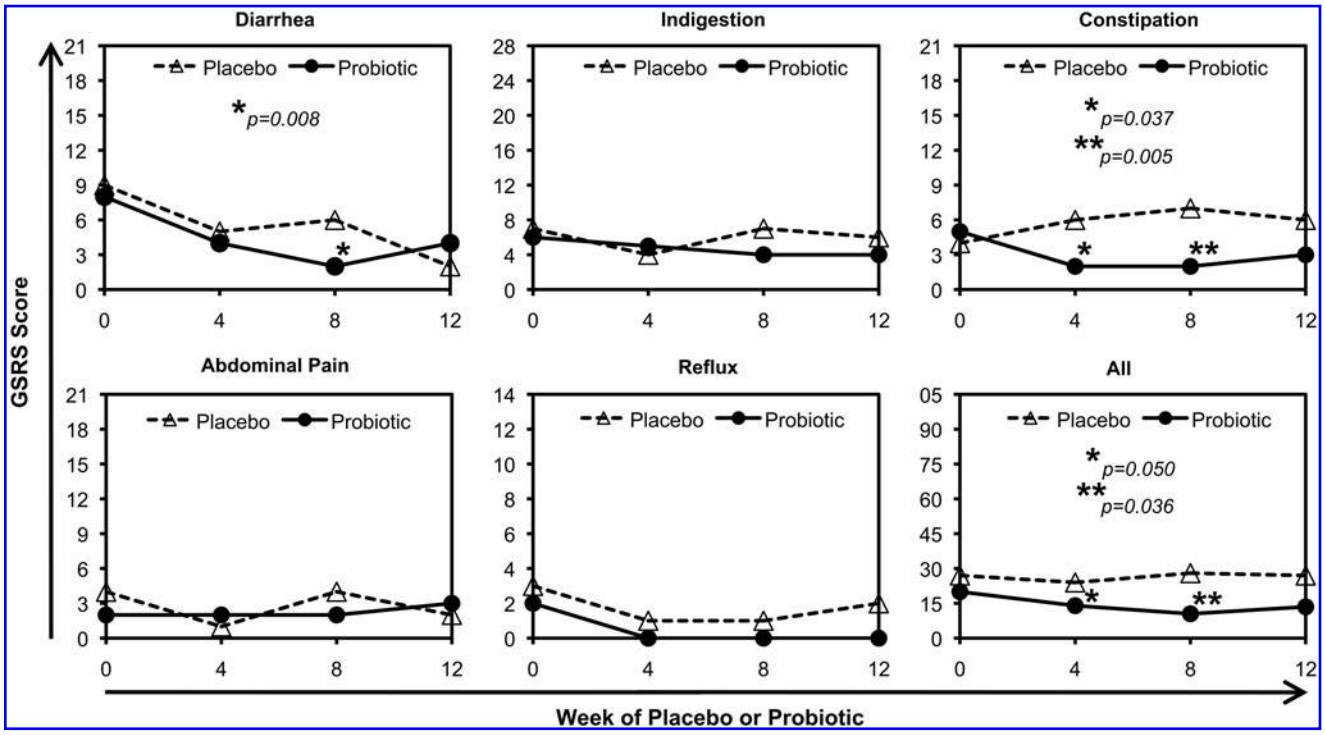

FIG. 1. Gastrointestinal symptoms. Median scores from the Gastrointestinal Symptom Rating Scale (GSRS) for five categories of symptoms and all combined are plotted for placebo ($n=7$) and probiotic ($n=9$) groups. Subject GAN 009 was excluded because survey information was not provided for two study visits. The maximal value on the *y*-axis is the highest possible score on the GSRS. *Asterisks* indicate probiotic group significant differences from week 0 (paired Student's *t*-test); there were no significant differences from week 0 in the placebo group. Comparisons of placebo versus probiotic groups showed significant differences only for the constipation category at week 8 ($p=0.049$) and the combination of all symptoms at week 8 ($p=0.049$).

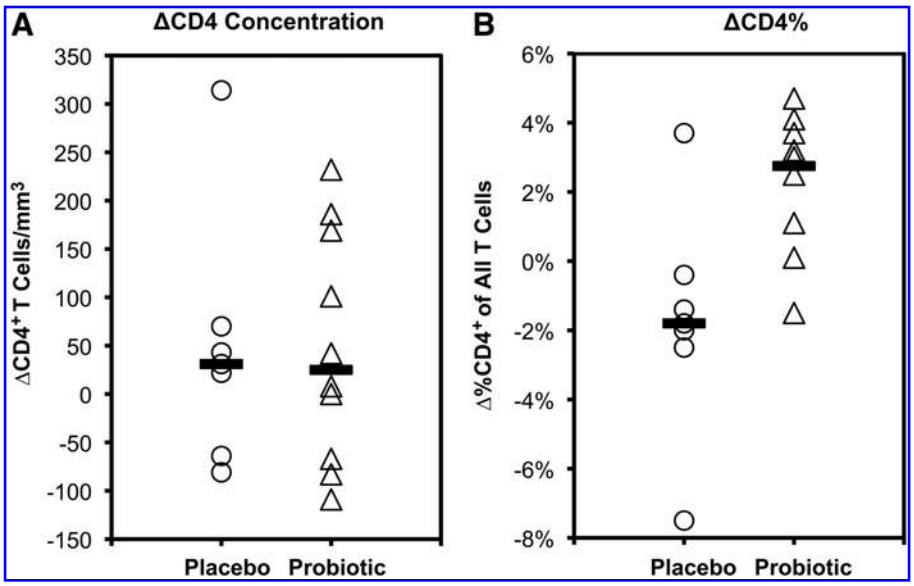

FIG. 2. Changes in blood $CD4^+$ T cell counts and percentages during the study. For each group, the changes in blood $CD4^+$ T cell counts **(A)** and percentages **(B)** during the 90 days of placebo or probiotic administration are plotted. Each point represents one individual, and the bars indicate the medians for each group.

T cell counts for both groups were minimally changed, although there was variability between individuals ranging from −81 to +315 (median +31) cells/mm^3 for the placebo group and −109 to +232 (median +25) cells/mm^3 for the probiotic group (Fig. 2A). However, changes in $CD4^+$ percentage of total T cells were significantly different between placebo and probiotic groups (Fig. 2B), with medians of −1.8% (range −7.5% to +3.7%) versus +2.8% (range −1.5 to +4.7%), respectively ($p=0.018$). Further taking $CD4^+$ T cell measurements over the prior year before the study into account for the baseline, the slopes of $CD4^+$ T cell counts were similar but the percentages increased more in the probiotic group compared to the placebo group (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/aid). Viremia remained <40 copies HIV-1 RNA/ml plasma in all subjects during the study, indicating that gross changes in viral replication did not explain these differences. These findings suggest that probiotic administration had an immunologic impact.

Proinflammatory blood biomarkers did not change consistently

To evaluate for potential inflammatory correlates, several blood biomarkers were evaluated (Fig. 3). There were no

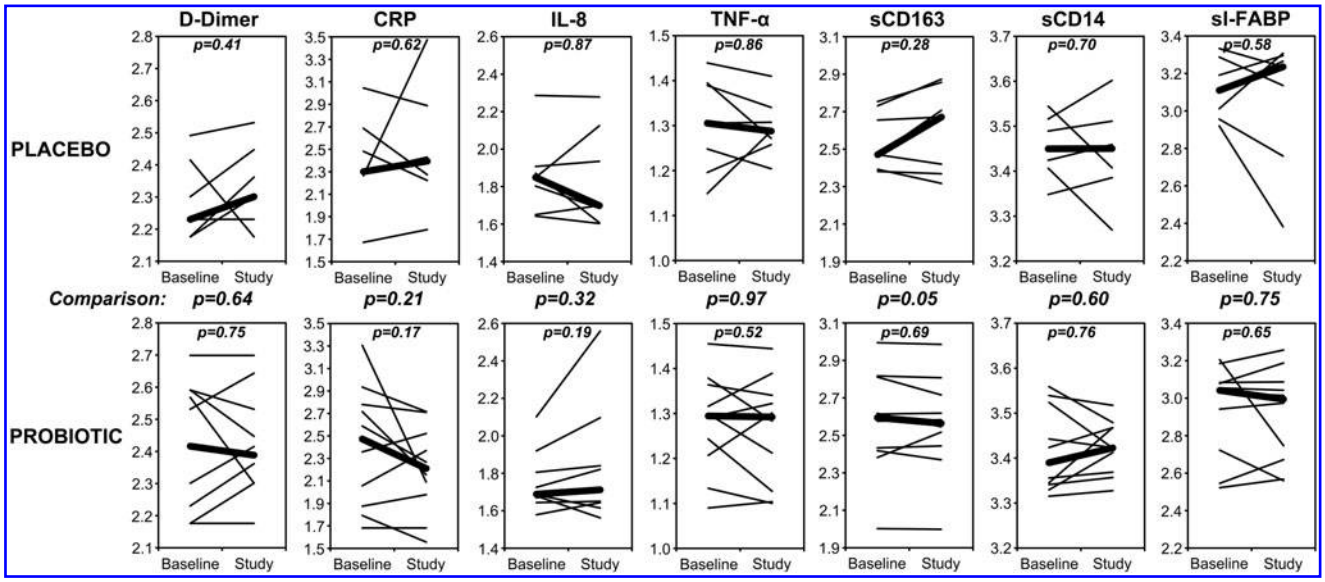

FIG. 3. Levels of blood biomarkers during the study. For each individual, the blood concentrations of the indicated biomarkers are plotted for values at start to end of placebo/probiotic administration (*thin lines*), on $\log_{10}$ scales. *Thick lines* indicate the medians for each group. Not shown are lipopolysaccharide (LPS), interleukin (IL)-1β, and IL-6 because these were not detected (<0.05 EU/ml, <7.1 pg/ml, and <0.6 pg/ml, respectively). Changes in these markers within groups were not statistically significant. Comparisons of changes between groups approached significance ($p<0.2$) only for soluble (s)CD163 ($p=0.05$). Units were μg/ml for D-dimer, mg/dl for C-reactive protein (CRP), pg/ml for IL-8, pg/ml for tumor necrosis factor (TNF)-α, ng/ml for sCD163, μg/ml for sCD14, and ng/ml for serum intestinal-type fatty acid binding protein (sI-FABP).

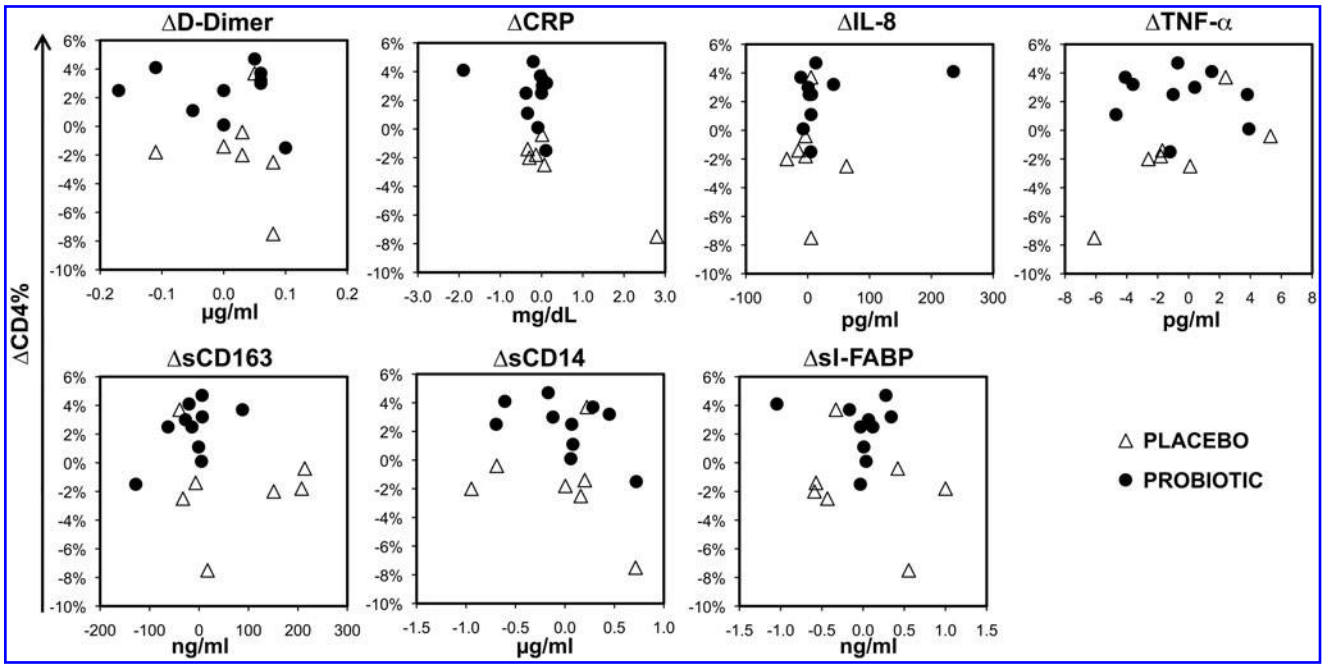

FIG. 4. Changes in percentage of $CD4^+$ T cells versus changes in proinflammatory blood biomarkers during the study. Changes in the biomarkers and CD4% T cells between the start and end of placebo/probiotic administration are plotted against each other.

statistically significant changes in D-dimer, C-reactive protein (CRP), or intestinal-type fatty acid binding protein (I-FABP) for either the placebo or probiotic group. Between group comparisons of changes in these markers approached statistical significance only for sCD163 ($p = 0.094$). Furthermore, comparisons of changes in these markers to changes in the percentage of $CD4^+$ T cells did not show any significant correlations (Fig. 4). These results indicate that the facets of systemic inflammation reflected by these markers are not overtly affected by administration of probiotic, and suggest that the observed change in percentage of $CD4^+$ T cells in the probiotic group is not simply relatable to changes in the inflammatory processes reflected by these blood markers.

Several proinflammatory blood biomarkers were associated with each other

Given the mixed literature regarding the prognostic value of different biomarkers in the pathogenesis and complications of HIV-1 infection, the data were analyzed for associations between biomarkers (Table 2 and Supplementary Fig. S2). Notably, several markers significantly correlated. Across measurements at both time points (Table 2A and

Table 2. Correlations Between Changes in Blood Biomarker Levels Over the Course of the Study

	D-dimer	*CRP*	*IL-8*	*TNF-α*	*sCD163*	*sCD14*	*I-FABP*
A. Absolute Values							
D-dimer	N/A	0.51 (0.002*)	0.00 (0.98)	0.21 (0.23)	0.26 (0.15)	−0.05 (0.80)	−0.35 (0.044*)
CRP	0.51 (0.002*)	N/A	0.28 (0.11)	0.30 (0.087)	−0.01 (0.97)	0.16 (0.37)	0.13 (0.47)
IL-8	0.00 (0.98)	0.28 (0.11)	N/A	0.28 (0.12)	−0.04 (0.82)	0.26 (0.12)	0.36 (0.035*)
TNF-α	0.21 (0.23)	0.30 (0.087)	0.28 (0.12)	N/A	0.51 (0.002*)	0.34 (0.050*)	0.12 (0.49)
sCD163	0.26 (0.15)	−0.01 (0.97)	−0.04 (0.82)	0.51 (0.002*)	N/A	0.31 (0.078)	−0.13 (0.46)
sCD14	−0.05 (0.80)	0.16 (0.37)	0.26 (0.12)	0.34 (0.050*)	0.31 (0.078)	N/A	0.11 (0.52)
I-FABP	−0.35 (0.044*)	0.13 (0.47)	0.36 (0.035*)	0.12 (0.49)	−0.13 (0.46)	0.11 (0.52)	N/A
B. Changes in Values							
D-dimer	N/A	0.84 (0.00002*)	0.08 (0.77)	−0.26 (0.31)	−0.14 (0.59)	0.62 (0.008*)	0.03 (0.90)
CRP	0.84 (0.00002*)	N/A	0.12 (0.64)	−0.15 (0.56)	−0.12 (0.65)	0.65 (0.004*)	0.33 (0.20)
IL-8	0.08 (0.77)	0.12 (0.64)	N/A	0.11 (0.69)	−0.39 (0.13)	0.10 (0.71)	0.07 (0.78)
TNF-α	−0.26 (0.31)	−0.15 (0.56)	0.11 (0.69)	N/A	−0.28 (0.27)	−0.50 (0.042*)	−0.06 (0.82)
sCD163	−0.14 (0.59)	−0.12 (0.65)	−0.39 (0.13)	−0.28 (0.27)	N/A	−0.26 (0.30)	0.46 (0.065)
sCD14	0.62 (0.008*)	0.65 (0.004*)	0.10 (0.71)	−0.50 (0.042*)	−0.26 (0.30)	N/A	0.02 (0.94)
I-FABP	0.03 (0.90)	0.33 (0.20)	0.07 (0.78)	−0.06 (0.82)	0.46 (0.065)	0.02 (0.94)	N/A

For each of the listed biomarkers, Spearman rank correlation rho values (p-value) are given for **(A)** intraindividual comparisons of blood levels including both time points at the start and finish of the study and **(B)** intraindividual comparisons of changes of blood levels between the start and finish of placebo/probiotic administration.

CRP, C-reactive protein; IL, interleukin; TNF, tumor necrosis factors sCD163, soluble CD163; sCD14, soluble CD14; I-FABP, intestinal-type fatty acid binding protein.

*$p \leq 0.05$.

Supplementary Fig. S2A), D-dimer was strongly correlated with CRP and TNF-α was strongly correlated with sCD163. Borderline correlations were seen for D-dimer with sI-FABP, sI-FABP with IL-8, TNF-α with sCD14, and TNF-α with sCD163; the two monocyte/macrophage markers sCD14 and sCD163 showed only a trend for a correlation ($p = 0.078$). However, comparison of changes in biomarkers between the start and end of the study (Table 2B and Supplementary Fig. S2B) showed strong correlations for D-dimer with CRP ($p = 0.00002$), CRP with sCD14 ($p = 0.004$), and sCD14 with D-dimer ($p = 0.008$), a borderline correlation for sCD14 with TNF-α, and no correlation for sCD14 with sCD163 ($p = 0.30$). These findings indicate that some of these markers reflect the same inflammatory processes.

Discussion

The gastrointestinal tract is the major reservoir of activated $CD4^+$ T cells and is centrally involved in HIV-1 pathogenesis. Regardless of infection route, this compartment is the first major site of viral replication in acute infection, sustaining massive loss of $CD4^+$ T cells.[2] It is believed that is a major contributor to the inappropriate chronic immune activation that drives progressive immunodeficiency. This activation does not fully normalize with cART, and contributes to increased morbidity such as premature atherosclerotic disease seen in treated persons. At least three mechanisms have been proposed for the role of the gastrointestinal tract: reduced containment of bowel flora and systemic bacterial translocation,[4] ongoing viral replication despite cART,[6] and loss of key regulatory $CD4^+$ T cells that downmodulate immune activation.[5]

Bowel flora play a key immunomodulatory role in the gastrointestinal tract. Bacterial components interact with pattern recognition receptors such as TLRs, which give proinflammatory or antiinflammatory immune signals depending on the receptor.[17] Certain normal flora promote mucosal integrity and reduce inflammation, and perturbations of flora have been associated with various immune disorders.[17] Thus altering the bacterial flora offers a potential avenue to intervene. This pilot study was undertaken to explore the safety and effects of a probiotic in persons with chronic HIV-1 infection who were on cART with viremia to <50 HIV-1 RNA copies/ml, considered to be optimally treated, given that such persons still have residual immune activation that likely mediates the heightened risk of complications such as premature atherosclerotic cardiovascular disease.

Bacillus coagulans is not known to be pathogenic, and the BC[30] probiotic utilized in our study was delivered in capsules as inert spores that survive stomach acid and bloom in the gastrointestinal tract,[18] and has been safe in human studies.[19,20] Moreover, this formulation is stable indefinitely at room temperature, in contrast to some other probiotics. Symptomatically, administration has been shown to improve postprandial gas-related intestinal symptoms[21] and to reduce abdominal pain, bloating, and diarrhea in patients with irritable bowel syndrome.[22] Given these properties, this probiotic was selected for testing. Our findings were similar in observing this probiotic to be safe and well-tolerated. In this pilot study, the improvement in constipation scores was consistent with prior observations,[21,22] although the small sample size and relatively low baseline gastrointestinal symptoms of these generally healthy individuals limited our power to detect a clinical impact.

A handful of prospective studies have examined other probiotics in persons with HIV-1 infection with mixed clinical and immunologic results. A placebo-controlled study of 39 American adults by Wolf *et al.* administered 3 weeks of freeze-dried *Lactobacillus reuteri* to persons with $CD4^+$ T cell counts >400 cells/mm^3 blood on zidovudine monotherapy or no treatment for HIV-1, finding no clinically significant impact.[23] Also negative was a 25-week placebo-controlled trial of oral capsules containing *Lactobacillus rhamnosus* and *Lactobacillus reuteri* in 65 Tanzanian women with $CD4^+$ T cell counts >350 cells/mm^3 blood not on cART, finding no change in blood $CD4^+$ T cell counts, IgG, IgE, IFN-γ, IL-10, or diarrhea symptoms.[24]

Another study by the same group included 112 Tanzanian women not on cART administered yogurt supplemented with *Lactobacillus rhamnosus* for 4 weeks; they demonstrated no change in blood $CD4^+$ T cell counts or clinical symptoms.[25] In contrast, a study of 77 Brazilian infected infants mostly not receiving cART given *Bifidobacterium bifidum* and *Streptococcus thermophilus* in milk demonstrated an increase in $CD4^+$ T cell counts over 2 months and less diarrhea.[26] Another recent pediatric study of 127 Indian children untreated with cART also found significant increases in $CD4^+$ T cell counts over 12 weeks.[27] An adult study of 24 cART-untreated Nigerian women receiving yogurt without or with supplementation with *Lactobacillus rhamnosus* and *Lactobacillus reuteri* for 30 days similarly noted increased $CD4^+$ T cell counts,[28] as well as a larger placebo-controlled study of about 100 Tanzanians administered yogurt supplemented with *Lactobacillus rhamnosus* and micronutrients.[25]

These studies, mostly in resource-limited settings and in persons not on effective cART, highlight the challenges of probiotic administration in such settings, such as lack of refrigeration required by many formulations and inadequate clinical study design, as discussed in a consensus statement by Monachese *et al.*[29] Besides the differing patient characteristics, a major caveat to comparing these studies is the diversity of probiotics tested. Although the precise interactions of bacterial flora with the gut immune system are not well understood, the diversity of both innate immune receptors and bacterial proteins indicates that different probiotics likely vary widely in their effects on gut and gut regulation of systemic immunity.

Our study is one of the first to evaluate double-blind placebo-controlled probiotic administration to persons with HIV-1 infection on optimal cART as defined by viremia below the limits of conventional clinical testing. In contrast to a recent probiotic study of *Lactobacillus rhamnosus* in a similar sized cohort of HIV-1-infected persons on cART,[30] we observed no significant rise in blood absolute $CD4^+$ T cell counts. However, we observed an increase in the $CD4^+$ percentage of total T cells, which is a strong independent predictor of immune status and disease progression.[31] Unlike prior studies, our subjects were on successful cART treatment with relatively high $CD4^+$ T cell counts, perhaps limiting the dynamic range to observe a rise. Furthermore, we cannot exclude the possibility that this change was not specific to probiotic administration.

Our results are consistent with increasing data suggesting that *Bacillus coagulans* can have immunomodulatory activity. *In vitro*, cell wall and metabolites from this bacterium

have several effects on immune cells, increasing or decreasing various functions (chemotaxis, phagocytosis, cytokine production).[12] Small clinical studies administering this probiotic *in vivo* have shown increased blood T cell reactivity (CD69 upregulation) and cytokine production (IFN-γ, TNF-α, IL-6, IL-8) after *ex vivo* exposure to strains of adenovirus and influenza virus,[13,14] as well as a reduction of blood CRP in persons with rheumatoid arthritis.[15] These apparently contradictory findings of increased inflammatory cytokine production versus reduction of an inflammatory marker suggest that the potential beneficial effects of this probiotic (and probably probiotics in general) are not due to simple down-regulation or up-regulation of immunity, but rather to immunomodulation for more appropriate responses.

Several plasma biomarkers known to be abnormal in HIV-1 infection were examined as potential correlates of immunomodulation by probiotic administration. Those related to gut microbial translocation included sCD14 associated with increased mortality[32] and premature cardiovascular disease,[33] I-FABP associated with gut mucosal endothelial damage,[32] and LPS associated with disease status and T cell activation.[34] More general markers of inflammation and immune activation included sCD163 associated with premature cardiovascular disease,[35] D-dimer associated with vascular dysfunction/disease[36] and increased mortality,[37] IL-6 associated with vascular dysfunction[36] and increased mortality,[37] CRP associated with increased mortality,[37] and other proinflammatory cytokines previously shown to be increased in HIV-1 infection including IL-8,[38] TNF-α,[38,39] and IL-1β.[39] Unfortunately, LPS, IL-1 β, and IL-6 were not reliably detectable in the commercial laboratories running those assays, and there were no cells available for direct evaluation of blood T cell activation. For the remaining plasma biomarkers, administration of probiotic was not associated with any significant difference compared to placebo, and none of these markers showed significant correlation with the observed change in percentage of $CD4^+$ T cells. It is unclear whether this lack of observed changes and correlations is related to the small cohort size, relatively short duration of follow-up, immunomodulation in the gut compartment not reflected in the blood, or a biological impact not reflected by these biomarkers.

To our knowledge, the only published data on the effect of probiotics on bacterial translocation and cytokine markers in HIV-1-infected persons involved a recent small study (five persons per arm) of cART-untreated subjects, showing increased $CD4^+$ T cell counts, reduced plasma bacterial RNA, and reduced plasma IL-6 (but unchanged IL-1β and TNF-α) in subjects receiving a ''synbiotic'' combination of prebiotic (*Agave tequilana* extract) and probiotic (*Lactobacillus rhamnosus* plus *Bifidobacterium lactis*) but not those receiving either prebiotic or probiotic alone.

Incidentally, it was interesting that we observed relatively strong associations between some of these parameters. Sandler *et al.* and Borges *et al.* also noted an association of D-dimer with CRP,[32,40] with the former additionally noting an association of D-dimer with sCD14. Overall, the biological relationships of these and other biomarkers remain unclear; for example, while D-dimer has been correlated with monocyte activation in HIV-1 infection,[41] we saw a correlation of D-dimer only with sCD14 and not CD163, and saw no correlation between sCD14 and sCD163 despite the fact that both are markers on activated monocyte/macrophages.

In summary, the *Bacillus coagulans* BC[30] probiotic preparation in our study was safe and well tolerated in persons with chronic HIV-1 infection on suppressive cART. Administration appeared to increase the percentage of $CD4^+$ T cells, an independent predictor of immune status. A panel of biomarkers of inflammation did not correlate with this change, suggesting either that these markers do not reflect the processes being affected by the probiotic or that the blood compartment does not reflect the processes in the gastrointestinal compartment, a major reservoir of total body T cells. Sampling of the gastrointestinal tract for immune assessment would be a useful adjunct, and further studies will be required to confirm our results and to define the potential clinical benefit and mechanism(s) behind our findings.

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Author Disclosure Statement

H.K. serves is a consultant and shareholder of Merck.

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Original Research: A Patented Strain of *Bacillus coagulans* Increased Immune Response to Viral Challenge

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Abstract

Background: Viral respiratory tract infection is the most common illness among humans. Probiotics have been known to enhance the immune system and, therefore, may represent a significant therapeutic advancement for treating viral respiratory tract infections. **Objective:** A controlled study was conducted to evaluate the effects of the patented GanedenBC^{30} probiotic (*Bacillus coagulans* GBI-30, 6086, marketed as Sustenex® [Ganeden Biotech, Inc., Mayfield Heights, OH]) on the immune system when exposed to adenovirus and influenza in otherwise healthy adults. **Methods:** Ten healthy men and women (average age, 44 years) were instructed to consume 1 capsule of GanedenBC^{30} with water once a day for 30 days. At baseline and after completion of the 30-day treatment, blood levels of cytokines were measured in vitro after T-cell exposure to adenovirus and influenza A. Each participant served as his/her own control with baseline blood draw. **Results:** The use of GanedenBC^{30} significantly increased T-cell production of TNF-α in response to adenovirus exposure ($P = 0.027$) and influenza A (H3N2 Texas strain) exposure ($P = 0.004$), but it did not have a significant effect on the response to other strains of influenza. No serious adverse events were reported throughout the study. **Conclusions:** The patented GanedenBC^{30} probiotic may be a safe and effective therapeutic option for enhancing T-cell response to certain viral respiratory tract infections.

Keywords: probiotics; immune response; respiratory tract infection; *Bacillus coagulans*; GanedenBC^{30}; lactic acid-producing bacteria

Introduction

Viral respiratory tract infections (eg, common cold, influenza) are the most common illnesses among humans.[1] They have significant health and economic consequences, especially among young children, the elderly, and people with underlying or chronic conditions. Every year, an average of 5% to 20% of people in the United States contract influenza, more than 200 000 people are hospitalized with influenza-related complications, and approximately 36 000 people die from influenza.[2] It is also estimated that 1 billion colds occur annually in the United States.[3] To date, only symptomatic medications and homeopathic remedies are available.[4] With such high incidence rates, a safe and effective alternative is clearly needed to reduce the burden of illness.

Probiotics are live microbial preparations that have the ability to modulate host physiological systems.[5] There are several probiotic strains, particularly *Lactobacillus* sp., available in commercial products today. Gram-positive, lactic acid-producing bacteria have received major consideration in the past decade.[6] They exhibit a variety

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of effects, including enhancement of the systemic immune response and mucosal immunity to defend against viral respiratory tract infection.[7,8]

Bacillus coagulans is a gram-positive, spore-forming rod, 0.9 μm by 3 μm to 5 μm in size, and is aerobic to microaerophilic. Because of forming spores, these bacilli can withstand the acidic environment of the stomach to reach the intestine where they germinate and proliferate, producing the favored L (+) optical isomer of lactic acid.[9] Traditional lactobacilli and bifidobacteria are much less likely to tolerate gastric and bile acid.[10]

GanedenBC^{30} (*B coagulans* GBI-30, 6086, marketed as Sustenex® [Ganeden Biotech, Inc., Mayfield Heights, OH]) is a patented strain of *B coagulans* that has the potential to improve the immune response to various pathogens. The aim of the present study was to evaluate the effects of GanedenBC^{30} on T-cell immune response after exposure to adenovirus and influenza in otherwise healthy adults.

Materials and Methods

This study was a controlled trial including 10 healthy volunteers. All participants gave signed informed consent before inclusion in the study. The study was approved by the institutional review board (IRB). It was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki and was consistent with Good Clinical Practice and applicable regulatory requirements. The study was also conducted in accordance with the regulations of the US Food and Drug Administration as described in 21 CFR 50 and 56, applicable laws, and the IRB requirements.

Participants

A total of 10 participants between the ages of 18 and 75 years were recruited for the study.

The exclusion criteria included the following: any chronic or current illness, pregnancy or breastfeeding, allergies to the test products or their ingredients, alcohol or drug abuse, immunization within 2 months of screening, and taking any investigational drug within 30 days of enrollment.

Participants were required to make 2 visits to the clinic during the study. At the first visit, an initial blood draw was taken. Participants were then instructed to consume 1 capsule of GanedenBC^{30} with water once daily for 30 days, at approximately the same time each day, regardless of meals. After 30 days, participants returned to the study site for a second blood draw. Compliance with the study treatment regimen was determined by caplet count.

Treatment

Participants were given capsules containing 2 billion CFU of GanedenBC^{30}. The probiotic bacteria used during the study period was tested by the manufacturer to ensure bacteria counts prior to shipping.

Laboratory Evaluation

In this study, each participant served as his/her own control, with 30-day results compared with baseline results. Blood drawn at baseline and after the 30-day treatment period was used in 2 separate assays to measure immune response.

For the first assay, 5 aliquots of whole blood were prepared in sterile microtubes. Antigens were added to the whole blood and a saline control. The antigens included adenovirus (Fitzgerald, 30-AA02; stock, 2.8 mg/mL) at a concentration of 10 μg/mL, influenza A Texas 1/77 (H3N2) (Fitzgerald, 30-A150; stock, 1 mg/mL) at a concentration of 50 μg/mL, influenza A New Caledonia/20/99 IVR 116 (H1N1) (ProSpec-Tany TechnoGene, IHA-003; stock, 1 mg/mL) at a concentration of 10 μg/mL, and lipopolysaccharide at a concentration of 10 ng/mL. After a 24 ± 2-hour incubation period at 37°C and 5% CO_2, supernatants were collected and cytokine concentrations were measured using the TNF-α and IFN-γ Luminex® cytokine assay per the manufacturer's instructions (Luminex Corp., Austin, TX).

For the second assay, whole blood was stimulated with phytohemagglutinin overnight. A CD4 test was conducted at baseline to screen for immunocompromised subjects. CD4 count was evaluated again at 30 days to ensure that the study treatment did not induce immunosuppression. CD4 cells were separated and immune response was measured using the ImmuKnow® immune function assay (Cylex® Inc., Columbia, MD) according to manufacturer's instructions.

Statistical Analysis

For each marker, the difference in viral-induced cytokine production at baseline and after 30 days of GanedenBC^{30} treatment was calculated to obtain a difference score. Wilcoxon signed-rank tests were performed to evaluate whether there was a change in marker levels at 30 days versus baseline. A P value < 0.05 was considered significant and indicated 95% confidence that the true parameter value was different than the null value. Analyses were performed using SAS Version 9.1 (SAS System, SAS Institute, Cary, NC) and StatXact Version 4.0 (Cytel Software Corporation, Cambridge, MA) statistical software.

Results

A total of 10 adults were screened for inclusion in the study. One participant was excluded from analysis because baseline values indicated a possible underlying infection. The remaining 9 subjects completed the study and were compliant with the treatment regimen. There was an even distribution of men and women participants. Ninety percent of participants were white and 10% were black. The average age was 44 years (range, 33–63 years) (Table 1).

Results showed a significant increase (250%) in the TNF-α response to adenovirus after 30 days of treatment with GanedenBC^{30} versus baseline ($P = 0.027$) (Table 2). There was also a significant increase (1709%) in the TNF-α response to influenza A (H3N2 Texas strain) after 30 days of treatment with GanedenBC^{30} versus baseline ($P = 0.004$) (Table 2). Treatment with GanedenBC^{30} did not have a significant effect on plasma TNF-α levels upon exposure to other strains of influenza, nor was there any effect on plasma IFN-γ production after exposure to any viral strains. No serious adverse events were reported throughout the study.

Discussion

The intestine is the largest immunological organ in the body and contains 70% to 80% of all IgA-producing cells.[11] It is known that gram-positive, lactic acid-producing bacteria improve the balance in the composition of the gut microflora and potentially modulate immune responses.[8]

Although the mechanism of action is not fully understood, it is postulated that probiotics may stimulate an immune response by increasing the number of IgA-secreting cells, which migrate from the Peyer's patches to distant mucosal sites such as the respiratory glands.[11]

In the present study, we investigated the effects of the patented GanedenBC^{30} probiotic on immune response after exposure to adenovirus and influenza. The use of GanedenBC^{30} significantly increased the production of TNF-α in response to exposure to adenovirus, which indicates a heightened immunological effect. Likewise, the use of GanedenBC^{30} significantly increased the production of TNF-α in response to exposure to influenza A (H3N2 Texas strain).

Among subjects treated with GanedenBC^{30}, there were no serious adverse events reported throughout this study. Studies have shown that lactobacilli and bifidobacteria may be associated with opportunistic infections such as bacteremia, sepsis, or endocarditis among immunocompromised patients.[12,13] The transience of GanedenBC^{30} in the gut reduces the risk of developing pathogenesis-conferring mutations and causing infection compared with other probiotics that colonize and adhere to the gut epithelium (eg, lactobacilli, bifidobacteria).

Studies investigating the effects of gram-positive, lactic acid-producing bacteria on viral respiratory tract infections are limited and results are conflicting. A double-blind, placebo-controlled intervention study by Winkler et al[14] found that probiotics including *Lactobacillus gasseri* PA 16/8, *Bifidobacterium longum* SP 07/3, and *B bifidum* MF 20/5 plus vitamins and minerals did not significantly reduce the incidence of the common cold and did not affect the duration of infection. An earlier study found significantly fewer respiratory infections in children attending day care who consumed milk containing *L rhamnosus* GG.[8] Likewise, elderly subjects who consumed milk with *L casei* showed a 20% reduction in the duration of gastrointestinal and respiratory infections during winter months.[15] A more recent study investigating the effects of probiotic bacteria on viral respiratory tract infections found that lactobacilli and bifidobacteria in tablet form had no effect on the incidence of colds, but did reduce the duration of episodes by almost 2 days.[16]

It is known that probiotics tend to exhibit various overlapping mechanisms, such as the regulation of intestinal microbial homeostasis, the stimulation of local and systemic immune responses, the prevention of pathogens infecting the mucosa, the stabilization or maintenance of the gastrointestinal barrier function, the inhibition of procarcinogenic enzymatic activity, and the competition for limited nutrients.[17,18] Given the diversity of effects, it is unlikely that any 1 probiotic strain can accomplish all of these functions.[17] Rather, probiotic effects appear to be strain specific.[6]

Regardless of the strain and its potential effect, probiotics must survive gastric and bile acids[19] in order to reach that intestinal tract, colonize the host epithelium, and exhibit a beneficial effect.[20] Most conventional forms of lactobacilli probiotics are inactivated by bile and low gastric pH.[10]

Table 1. Participant Demographics

Participants (N = 10)	
Race	**n (%)**
White	9 (90%)
Black	1 (10%)
Gender	**n (%)**
Women	5 (50%)
Men	5 (50%)
Age	**Years**
Average	44
Range	33–63

Table 2. Means and Standard Errors for the Marker Difference Scores (day 30 minus day 0) and *P* Values for the (exact) Wilcoxon Signed-Rank Test

Marker	Mean (Standard Error)	*P* Value (Wilcoxon)
TNF-α		
Adenovirus VI	245.3 (107.6)	0.027
Influenza A (H3N2 Texas strain)	304.3 (142.2)	0.004
Influenza A (H1N1 New Caledonia)	−262.9 (330.1)	0.50
INF-γ		
Adenovirus VI	1.52 (4.10)	0.81
Influenza A (H3N2 Texas strain)	2.84 (2.21)	0.38
Influenza A (H1N1 New Caledonia)	−3.96 (2.85)	0.062
CD4[a]	−5.56 (5.22)	0.44
PHA CD4	15.6 (33.7)	0.65

Abbreviation: PHA, phytohemagglutinin.
[a]CD4 was evaluated at baseline to screen for immunocompromised subjects and at 30 days to ensure that the study treatment did not induce immunosuppression.

However, spore-bearing, lactic acid-forming bacteria, such as GanedenBC[30], are protected by a hardened spore coating that can withstand gastric and bile acid for delivery to the small and large intestines.[10] This may partly explain why GanedenBC[30] stimulated a significant immune response while other forms of probiotics in the studies mentioned above have not.

In addition, probiotics selected for commercial use must survive industrial manufacturing and storage to ensure long-term viability and activity.[6] Most cells of conventional lactobacilli die at 70°C, while spore-bearing, lactic acid-forming bacteria do not show a decrease in viable cells even after heating in saline at 85°C for 30 minutes.[10] In addition to surviving heat, gastric acidity, and bile, GanedenBC[30] maintains spore viability without the need for refrigeration, making it an ideal product for commercial use.

The present study provides evidence that the patented GanedenBC[30] probiotic has the ability to boost the immune response upon exposure to adenovirus and a strain of influenza A. Therefore, in addition to its other therapeutic effects, GanedenBC[30] may be considered a safe and effective option for enhancing immune defense against certain viral infections of the respiratory tract. Only TNF-α and IFN-γ responses were measured because of financial limitations. Larger clinical studies that include additional markers are warranted.

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Conflict of Interest Statement

Mira Baron, MD discloses no conflicts of interest.

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ORIGINAL ARTICLE

Probiotic metabolites from *Bacillus coagulans* GanedenBC30™ support maturation of antigen-presenting cells *in vitro*

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Abstract

AIM: To study the effects of probiotic metabolites on maturation stage of antigen-presenting immune cells.

METHODS: Ganeden *Bacillus coagulans* 30 (GBC30) bacterial cultures in log phase were used to isolate the secreted metabolite (MET) fraction. A second fraction was made to generate a crude cell-wall-enriched fraction, by centrifugation and lysis, followed by washing. A preparation of MET was subjected to size exclusion centrifugation, generating three fractions: < 3 kDa, 3-30 kDa, and 30-200 kDa and activities were tested in comparison to crude MET and cell wall in primary cultures of human peripheral blood mononuclear cell (PBMC) as a source of antigen-presenting mononuclear phagocytes. The maturation status of mononuclear phagocytes was evaluated by staining with monoclonal antibodies towards CD14, CD16, CD80 and CD86 and analyzed by flow cytometry.

RESULTS: Treatment of PBMC with MET supported maturation of mononuclear phagocytes toward both macrophage and dendritic cell phenotypes. The biological activity unique to the metabolites included a reduction of CD14+ CD16+ pro-inflammatory cells, and this property was associated with the high molecular weight metabolite fraction. Changes were also seen for the dendritic cell maturation markers CD80 and CD86. On $CD14^{dim}$ cells, an increase in both CD80 and CD86 expression was seen, in contrast to a selective increase in CD86 expression on $CD14^{bright}$ cells. The co-expression of CD80 and CD86 indicates effective antigen presentation to T cells and support of T helper cell differentiation. The selective expression of CD86 in the absence of CD80 points to a role in generating T regulatory cells.

CONCLUSION: The data show that a primary mechanism of action of GBC30 metabolites involves support of more mature phenotypes of antigen-presenting cells, important for immunological decision-making.

Key words: Mononuclear phagocytes; Dendritic cell maturation; Co-stimulatory molecules; Antigen-presentation; Probiotics; Metabolites

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INTRODUCTION

Bacteria are ubiquitous in the environment, having colonized every extreme of nature. This includes the human body where they outnumber human cells by an order of magnitude. The biggest reservoir of these symbiotic bacteria on the human body is the lower gastrointestinal tract[1] where large numbers of coexisting (commensal) bacteria participate in nutrient assimilation including the breakdown of indigestible carbohydrates. They also produce amino acids and vitamins for their host and play a key role in healthy immune system development.

The immune system recognizes both pathogenic and commensal bacteria through a family of pattern recognition receptors known as the toll-like receptor (TLR) family[2]. These receptors interact with molecules present on the exterior surface of bacteria and include lipopolysaccharide (LPS), flagellin, lipoteichoic acid and lipoproteins as well as bacterial DNA. Toll-like receptors are present on cells participating in both innate and adaptive immunity such as monocytes/macrophages and dendritic cells (DC) as well as epithelial cells of the intestinal mucosa.

The emerging picture is that commensal bacteria have an enormous impact on health. While a healthy microbiota can aid the host by increasing nutrient absorption and training the immune system to not respond to self, conversely an unhealthy (i.e., unbalanced) microbiota can lead to malabsorption, inflammation and disease[3-5]. A growing body of evidence suggests that these effects, both positive and negative, of the microbiota on the host are mediated by the immune system. Probiotics are defined as microorganisms that when ingested in a sufficient amount confer a health benefit upon the host and are known to interact with the immune system. Probiotic microorganisms have a long history of human consumption in the form of fermented foods and have shown health benefits in treating dysbiosis, irritable bowel syndrome, and eczema[6]. GanedenBC30™(*Bacillus coagulans* GBI-30, 6086) (GBC30) is a proprietary strain of the gram positive, lactic acid producing spore-forming bacteria known as *Bacillus coagulans*. This strain of *B. coagulans* can survive extremes of heat and pressure in manufacturing as well as the harsh, acidic environment of the human gastrointestinal tract, leading to a very high survival rate and germination in the lower intestinal tract. The safety of consumption of this strain was documented in acute and sub-chronic studies in rats[7].

One way in which commensal bacteria modulate the immune response is by the secretion of certain bioactive compounds. This suggests that metabolites of commensal bacteria have effects of their own and that there may be unique health benefits to be derived from the consumption of live probiotic cultures or probiotic metabolite preparations. Recent studies on the bacterial compound polysaccharide A from *Bacteroides fragilis* have shown the ability of this molecule to prevent intestinal inflammation caused by *Helicobacter pylori* infection and to correct the symptoms of encephalomyelitis in mice, an animal model for human multiple sclerosis[8-10]. The recent sequencing data from 178 commensal microbial genomes has identified over 30 thousand potential protein-coding sequences of which 97% are unique[11]. This suggests a vast untapped reservoir of novel genes including those coding for potential secreted compounds.

The work presented here build on a previous study that showed both enhancement of innate immune responses as well as anti-inflammatory effects of GBC30 *in vitro*[12]. In particular, the data presented here has aimed at investigating the differences between a crude preparation versus the metabolite fraction in more detail with a particular focus on modulation of key regulatory immune cells by specific size-selected fractions of GBC30 metabolite (MET) compounds.

MATERIALS AND METHODS

Reagents

The following buffers and reagents were obtained from Sigma-Aldrich (St. Louis, MO): Histopaque 1077 and 1119, phosphate-buffered saline (PBS), RPMI-1640 culture medium, fetal calf serum, L-glutamine 200 mmol/L, penicillin-streptomycin 100X solution, and bovine serum albumin. CD80-FITC, CD86-PE, CD16-PE and CD14-PerCP were obtained from BD Biosciences (San Jose, CA). Sodium Azide (NaN_3) was obtained from LabChem Inc. (Pittsburgh, PA). Low-binding 100 μm zirconium beads were obtained from OPS Diagnostics (Lebanon, NJ) and 0.2 μm cellulose acetate filters from Whatman (Florham Park, NJ). The *Bacillus coagulans* strain (GanedenBC30™) was obtained from Ganeden Biotech Inc. (Mayfield Height, OH).

Preparation of Bacillus coagulans metabolite fractions

Using sterile technique, two separate samples of 2.0 g of GanedenBC30™ spores were each placed into 25 mL PBS and heated at 70 ℃ for 30 min. Spores were then centrifuged at 2400 rpm for 5 min, PBS was removed and each tube of spores re-suspended and placed in culture flasks containing 25 mL of RPMI-1640 culture medium. The cultures were incubated at 37 ℃ for 24 h at which time an additional 20 mL of RPMI-1640 was added and the cultures incubated for an additional 24 h. Following 48 h of incubation, the bacterial cultures contained 5×10^7 bacteria/mL.

Preparation of GanedenBC30™ culture supernatant as

a source of metabolites (MET): Cultures were transferred to 50 mL centrifuge tubes and initially spun at 1000 rpm for 2 min to remove any remaining spores. The liquid containing the bacteria and metabolites was decanted into new tubes and centrifuged at 3500 rpm for 20 min. The supernatant was decanted from the large bacterial pellets and combined in a single tube followed by filtration twice through a 0.2 μm cellulose acetate syringe filter. This filtrate was either frozen directly as 250 μL aliquots (crude metabolite fraction) or spun through Amicon Ultra protein size separation columns from Millipore (Bedford, MA) followed by aliquot preparation and storage at -20 ℃. Separation of the metabolites into different molecular weight fractions was performed in the following manner: for the fraction that is < 3 kDa, the crude metabolite preparation was placed onto a 3 kDa molecular weight cutoff centrifuge column and spun at 2500 rpm for 10 min. The filtrate that passed through the filter contained material that was less than 3 kDa. Aliquots were made from this material and frozen (metabolites < 3 kDa fraction). The material that did not pass through the column was then placed into a tube containing a 30 kDa molecular weight cutoff filter and spun at 2500 rpm for 10 min. The filtrate that passed through this filter contained material that was 3-30 kDa. Aliquots were made from this material and frozen (metabolites 3-30 kDa fraction). The remaining metabolite material that did not pass through the 30 kDa molecular weight filter was also aliquoted and frozen and this fraction was called metabolites 30-200 kDa fraction. It is important to note that these size separations are not exact and that while large molecules are excluded from the fractions containing the smaller molecules, some small molecules may still remain in the fractions containing the larger molecules.

Preparation of GanedenBC30™ crude cell wall (CW): The two bacterial pellets were each processed separately and then combined after the final bead milling step. Following centrifugation of the bacterial culture at 3500 rpm for 20 min and decanting of the supernatant, the bacterial pellets were washed twice in 45 mL of PBS with subsequent pelleting by centrifugation at 2500 rpm for 10 min. The washed pellets went through one freeze/thaw cycle followed by multiple bead milling cycles. In brief, the pellets were resuspended in 4 mL of PBS and then 4 mL of 100 μm low-binding zirconium beads were added. One cycle of bead milling consisted of 60 one-second pulses of the bacteria/bead mixture on a vortex mixer. Ten of these cycles were performed. The bacteria/bead mixture was placed on ice in between bead milling cycles. At the end of the 10 bead-milling cycles, the beads were allowed to settle in the tubes and the liquid removed from the two tubes and combined. The liquid containing the fragmented bacteria was spun at 3500 rpm for 20 min followed by transfer of the liquid to Eppendorf tubes and centrifugation at 14 000 rpm for 5 min. The high speed centrifugation was necessary to remove any large fragments of bacteria that were not disrupted by the bead milling. The final solution was brought up to 45 mL with PBS and filtered through a 0.2 μm cellulose acetate filter and stored directly at -20 ℃ as 250 μL aliquots (crude cell wall). It is important to note that the cell wall preparation will also contain some GBC30 cellular contents in addition to cell wall components.

Purification of peripheral blood mononuclear cells

Healthy human volunteers between the age of 20 years and 60 years served as blood donors after obtaining written informed consent, as approved by the Sky Lakes Medical Center Institutional Review Board. Isolation of peripheral blood mononuclear cell (PBMC) was performed as previously described[13].

Cell surface staining of CD14 positive mononuclear phagocytes

Complete cell culture media used for the culture of PBMC consisted of RPMI-1640 supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Peripheral blood mononuclear cells were cultured for 3 d in the presence of serial dilutions of BC30 metabolite fractions or crude cell wall followed by cell surface immunostaining with CD14, CD80, CD86 and CD16 monoclonal antibodies. Processing of cells for immunostaining was performed as previously described[13] with the following modifications: optimal amounts of monoclonal antibodies per sample were 3 μL for CD14-PerCP and 4 μL for CD80-FITC, CD86-PE and CD16-PE. Experiments were performed three times using PBMC isolated from three different blood donors. Each test condition was performed in duplicate and untreated and LPS-treated controls were tested in quadruplicate and triplicate, respectively.

Statistical analysis

Statistical significance was tested using Student's *t*-test performed with Microsoft Excel. All *P* values were two-sided and were considered significant when $P < 0.05$ and highly significant when $P < 0.01$. Only statistically significant *P* values are reported.

RESULTS

GBC30 effects on mononuclear phagocyte differentiation

Mononuclear phagocyte differentiation in 3-d primary PBMC cultures was examined by cell surface staining for proteins expressed by monocytes/macrophages and dendritic cells. These included CD14 (Figure 1) and CD80 and CD86 (Figures 2 and 3). CD14 expression on CD-14^{bright} cells was increased following exposure of cells to GBC30 crude MET and CW fractions (Figure 1A). Treatment of cells with crude MET showed a strong dose-dependent response with statistically significant increases occurring with the 4 highest doses. As expected, LPS treatment of cells greatly increased CD14 expression[14]. Crude CW also led to statistically significant increases in CD14 expression. When the effects of crude and size-selected MET fractions of GBC30 at a 1:200 dilution

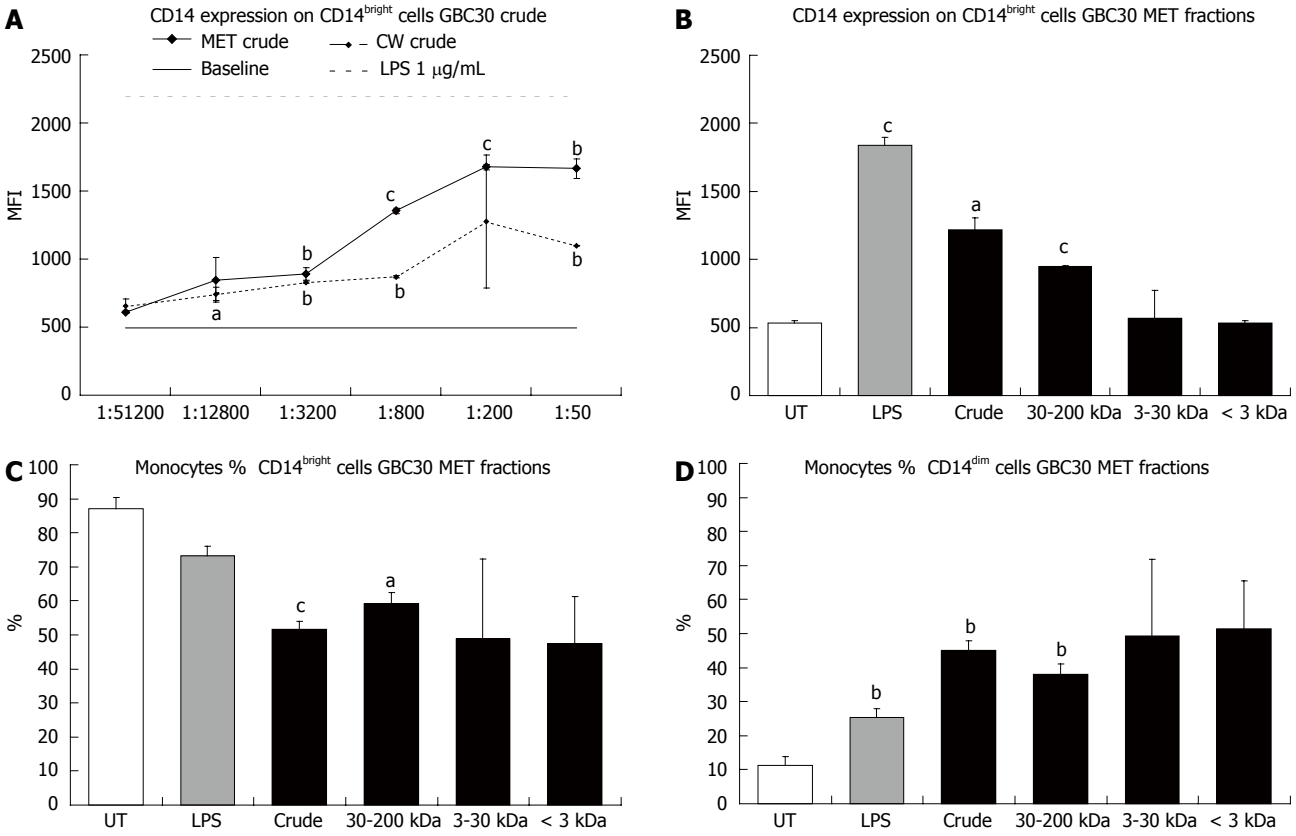

Figure 1 CD14 expression on mononuclear phagocytes. Mononuclear phagocytes present in 3-d peripheral blood mononuclear cell cultures exposed to either the Ganeden *Bacillus coagulans* 30 (GBC30) metabolites (MET), cell wall-enriched (CW), or MET fractions, were identified using electronic gating of the flow cytometry data by gating on forward scatter/side scatter followed by gating for CD14 positivity. A comparison was made between cells that were untreated (UT), exposed to lipopolysaccharide (LPS) or to the different GBC30 fractions. A: Comparison of CD14 mean fluorescence intensity showed a dose-dependent increase in CD14 expression in cells treated with crude MET. A milder increase was seen for cells treated with crude CW. The baseline indicates CD14 expression on untreated cells; B: The increase in CD14 expression was primarily caused by high molecular weight compounds present in MET; C: The percent of $CD14^{bright}$ cells in the mononuclear phagocyte population was decreased by all fractions of MET; D: The percent of $CD14^{dim}$ cells in the mononuclear phagocyte population was increased by treatment of cells with all MET fractions. Bar graphs show data from 1:200 dilutions of each MET fraction and lipopolysaccharide (1 μg/mL). $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$. For each data point, the mean ± SD are shown for each duplicate data set. Graphs show data representative of 1 out of 3 experiments. MFI: Mean fluorescence intensity.

were compared (Figure 1B), high molecular weight fractions (crude and 30-200 kDa) increased CD14 expression while PBMC treated with either the 3-30 kDa or < 3 kDa fractions showed CD14 expression levels similar to untreated cultures.

Reduction of $CD14^{bright}$ cells

Because CD14 expression on mononuclear phagocytic cells varies and expression levels have been correlated with different cell populations, the percent of $CD14^{bright}$ versus $CD14^{dim}$ cells was determined for PBMC cultures exposed to different GBC30 MET fractions. All fractions of MET at a 1:200 dilution led to decreased numbers of $CD14^{bright}$ cells (Figure 1C) while an inverse pattern of response was seen regarding changes in $CD14^{dim}$ cell numbers (Figure 1D). In this case all fractions of MET led to increases in the percent of $CD14^{dim}$ cells.

$CD14^{bright}$ cells: Effects of GBC30 metabolite and cell wall on CD80 and CD86 expression

Next, CD80 and CD86 expression was determined for the $CD14^{bright}$ cell population. Both MET and CW crude fractions led to statistically significant decreases in CD80 expression on $CD14^{bright}$ cells (Figure 2A) while only MET crude increased CD86 expression (Figure 2C). When crude and size selected fractions of MET were compared at the 1:200 dilution all fractions of MET led to similar statistically significant decreases in CD80 expression (Figure 2B). A comparison of the effect of MET fractions on CD86 expression showed that the 3-30 kDa and < 3 kDa fractions led to a decrease but these changes were not statistically significant (Figure 2D).

$CD14^{dim}$ cells: Effects of GBC30 metabolite and cell wall on CD80 and CD86 expression

When expression of the co-stimulatory molecules CD80 and CD86 was determined for the $CD14^{dim}$ cell population, both MET and CW crude increased CD80 (Figure 3A) and CD86 (Figure 3C) expression with MET crude having the biggest effect, particularly on CD86 expression. When crude and size selected fractions of MET were compared at the 1:200 dilution, only the crude and 30-200 kDa fractions led to statistically significant increases in CD80 expression (Figure 3B). A comparison

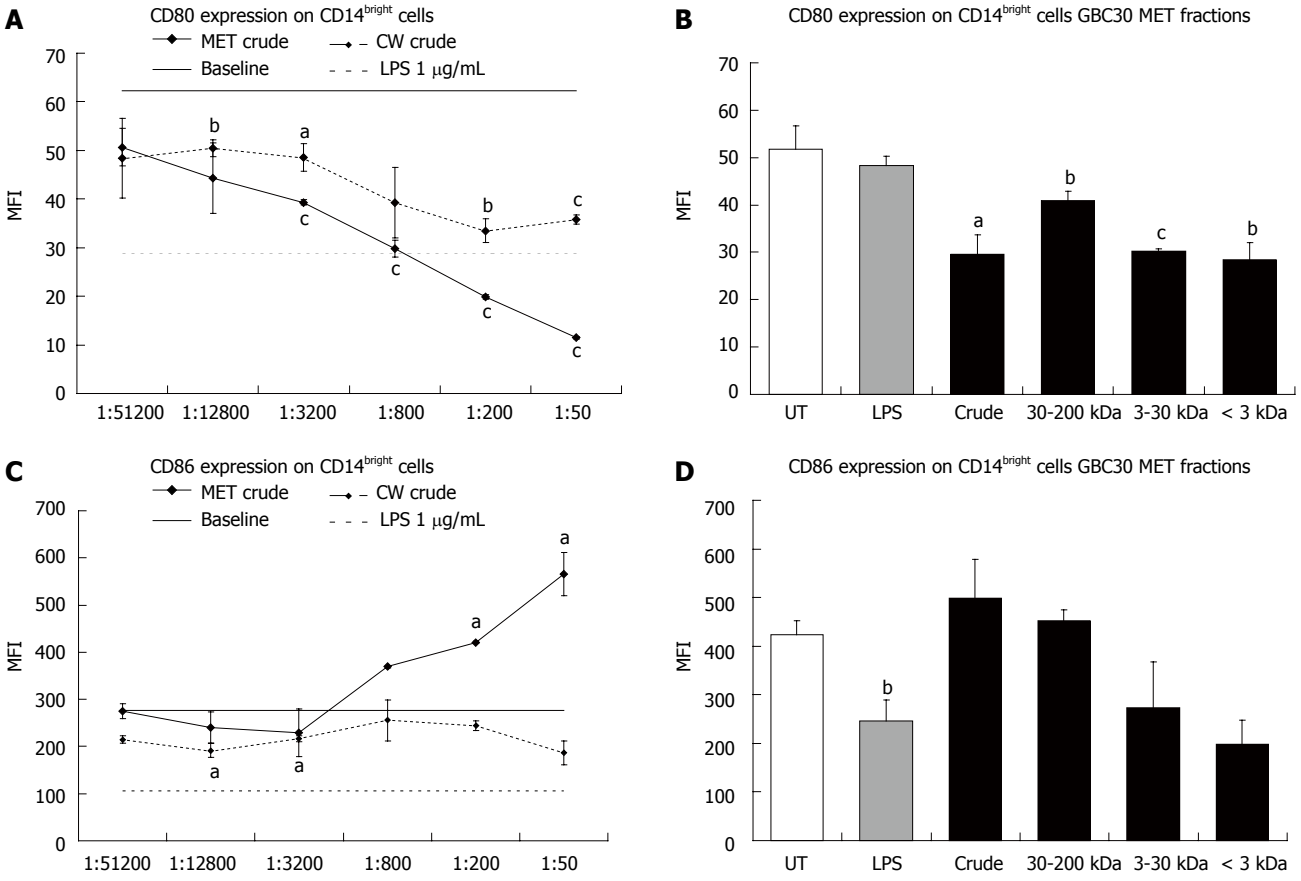

Figure 2 Expression of the co-stimulatory molecules CD80 and CD86 on CD14^{bright} mononuclear phagocytes from 3-d peripheral blood mononuclear cell cultures. A: Comparison between the effects of serial dilutions of Ganeden *Bacillus coagulans* 30 (GBC30) crude metabolites (MET) or cell wall enriched (CW) fractions on CD80 expression on CD14^{bright} cells showed dose-dependent decreases in CD80 expression. Both MET and CW reduced CD80 expression to levels similar to those seen with Lipopolysaccharide (LPS) treatment; B: A comparison of the effects of size-fractionated MET on CD80 expression on CD14^{bright} cells shows that all MET fractions reduce expression; C: Comparison between the effects of serial dilutions of crude MET or CW on CD86 expression on CD14^{bright} cells showed dose-dependent increases in CD86 expression when cells were exposed to the three most concentrated dilutions of MET. Treatment of cells with CW resulted in a uniform modest decrease in CD86 expression; D: The effect on increased CD86 expression is present only in the crude preparation of MET. Bar graphs show data from 1:200 dilutions of each MET fraction and lipopolysaccharide (1 μg/mL). $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$. For each data point, the mean ± SD are shown for each duplicate data set. Graphs show data representative of 1 out of 3 experiments. MFI: Mean fluorescence intensity; UT: Untreated.

of the effect of MET fractions on CD86 expression showed that crude MET increased CD86 expression while the 3-30 kDa and < 3 kDa fractions decreased expression (Figure 3D).

Reduction in CD14+ CD16+ cells

Mononuclear phagocytes have also been classified according to expression of the cell surface protein CD16 with CD14+ CD16+ cell subsets considered to be pro-inflammatory[15]. The effect of crude MET and CW fractions on the percent of CD14+ CD16+ and CD14+ CD16- cells in 3-d PBMC cultures was investigated. Crude MET treatment of cells resulted in a dose dependent decrease in CD14+ CD16+ cells (Figure 4A) and an increase in CD14+ CD16- cells (Figure 4C). Crude CW had a much milder effect that mirrored that of crude MET. When cells were exposed to the crude or size fractionated preparations of MET at a 1:200 dilution, it was the fractions with the largest compounds (crude and 30-200 kDa) that showed the greatest effect on CD14+ CD16+ (Figure 4B) and CD14+ CD16- (Figure 4D) cell numbers although the 3-30 kDa and < 3 kDa fractions also produced statistically significant reductions in the number of CD14+ CD16+ cells.

DISCUSSION

The work presented here investigated the effects of MET and CW fractions of the GBC30 probiotic strain on mononuclear phagocyte phenotypes in primary PBMC cultures. The cellular model for examining the immune effects was carefully chosen, and primary PBMC cultures were used because this allows the simultaneous interaction of multiple cell types and has been shown to support the survival of blood dendritic cells without the addition of exogenous cytokines[16]. One of the main findings was the biological activities of the metabolites and the data showed that a primary mechanism of action of BC30 metabolites involved support of more mature phenotypes of antigen-presenting cells, important for immunological decision-making.

Compounds present in the MET crude fraction con-

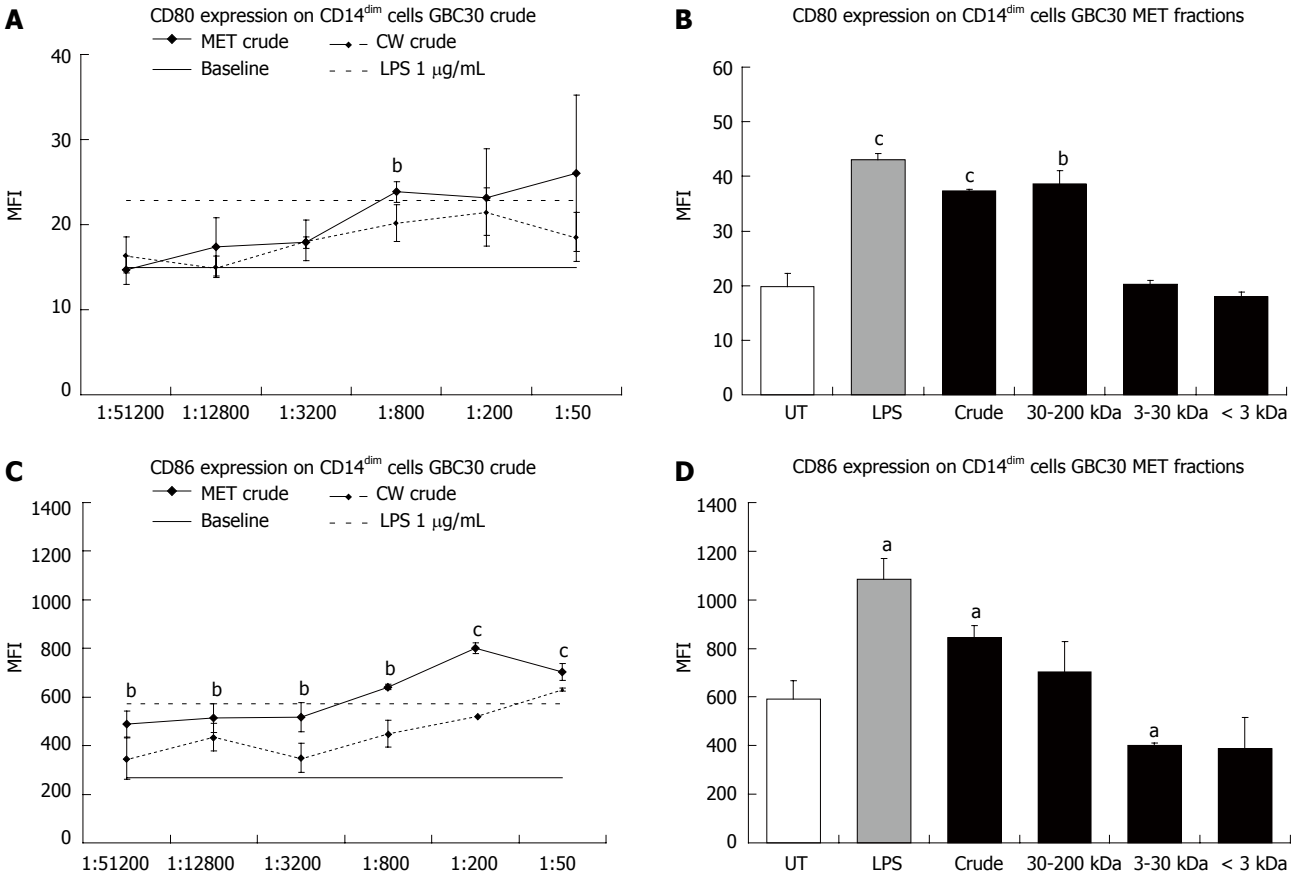

Figure 3 Expression of the co-stimulatory molecules CD80 and CD86 on CD14^{dim} mononuclear phagocytes from 3-d peripheral blood mononuclear cell cultures. A: Comparison between the effects of serial dilutions of Ganeden *Bacillus coagulans* 30 (GBC30) crude metabolites (MET) or cell wall enriched (CW) fractions on CD80 expression on CD14^{dim} cells showed that both MET and CW led to increased expression; B: The increase in CD80 expression following treatment of cells with MET was due to high molecular weight compounds; C: Comparison of CD86 expression on CD14^{dim} cells exposed to crude fractions of MET or CW resulted in increased CD86 expression; D: Size-selected fractions of MET did not have uniform effects on CD86 expression on CD14^{dim} cells. Crude MET increased expression while 3-30 kDa and < 3 kDa fractions decreased expression. Bar graphs show data from 1:200 dilutions of each MET fraction and lipopolysaccharide (1 μg/mL). $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$. For each data point, the mean ± SD are shown for each duplicate data set. Graphs show data representative of 1 out of 3 experiments. MFI: Mean fluorescence intensity; UT: Untreated; LPS: Lipopolysaccharide.

sisted entirely of compounds that were secreted by GBC30 into the culture media. The CW crude fraction was isolated from whole bacteria and may contain some compounds present in the MET preparation in addition to compounds unique to the cell wall. Size fractionation of crude MET was used to evaluate immune modulating compounds based on MW and their association with one or more fractions.

Probiotic organisms support mucosal immunity and similar to commensal bacteria in the human gut, they interact with mononuclear phagocytic cells such as dendritic cells and macrophages[17-19]. The expression levels of CD80 and CD86 co-stimulatory molecules can be used to indicate the differentiation of mononuclear phagocytes to that of antigen presenting cells such as dendritic cells. While CD14 is still present on some subsets of dendritic cells, typically when mononuclear phagocytes adopt a dendritic cell identity, CD14 expression is down regulated with the concurrent up regulation of CD80 and CD86[20]. The differential roles of the co-stimulatory molecules CD80 and CD86 suggests that co-expression of both molecules on dendritic cells leads to T helper cell differentiation, whereas the predominant expression of CD86 support T regulatory cells, and supports an anti-inflammatory cytokine profile by decreasing Interferon-gamma production and increasing interleukin (IL)-4 production[21]. Since the current literature suggests that mononuclear phagocytes present in the circulation are already committed in their developmental path[22], the changes seen in CD14 expression suggest that MET and CW simultaneously enhance the maturation of two separate subpopulations of mononuclear phagocytic cells (CD14^{bright} and CD14^{dim}) towards their corresponding macrophage and dendritic cell phenotypes. The effect of GBC30 on putative DC maturation in PBMC cultures, suggests that DC may be responsible for the IL-6 production that was previously shown *in vitro*[12], and this increased IL-6 production may reflect normal physiological interactions between DC and commensal bacteria in the human gut[17,23].

The data suggest that live GBC30 in the gut lumen would provide metabolites from GBC30, different from the immune modulating compounds associated with the cell wall enriched fraction, and support the interpretation that the live metabolically active GBC30 has stronger immune modulating activity than accounted for by its cell wall alone. Immune modulating activity has been identified from the supernatant of the probiotic strains *Lactoba-*

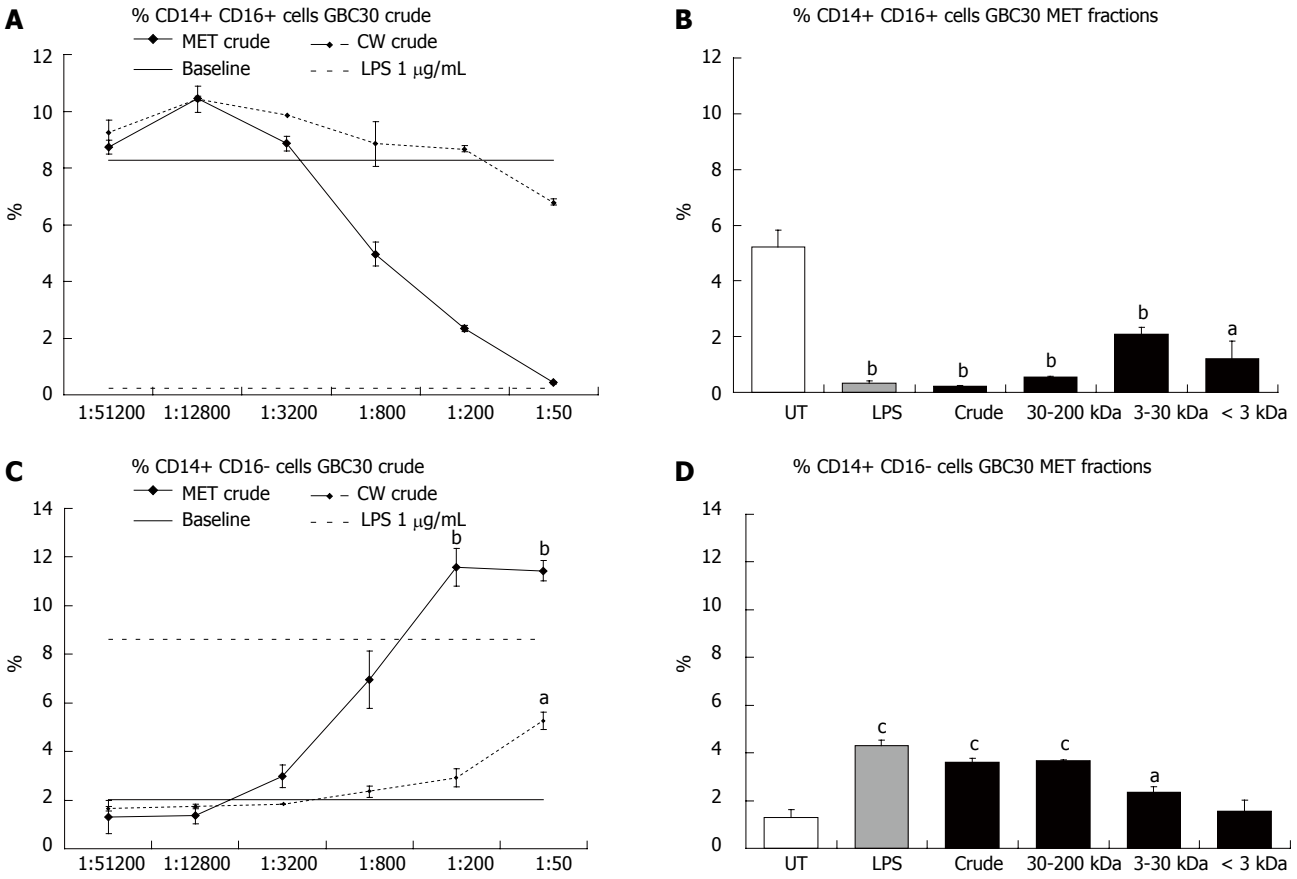

Figure 4 Changes in the percent of CD14+ CD16+ and CD14+ CD16- cell populations following exposure of 3-d peripheral blood mononuclear cell cultures to Ganeden *Bacillus coagulans* 30. A: Exposure of cells to serial dilutions of Ganeden *Bacillus coagulans* 30 (GBC30) crude metabolites (MET) led to a strong dose-dependent decrease in CD14 CD16 double positive cells while exposure to cell wall enriched (CW) did not reduce this cell population; B: Treatment of cells with size-selected MET fractions show that all fractions of MET reduce the number of CD14+ CD16+ cells; C: The percent of CD14+ CD14- cells in peripheral blood mononuclear cell cultures increased in cultures treated with crude MET and CW. Treatment with MET resulted in a very strong dose-dependent increase while CW treatment produced a milder increase at the two highest concentrations; D: Treatment of cells with size-selected MET fractions show that only fractions containing high molecular weight compounds increase the number of CD14+ CD16- cells. Bar graphs show data from 1:200 dilutions of each MET fraction and lipopolysaccharide (1 μg/mL). $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$. For each data point, the mean ± SD are shown for each duplicate data set. Graphs show data representative of 1 out of 3 experiments. UT: Untreated; LPS: Lipopolysaccharide.

cillus casei Shirota[24] and *Bifidobacterium breve*[25], the probiotic yeast *Saccharomyces boulardii*[26], the commensal bacterium *Faecalibacterium prausnitzii*[27] and gut-derived lactobacilli and bifidobacteria[28]. In the case of *Faecalibacterium prausnitzii*, injection of the supernatant completely protected mice from trinitrobenzenesulphonic acid induced colitis while live bacteria provided only partial protection[27]. Most of these studies focused on cytokine production in monocyte-derived dendritic cell cultures[26,27] and have determined this to occur through a TLR2 dependent mechanism. In one study, it was determined that the active component in the supernatant from *Lactobacillus casei* was a polysaccharide peptidoglycan complex[24] while another study has suggested that the immune boosting effect of common botanical extracts is through effects of bacterial lipoproteins and lipopolysaccharides (derived from endophytes, the resident bacteria present in all plants) on macrophage activation[29].

Thus, due to direct effects on mononuclear phagocyte differentiation, GBC30 metabolites lend support to two important cell types responsible for antigen recognition, presentation to cells within the adaptive immune system, and execution of regulatory functions, including immunological memory. The effect of dried/reconstituted material was tested in three different bioassays previously reported to show bioactivity[12], including anti-inflammatory effects (data not shown), and no significant difference was seen between this and frozen/thawed material. The stability of the bioactive compounds in the metabolite fraction holds promise for development of a consumable product.

Results from the GBC30 MET fractions suggest that the metabolic activity of this probiotic organism is an intregral part of its immune modulating functions, and that multiple different compounds act in synergy to support key aspects of mucosal immune protection. These results suggest specific mechanisms of action and may give insight into some aspects of previous clinical studies showing reduced symptoms from irritable bowel syndrome[30]. We suggest that further studies include *ex vivo* evaluation of mononuclear cells isolated from lamina propria and Peyer's patches, in terms of antigen presentation, dendritic cell and B lymphocyte maturation, and IgA production. Further clinical work is warranted, not only

in populations with inflammatory syndromes, but also in populations with reduced mucosal immune protection, and should include assessment of inflammatory markers in serum, as well as secretion of IgA.

In conclusion, the biological activities reported here for the metabolites point to a unified mechanism of action directed at the differentiation and maturation state of antigen-presenting cells such as the macrophage/dendritic cells. In terms of immune regulation, this plays a pivotal role in decision-making, for example in whether T lymphocytes are induced into immunological anergy (unresponsiveness, tolerance) or whether they are triggered into proliferation, cytokine production, and other mechanism of inter-cellular communication. It is conceivable that metabolites are absorbed into the mucosal immune tissue along the intestinal track and help direct more efficient antigen-recognition, while reducing immune reactivity towards harmless food-borne antigens. This may provide a mechanism to explain the improved immune protection, while also seeing a reduction in food allergies and associated inflammatory reactions with consumption of certain probiotic strains.

COMMENTS

Background

The mucosal surface of the human gastrointestinal tract is an interface between the external and internal environments, separated by a single epithelial cell layer. On the one side are food antigens, commensal bacteria and potential pathogens while cells of the immune system reside on the other. Oral tolerance refers to the ability of the immune system to not react towards food and commensal bacterial antigens while still evoking a robust immune response towards pathogens. Probiotic bacteria interact with the host immune system and elicit beneficial immune modulating effects that include a reduction in inflammation in inflammatory bowel disease, amelioration of antibiotic-induced diarrhea, and protection from pathogen infection.

Research frontiers

Recent evidence suggest that the interaction of commensal bacteria and probiotics with the immune system is more than a mechanical engagement of bacterial cell wall components with immune cell receptors and includes an active cross-talk between live bacteria and the host through secreted substances (metabolites). This is an active area of research and data from microbiome genomic sequencing suggests that the majority of predicted genes encode proteins with unknown functions.

Innovations and breakthroughs

Most of the published work on probiotics interacting with the immune system has focused on the bacterial cell wall activating the immune system through engagement of the Toll-like receptor (TLR) family, in particular TLR2 and TLR4. Much less research has focused on secreted metabolites and very little is known about what these secreted compounds are. The data presented here showed that a primary mechanism of action of Ganeden *Bacillus coagulans* 30 (GBC30) metabolites involved support of more mature phenotypes of antigen-presenting cells, important for immunological decision-making. An immature antigen presenting cell may fail in triggering an appropriate immune defense reaction, while either inducing immunological unresponsiveness (anergy) towards the antigen, or induce an allergic reaction to the antigen.

Applications

The support of antigen-presenting cells *in vitro* by GBC30 metabolites suggests that consumption of GBC30 may lead to *in vivo* effects of improved decision-making in the gut-associated lymphoid tissue (GALT), translating into clinical observations of improved immunity against infections, and reduced immunological anergy and allergy.

Terminology

Cluster of differentiation 14 is a monocyte marker and functions as a co-receptor for bacterial lipopolysaccharide recognition. It is highly expressed on the cell surface of monocytes and macrophages; GALT is a mucosa-associated lymphoid tissue lining the gastrointestinal tract from the esophagus to the colon. It contains immune cells and plays an important part in preventing the immune system from reacting to the resident microflora as well as defence from pathogens; Anergy refers to the absence of a normal immune response to a specific antigen or allergen.

Peer review

The authors present a paper that aimed to investigate any differences between the effects of a crude preparation of GBC30 bacterial culture metabolites compared to the fractionated preparations on the maturation of peripheral blood mononuclear cell. It demonstrates that probiotic bacteria produce metabolites that activate cells of the immune system, beyond what is expected from simple bacterial cell wall components.

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ORIGINAL ARTICLE

EFFECTS OF A PROPRIETARY *BACILLUS COAGULANS* PREPARATION ON SYMPTOMS OF DIARRHEA-PREDOMINANT IRRITABLE BOWEL SYNDROME

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SUMMARY

Symptoms of irritable bowel syndrome (IBS) have a profound impact on quality of life for many patients and current treatments are sometimes unsatisfactory. This controlled pilot study was conducted to evaluate effects of the proprietary GanedenBC^{30} (Bacillus coagulans GBI-30, 6086) probiotic on IBS symptoms, in a randomized, double-blind, placebo-controlled clinical trial including patients with diarrhea-predominant IBS (IBS-D). Patients were randomized to receive either B. coagulans GBI-30, 6086 or placebo once a day for 8 weeks. Patients filled out a quality-of-life questionnaire, and self-assessment diaries were provided to record stool count and consistency, symptom severity, and medication consumption. Of the 61 patients enrolled, six did not meet the inclusion criteria and three were lost to follow-up. Of the remaining 52 patients with IBS-D, the average number of bowel movements per day was significantly reduced for patients treated with B. coagulans GBI-30, 6086 when compared to placebo (P = 0.042). Large variability in baseline scores prevented the assessment of severity scores and quality of life. This small pilot study provides evidence that the proprietary B. coagulans GBI-30, 6086 probiotic is safe and effective for reducing daily bowel movements in patients with IBS-D.

Key words: Probiotics - Irritable bowel syndrome - *Bacillus coagulans* - Lactic acid-producing bacteria - Diarrhea

INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic gastrointestinal (GI) disorder that affects 7 to 14% of the US population, with a higher prevalence among women (1, 2). It is the most common diagnosis made by gastroenterologists (3). IBS is not life threatening; however, the symptoms can severely impact the emotional health, functional ability and quality of life of the patient. According to a US household survey, patients with IBS are twice as likely to report that they are too sick to go to work or school when compared with those without IBS (11.3 vs 4.2%) and they miss nearly three times as many days of work or school per year (13.4 vs 4.9 days) (4). According to a large internet-based survey, IBS has a consistently negative impact on quality of life (1). Likewise, social activities such as eating out, taking long trips and holidays were hindered by symptoms of IBS (2). Unfortunately, there are currently few therapeutic options available for the treatment of IBS (5). This lack of treatment options, together with the high prevalence of IBS and its associated debilitating disease burden, have sparked considerable interest in the use of probiotics, live bacteria that are beneficial to the host, to help relieve symptoms of IBS.

Patients with IBS are classified as possessing diarrhea-predominant IBS (IBS-D), constipation-predominant IBS (IBS-C) or alternating symptoms. The symptoms associated with IBS include cramping, abdominal pain, bloating, constipation and/or diarrhea (6). Symptoms can occur in response to infection, changes in diet, lifestyle changes or psychological stress (6). A significant number of IBS patients report postprandial symptoms; however, the interaction of food and intraluminal content with secretory, motor and sensory mechanisms is poorly understood (7).

While the exact pathophysiology of IBS is unknown, it is believed to result from a dysregulated and excessive immune response to components in the GI microflora, which leads to a sustained inflammatory response and mucosal damage (8, 9). Both retrospective and prospective studies have documented the onset of IBS following bacterial gastroenteritis, which is more common among patients with IBS-D (10-12). Other studies have provided evidence of low-level mucosal inflammation and immune activation in patients with IBS (13, 14). In addition, it has been shown that the fecal flora of IBS patients differs from that of normal patients (15). Patients with IBS may harbor bacterial overgrowth and their symptoms may be ameliorated by its eradication (16).

Probiotics have been demonstrated to normalize or rebalance the GI microflora status quo, restoring gut epithelial function and the mucosal immunological barrier (9, 17). GanedenBC^{30} (*Bacillus*

coagulans GBI-30, 6086, Ganeden Biotech, Inc., Mayfield Heights, OH, USA) is a proprietary strain of lactic acid-producing bacteria. The objective of this preliminary study was to evaluate the safety and efficacy of *Bacillus coagulans* (*B. coagulans*) GBI-30, 6086 probiotic in treating symptoms of IBS-D.

MATERIALS AND METHODS

Study design

A double-blind, placebo-controlled clinical pilot study was conducted to evaluate the effects of the patented probiotic *B. coagulans* GBI-30, 6086 on the symptoms of IBS-D. A 2-week observation period was followed by an 8-week treatment period. The study protocol was in accordance with the World Medical Association Helsinki Declaration on the Ethical Principles for Medical Research Involving Human Subjects.

Patient population

Otherwise healthy male and female patients between 18 and 75 years of age with documented IBS-D as assessed by the Rome III criteria were recruited and then randomized to receive *B. coagulans* GBI-30, 6086 or placebo. All patients agreed to refrain from taking other medications or supplements (prescribed and/or over-the-counter) for relief of IBS symptoms throughout the study period. Individuals with a history of other gastrointestinal conditions or diseases, nonfunctional bowel disease, previous abdominal surgery, immunodeficiency or drug/alcohol abuse were excluded from the study. Individuals who were pregnant (or wanted to become pregnant), who were lactating or had received antibiotics within the 28 days prior to start of the study were also excluded.

Treatment and follow-up

The active study capsule contained 2.0 x 10^9 colony forming units (CFU) of *B. coagulans* GBI-30, 6086. Active capsules also contained inert carriers which comprised microcrystalline cellulose, lysine, silicon dioxide and magnesium stearate contained within a vegetarian capsule (hypromellose, water, titanium dioxide (color), caramel (color), and propylene glycol). Identically appearing placebo capsules contained microcrystalline cellulose. Patients were instructed to take one capsule a day for 8 weeks.

Patients who provided informed consent and met the inclusion criteria were given daily diary cards to complete and scheduled for a return visit at the end of an initial 2-week observation period. No treatment was given during this initial 2-week period, but patients were asked to record their daily stool count and consistency (using the Bristol Stool Scale) on the diary cards. At the end of the 2-week observation period, patients returned to the clinic and completed a quality of life questionnaire. The questionnaire contained 26 questions on the impact of IBS on physical function, daily activities and psychological status, with answers ranging from "all the time" to "none of the time."

Patients were then randomized to receive either the treatment or placebo once a day for 8 weeks. Again, patients were given daily diary cards and asked to record daily stool count and consistency and medication consumption as well as rate any abdominal pain, abdominal tightness, flatulence and/or urgency symptoms at the end of each 7-day period via a visual analog scale ranging from 0% (no symptoms) to 100% (very severe symptoms). Patients were also asked about any adverse experiences they may have had and about any change in medications since the previous visit. Patients returned for follow-up visits after 4 and 8 weeks. At the 4-week follow-up visit, capsule count was used to assess compliance. A new supply of the same study medication was dispensed and a return visit was scheduled 4 weeks later. At each follow-up visit, patients were asked to complete a quality of life questionnaire as described above.

Statistical Analysis

The daily diary data were used to obtain an average count of bowel movements per week (over days in which a count was provided) for the subsequent visit. This provided up to eight post-randomization measures for each patient. A missing value for the visit was recorded if no diary counts were provided for the preceding week. There were also two pre-randomization baseline bowel-movement scores obtained at 2 different time points. The weekly symptom evaluations from the visual analog scales were converted to numerical scores by use of a ruler.

The repeated measures were modeled using a general (multivariate) linear model. Covariates included the baseline average count (the average of the two baseline measures) and patient age at the time of randomization. Correlations among repeated measures within each patient were accounted for by assuming a first-order autoregressive correlation model. Residual variances were assumed to be homogenous. Model parameters were estimated using the method of restricted maximum likelihood, which assumes that residuals for each patient follow a multivariate normal distribution. Differences were considered statistically significant at $P \leq 0.05$. Analyses were performed using SAS Version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Patient demographics and regimen compliance

Sixty-one patients signed informed consent agreements and were screened for inclusion in the study. Of these patients, six did not meet the inclusion criteria and were excluded from the study. The remaining 55 patients enrolled and completed the initial 2-week phase of the study and were then randomized for treatment or placebo for 8 weeks. Patients in the placebo group had a mean age that was significantly younger than that of the *B. coagulans* GBI-30, 6086 treated group ($P < 0.044$), primarily due to a few younger subjects who were enrolled in that group (Table I). Patients were predominantly female and most (81.8%) were Caucasian. Three patients in the placebo group were lost to follow-up.

Stool count

In a post hoc analysis, 375 weekly stool counts were gathered from 52 patients (26 patients on active treatment, 26 on placebo), representing an average of 7.2 weekly measurements per patient. After adjusting the treatment effect for baseline counts and age, the average number of bowel movements per day was significantly reduced in the *B. coagulans* GBI-30, 6086 group when compared to placebo ($P = 0.042$; Figure 1).

Table I. *Patient demographics.*

Demographic characteristic		*B. coagulans* GBI-30, 6086 (n=26)	Placebo (n=29)	*P* Value
Age:	Mean ± SD	52.3 ± 11.1	44.0 ± 17.9	< 0.044
	Range	30-67	18-73	NS
Sex:	Male: n (%)	7 (26.9%)	6 (20.7%)	NS
	Female: n (%)	19 (73.1%)	23 (79.3%)	NS
Race:	Asian	1 (3.9%)	1 (3.5%)	NS
	African American	4 (15.4%)	2 (6.9%)	NS
	Caucasian	21 (80.8%)	24 (82.8%)	
	Hispanic	0 (0%)	2 (6.9%)	

NS, not significant

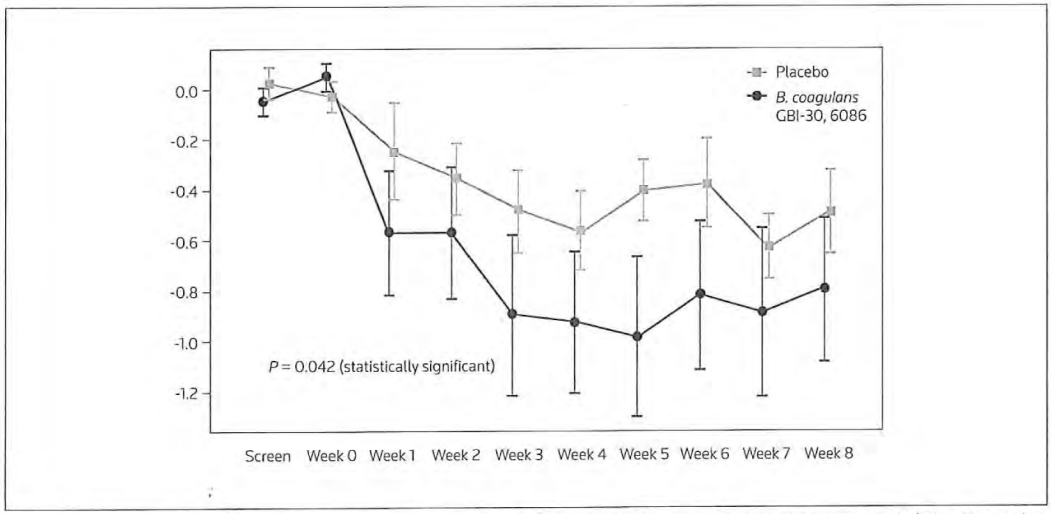

Figure 1. Average number of bowel movements per day. In a post-hoc analysis, 375 weekly stool counts were gathered from 52 patients (26 on *B. coagulans* GBI-30, 6086; 26 on placebo). The change from baseline in the average number of bowel movements per day was significantly reduced in the active treatment group (circles) compared to placebo (squares). $P = 0.042$.

Patient self-assessment

In the analysis of patient-assessed measures, including severity scores and quality of life, the large variability in baseline scores prevented assessment of treatment differences. Although there were no significant differences in patient-assessed measures between the placebo and active treatment groups, baseline variance is likely to have skewed the between-group analysis.

Safety

Adverse events reported were, for the most part, mild to moderate and were generally self limiting. Five patients who received *B. coagulans* GBI-30, 6086 reported six adverse effects and six patients receiving placebo reported six adverse effects. One severe adverse effect (headache) was reported in the placebo group. In general, *B. coagulans* GBI-30, 6086 was well tolerated.

DISCUSSION

Patients with IBS-D currently have limited therapeutic options; however, this study provides evidence that treatment with *B. coagulans* GBI-30, 6086 has the ability to reduce the number of daily bowel movements. In addition, *B. coagulans* GBI-30, 6086 was well tolerated, suggesting it is a viable option for symptom relief.

There are only a small number of studies that have evaluated the benefits of probiotics for the relief of IBS symptoms. The results of these studies are difficult to compare because of differences in study design, dose and bacterial strains used (18). A study by Friedman et al. evaluated the effects of a multi-strain probiotic in relieving diarrhea in a randomized, double-blind, placebo-controlled study of 84 patients with IBS-D (19). The multi-strain probiotic included 2×10^9 CFU each of *Lactobacillus acidophilus* (*L. acidophillus*) LA-5, *Bifidobacterium* BB-12, *Lactobacillus paracasei* CRL-431 and

Streptococcus thermophilus (*S. termophilus*) STY-31, and was administered once daily for 28 days. Results demonstrated that the average number of daily diarrheal episodes decreased significantly in the probiotic group (2.81 episodes) when compared to placebo (3.91 episodes; $P = 0.003$). The probiotic was well tolerated with no significant side effects reported throughout the study.

The effect of a composite probiotic, VSL#3, on IBS-D was evaluated by Kim et al in a small, randomized trial (20). Twenty-five patients received either VSL#3 or placebo twice daily for 8 weeks after a 2-week run-in period. Daily diaries were used to track symptoms. The results showed no significant differences in abdominal pain, gas or urgency from baseline or when compared with placebo. However, compared to baseline values, abdominal bloating was significantly reduced ($P = 0.046$).

An earlier case-control study investigated the efficacy of a probiotic preparation containing *Lactobacillus sporogenes* (*B. coagulans*), *L. acidophilus* and *S. thermophilus* for alleviating the symptoms of IBS (21). Thirty-seven patients were given the probiotic preparation and followed for 6 months. Compared with baseline values, those patients who received the probiotic treatment reported significantly reduced abdominal pain ($P < 0.000001$), abdominal distention ($P = 0.003$), constipation ($P = 0.03$) and alternating constipation and diarrhea ($P = 0.01$).

Similar to the current study, a randomized, double-blind, placebo-controlled clinical trial evaluated the effects of *B. coagulans* GBI-30, 6086 on abdominal pain and bloating (22). The study included 44 patients with IBS-D who received either placebo or *B. coagulans* GBI-30, 6086 once a day for 8 weeks. Self-assessment of the severity of IBS symptoms was recorded daily. Results displayed statistically significant changes in abdominal pain and bloating scores from baseline for patients treated with *B. coagulans* GBI-30, 6086 when compared after 7 weeks ($P < 0.01$). In patients who received placebo, changes in abdominal pain scores on weeks 6 and 8 achieved statistical significance ($P < 0.05$), a result not dissimilar to other clinical studies that have demonstrated a high placebo effect among an IBS patient population (23). No adverse events or serious adverse events were reported during the 8-week study period, and the investigator concluded that *B. coagulans* GBI-30, 6086 may be a safe and effective adjunct therapy for the relief of abdominal pain and bloating for patients with IBS (22).

The mechanism of action of probiotics is likely multifactorial and varies with bacterial strain. Overall, it is believed that probiotics impact the mucosal immune response via: (i) exclusion and inhibition of adherence of pathogenic antigens; (ii) support of epithelial cell survival and barrier integrity; (iii) stimulation of antibody and anti-inflammatory cytokine production; and (iv) dampening of immunologic responses to commensal bacteria (8, 24). Despite the lack of a more complete understanding of the molecular mechanisms of probiotic action, the interaction between specific probiotic bacterial surface antigens and host cell receptors likely mediate many effects of probiotics on various aspects of the host's mucosal barrier function (8, 25).

Probiotics have been demonstrated to exert anti-inflammatory effects at the mucosal surface (26, 27). By reducing mucosal inflammation, probiotics may decrease immune-mediated activation of intestinal motor and sensory neurons and modify neural traffic between the gut and the central nervous system (26). Furthermore, probiotics may also alter the volume and/or composition of stool and gas (28) or increase intestinal mucus secretion (29). These effects may, in turn, influence intestinal handling of its contents and thus modulate symptoms of IBS, such as constipation and diarrhea (21) and potentially improve quality of life (30). In a recent study of 30 adults with post-prandial intestinal gas-related symptoms, *B. coagulans* GBI-30, 6086 reduced the symptoms compared with placebo (31).

B. coagulans GBI-30, 6086 has additional advantages over currently available probiotic preparations. Most conventional forms of lactobacilli-type probiotics are inactivated by bile and at low gastric pH (32, 33). Spores of *Bacillus*, however, are resistant to heat and hostile gastrointestinal conditions and therefore are able to reach the intestine where they can germinate and proliferate within the host (32, 34, 35). In addition, spore-bearing lactic acid-forming bacteria do not show a decrease in viable cells even after heating in saline at 85°C for 30 minutes (33). Maathius et al recently demonstrated in an *in vitro* model system that *B. coagulans* GBI-30, 6086 aids in protein, lactose and fructose digestion (36), a factor which may impact gastrointestinal motility. Future studies will seek to uncover the functional role of *B. coagulans* GBI-30, 6086 in motility in patients with IBS-D.

This study was limited by the need to use self-assessment diaries to measure outcomes, which contributed to the wide variations in reported symptom changes before and after treatment. Nevertheless, the present study provides some evidence that *B. coagulans* GBI-30, 6086 probiotic is well tolerated and has the ability to reduce the number of daily bowel movements in patients with IBS-D. Therefore, *B. coagulans* GBI-30, 6086 may be a safe and effective adjunct therapy for patients with IBS-D who currently have limited therapeutic options. However, larger and longer-term studies of the effects of this probiotic in patients with IBS are needed to confirm these preliminary study results.

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DISCLOSURE

The author states no potential conflicts of interest.

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One-year chronic oral toxicity with combined reproduction toxicity study of a novel probiotic, *Bacillus coagulans*, as a food ingredient

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ABSTRACT

Some strains of *Bacillus coagulans* can survive extremes of heat, stomach acid and bile acids, to which commonly consumed probiotics are susceptible. A toxicological safety assessment was published in 2009 on a proprietary preparation of *B. coagulans* – GanedenBC$^{30™}$ – a novel probiotic. It was concluded that GanedenBC$^{30™}$ is safe for chronic human consumption based upon scientific procedures, supported by a safe history of use (Endres et al., 2009).

A one-year chronic oral toxicity study combined with a one-generation reproduction study was conducted to further investigate safety of long-term consumption. The one-year study of GanedenBC$^{30™}$ administered to male and female HsdBrlHan: Wistar rats in their diet showed no signs of toxicity at the highest dose tested. The conclusion from the reproduction toxicity study is that administration of GanedenBC$^{30™}$ in the diet caused no signs of toxicity in the parental generation (male or female) nor the F1 offspring. Using the lowest NOEL of 1948 mg/kg concluded at the end of the 1-year feeding study, a 100-fold safety factor, a test article concentration of 6.88×10^{10} CFU (colony forming units) per gram, and an average 70 kg human, it is determined that GanedenBC$^{30™}$ is safe for chronic consumption at up to 9.38×10^{10} CFUs per day.

1. Introduction

Probiotics are defined as viable organisms (generally bacteria or yeast) that have demonstrated beneficial effects on the health of a host (Lee, 1999). Interest in probiotics for health promotion is at an all time high in more than 100 years they have been used (De Vecchi and Drago, 2006). The primary use of probiotics has been suggested for the prevention and/or mitigation of specific disorders such as irritable bowel syndrome (IBS), eczema, allergies, *Helicobacter pylori* infection, as well as for support of intestinal and immunological health (Tappenden and Deutsch, 2007; Quigley, 2007; Rastall et al., 2005; Hyronimus et al., 2000; Spiller, 2008; McFarland and Dublin, 2008; Lesbros-Pantoflickova et al., 2007; Ouwehand, 2007). Since 2009, the results of six clinical trials with GanedenBC$^{30™}$ have been published (Mandel et al., 2010; Kimmel et al., 2010; Dolin, 2009; Hun, 2009; Kalman et al., 2009; Baron 2009).

In general, probiotic organisms are sensitive to normal physiological conditions such as the very low pH of the stomach and exposure to bile salts in the gastrointestinal tract. The viability of these bacteria can also be affected by manufacturing processes, storage, and shipping conditions. (Bezkorovainy, 2001; Ljungh and Wadstrom, 2006; Graff et al., 2008). Because *Bacillus coagulans* is a spore-forming bacterium, certain strains are able to survive the extremes of heat, acidity of the stomach, and bile acids, and thrive at physiological conditions (Patel et al., 2006; De Clerck et al., 2004; De Vecchi and Drago, 2006; Hyronimus et al., 2000; Katsutoshi et al., 2003). This increased hardiness, in comparison to typical probiotic organisms, results in a greater probability for survival through, and hence is able to transiently populate, the gastrointestinal tract (Adami and Cavazzoni, 1999). *B. coagulans*, when taken orally, has been demonstrated in a series of experimental studies to have beneficial effects on the gastrointestinal environment, stool frequency, and dermal appearance in animals and humans (Adami and Cavazzoni, 1999; Donskey et al., 2001; Katsutoshi et al., 2003). Because it lacks the ability to adhere to the intestinal epithelium, it is completely eliminated in four to five days unless chronic administration is maintained (Donskey et al., 2001). An unpublished study conducted by Goodner et al., reports the results of a PCR-based assay investigating GanedenBC$^{30™}$ for the potential *B. cereus* enterotoxin and haemolysin genes. The conclusion from the study

Abbreviations: BC30 or GBI-30, GanedenBC^{30}.

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was that GanedenBC^{30}™ does not produce any sort of enterotoxin or haemolysin (Goodner, in press).

The history of *B. coagulans* dates back to 1915, when the discovery of curdled canned evaporated milk was first described by the Iowa Agricultural Experiment Station (Sarles and Hammer, 1932). In 1932, *B. coagulans* was first isolated and described by Horowitz-Wlassowa and Nowotelnow (Gandhi, 1994).

In 2009 we presented the first comprehensive safety assessment for the strain of *B. coagulans* known as GanedenBC^{30}™ (*B.* coagulans GBI-30, 6086). That assessment was based upon not only a history of human exposure, but more importantly on the results of the following FDA, OECD, and GLP compliant toxicological studies: bacterial reverse mutation assay; chromosomal aberration and mouse micronucleus studies, skin and eye irritation study in the rabbit, acute oral toxicity (the limit test) study in the rat, and a 90-day subchronic repeat oral toxicity study in the rat (Endres et al., 2009). This paper presents the results of a *US FDA Redbook* compliant one-year chronic repeated oral toxicity study combined with a one-generation reproduction toxicity study in the rat. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of Toxi-Coop Zrt., (Budapest, Hungary).

2. Materials and methods

2.1. Test product

The test product, GanedenBC^{30}™, supplied by Ganeden Biotech, Inc., (Mayfield Heights, OH, USA) is sold as a dietary ingredient for use in functional foods and dietary supplements. The organism is a gram-positive spore-forming rod that is aerobic to microaerophilic in nature. GanedenBC^{30}™ is manufactured as a pure cell mass consisting solely of *B. coagulans*. The pure cell mass is either spray-dried with maltodextrin or freeze-dried and mixed with maltodextrin to achieve the desired concentration of 15×10^9 CFU/g for the finished product. For the purpose of these toxicological studies, pure, GanedenBC^{30}™ (Lot# 90BC008–6.88 $\times$ 10^{10} CFU/g) was used.

The test article was mixed into standard rodent diet (Ssniff® SM R/M-Z+H, Ssniff Spezialdiäten GmbH, D-59494 Soest, Germany) on a weight per weight basis and administered orally to animals at predetermined concentration levels. Fresh preparations of the mixture were prepared every three months. Concentration and homogeneity of the GanedenBC^{30}™ cell mass were examined at each dose level and from each batch of the test diet. Control animals received only the standard rodent diet.

2.2. Chronic one-year oral toxicity study in rats

This study was conducted in accordance with procedures indicated by OECD Guideline for the Testing of Chemicals, No. 452 (12 May 1981) and *US FDA Redbook 2000* standards IV.C.5.a., "Chronic Toxicity Studies with Rodents" (July 2007).

Male and female HsdBrlHan rats of Wistar origin (7–9 weeks old) were obtained from Toxi-Coop Zrt. (Budapest, Hungary). The animals were housed in individual cages, at a temperature of 22 ± 3 °C, a relative humidity of 30–70%, and a 12-h light-dark cycle. Animals were acclimated for 14 days prior to initiation of treatment.

Animals received Ssniff® SM R/M-Z + H complete diet for rats (Ssniff Spezialdiäten GmbH, Soest, Germany). Tap water was available *ad libitum*. Drinking water was analyzed once after the study and determined to be free of contaminants. An equal number of animals from each weight and sex group were randomized to the four experimental groups. Body weights of the animals at randomization were 175–217 g for males and 136–189 g for females. Each group consisted of 40 rats (20/sex). Animals were sorted according to body weight. An equal number of animals from each weight group were randomized to each of four experimental groups per sex.

A high target dose level of 2000 mg/kg bw/day was established with additional dosage levels of 0, 600 and 1200 mg/kg bw/day. These levels corresponded to a test article concentration in rat feed of 0, 10,000, 20,000 and 33,300 mg/kg. The highest target dose was chosen based on the results of previous toxicology studies showing a lack of toxicity of GanedenBC^{30}™ (Endres et al., 2009). Dietary concentrations were not adjusted for body weight or food consumption during the study. All groups were fed diets containing the stipulated concentration of GanedenBC^{30}™ for 52 to 53 weeks on a daily dosing schedule of a 7-day per week basis.

Observations for signs of morbidity and mortality were made twice daily, while general clinical observations were conducted once daily following treatment at approximately the same time each day. Detailed clinical observations were made on all animals outside the cage using a standard method both before the first exposure, and once a week thereafter. Observations focused on skin, fur, eyes, mucous membranes, autonomic activity, circulatory and central nervous system, somatomotor activity, behavior pattern, tremors, convulsions, salivation, stool consistency, lethargy, sleep, changes in gait, posture and response to handling.

All animals were weighed on the day of randomization, on day 0 before the start of treatment, once weekly during the first 13 weeks of the study, and once every four weeks thereafter.

Food was weighed weekly, and the average food consumption per animal was calculated. Ophthalmological examination was performed before the study and at the end of the study. Clinical pathology examinations, including hematology and chemistry screens, were conducted in all animals at the end of the first 3 weeks, and at study months 3, 6, and 12. Urinalysis was performed the week prior to initiation of treatment, and at the end of study months 3, 6, and 12. Moribund animals were necropsied as soon as possible after observation. Any animals found dead were necropsied in the same manner as animals that were sacrificed at the end of the study. All surviving rats were sacrificed under Euthasol® anesthesia (Produlab Pharma, Raamsdonksveer, Netherlands) and necropsied during week 53. External appearance of each animal was examined, followed by dissection of the cranial, thoracic and abdominal cavities to observe the appearance of tissues and organs. All abnormalities were recorded in detail. Organs were preserved in 10% formalin solution, while testes were preserved in Bouin's solution.

Measurements of organ weights were recorded, including the liver, brain, heart, thymus, spleen, kidneys, testes, epididymis, uterus, thyroid/parathyroid, adrenal glands and ovaries. Full histopathological exam was performed in the control (0 mg/kg bw/day) and high dose (2000 mg/kg bw/day) groups, and in animals found dead or moribund. Any observations of abnormalities in organs from the mid and low-dose groups resulted in histological examination of the relevant tissues.

Statistical analyses were performed to assess body weight and body weight gain, food consumption, hematology parameters, clinical chemistry parameters, urine parameters and organ weights. Evaluation of data was performed using SPSS PC+4.0.

2.3. One-generation reproductive toxicity study in rats

This study was conducted in accordance with procedures indicated by OECD Guideline for the Testing of Chemicals, No. 415 (26 May 1983) and *US FDA Redbook 2000* standards IV.C.9.a., "Guidelines for Reproduction Studies" (July 2000).

The study was conducted in parallel to the one-year chronic oral toxicity study described above. Male and female HsdBrlHan rats of Wistar origin (7–9 weeks old) were obtained from Toxi-Coop Zrt. (Budapest, Hungary) and housed in the same environmental conditions described above. Animals were acclimated for 14 days prior to initiation of treatment.

For this study, parental animals were identified with numbers written in permanent marker on their tails following randomization into four dose groups (0 mg/kg bw/day (control), 600 mg/kg bw/day, 1200 mg/kg bw/day and 2000 mg/kg bw/day) as above. Each group consisted of 10 males and 20 females. Animals received the test article mixed into standard rodent diet and tap water *ad libitum*, as described above.

The test article-enriched diet, or control diet, in the appropriate concentration was dosed for ten weeks in all groups and during a three-week mating period. Male rats were dosed for 70 days before mating (corresponding to one complete spermatogenic cycle) and during the three-week mating period. Female rats were dosed with the test article mixed in the diet for ten weeks prior to mating, during the three-week mating period, throughout pregnancy and lactation and up to weaning of the F1 offspring.

The mating procedure consisted of one female being placed with a single male. Mating time was three hours daily in the morning for three weeks. The female remained with the same male until pregnancy occurred, or until the three weeks was over. Each morning a vaginal smear was prepared and stained with 1% aqueous methylene blue solution. The day of mating (identified by the presence of a vaginal plug or sperm on the vaginal smear) was considered as day 0 of the pregnancy. Sperm positive females were then separated from the males and housed singly.

Females that failed to mate within two estrous cycles were re-mated with proven males of the same test group. Pairs still failing to mate were evaluated to determine the cause of apparent infertility. This included additional opportunities to mate, microscopic examination of reproductive organs, and examination of the estrous cycle. Pregnant females were allowed to litter normally and rear progeny to the stage of weaning without standardization.

Observations for signs of morbidity and mortality, as well as detailed clinical observations, were made in the manner described above for the one-year study.

All animals were weighed on the day of randomization and on day 0 before the start of treatment. Male animals were weighed weekly until their necropsy. Female animals were weighed and evaluated weekly (on the same day) prior to and during mating, on gestation days 0, 7, 14 and 21, and on lactation days 0, 7, 14 and 21. Live pups were counted and weighed on the morning after birth and on days 4 and 7, and then weekly thereafter until termination of the study.

Food consumption was measured weekly during the pre-mating and mating periods. After parturition, and during lactation, food consumption was measured on the same day the litters were weighed.

Reproductive parameters of dams were assessed as follows: vaginal smears of all females were prepared daily during the pre-mating period and during mating. The smears were stained with 1% aqueous methylene blue solution and examined with a light microscope. Females were allowed to litter and rear their offspring. Dams were observed on the basis of whether or not they made a nest from provided bedding material and covered their newborns. Efficiency of nursing was observed by the presence of milk in the pups' stomachs. All observations were recorded.

The day of delivery of the F1 pups was defined as postnatal day 0. Two hours after delivery (following the first feeding), the offspring were individually identified by cutting off fingertips, were weighed, and sex was determined. Any observed abnormalities were recorded after identification.

All litters were checked daily for the number of viable and dead pups. Any dead pups were subjected to necropsy to identify the potential cause of death. F1 animals were weighed with an accuracy of 0.01 g up to the seventh postnatal day, and with an accuracy of 0.1 g until the 21st postnatal day. Weights were taken on days 0, 4, 7, 14 and 21. Pup development was assessed using surface-righting reflex, pinna detachment and eye opening.

Necropsy of the animals in these groups was conducted as follows: Males and non-mated females were sacrificed under anesthesia (as in the one-year study above) after the mating period and necropsied; animals failing to deliver up to gestation day 24, and dams with total litter death, were sacrificed and necropsied; dams with viable pups on lactation day 21 were necropsied, and the number of implantations in the uterus and the number of corpora lutea were counted. Any dead pups were examined to ascertain the cause of death, and were designated as live-born or stillborn. Offspring were sacrificed without necropsy following postnatal day 21.

Organ weights were measured and recorded for the parental animals, including the liver, brain, thymus, spleen, kidneys, testes, epididymis, uterus, adrenals and ovaries. Microscopic examinations were conducted on ovaries, uterus, cervix, vagina, testes, epididymis, seminal vesicles, prostate, coagulating gland and pituitary gland.

3. Results

3.1. Chronic one-year oral toxicity study in rats

The chronic one-year oral toxicity study on GanedenBC^{30}™ was performed to investigate for potential health hazards that are likely to arise as a result of repeated oral administration in the diet over a considerable part of the lifespan of HsdBrlHan rats of Wistar origin.

Analysis of the dietary intake of the rats and actual dose levels of GanedenBC^{30}™ in the various dose groups revealed the following: 600 mg/kg bw/day target dose group (males received a calculated mean daily intake of 585 mg/kg bw/day, females received 761 mg/kg bw/day); 1200 mg/kg bw/day target dose group (males: 1147 mg/kg bw/day, females: 1467 mg/kg bw/day); 2000 mg/kg bw/day target dose group (males: 1951 mg/kg bw/day, females: 2525 mg/kg bw/day).

No mortality occurred in any of the male groups (control, 600, 1200 and 2000 mg/kg bw/day) or the female group receiving 600 mg/kg bw/day of the test article. One female treated with 1200 mg/kg bw/day was found dead on day 137. Signs of irritability, stereotyped movement, abnormal gait, and eye and nasal drainage were observed on the preceding days. Gross pathology examination revealed autolysis of organs and tissues; therefore, histopathological examination was not feasible. The cause of death was attributed to an individual disorder. In the control group, one female was euthanized on day 104, while a female in the 2000 mg/kg bw/day group was euthanized on day 331. In the female in the control group, diffuse subacute dermatitis and cachexia were observed, along with an eventual decrease in body weight. In the female euthanized from the 2000 mg/kg bw/day group, an enlarged abdomen and skin scarring were noted. Pathological examination revealed generalized fibrosarcoma as the cause of the moribund condition. In accordance with historical control data of rats in the same age group, these alterations were considered to be individual occurrences, unrelated to consumption of GanedenBC^{30}™.

In terms of clinical observations over the course of the study, one male animal in the control group exhibited signs of irritability for one day (day 315), while in the female animals of this group, one had decreased body tone and four had alopecia, while two showed signs of skin scarring. One male animal in the 600 mg/kg bw/day group exhibited periods of alopecia between days 46 and 153 of the study, interrupted with normal periods. In the females of this group, four exhibited alopecia and one animal showed signs of orbital swelling and eye drainage. In the 1200 mg/kg bw/day group, no clinical findings were noted in the males, while one female exhibited decreased body tone and another exhibited alopecia. No clinical signs were noted in males in the 2000 mg/kg bw/day group. In females of this group, one had sporadic body scarring, two showed a transient decrease in body tone, one exhibited a decreased grip tone, and another showed a decreased righting reflex. Overall, detailed clinical observations did not reveal any signs of toxicity related to chronic administration of the test article. The signs and symptoms occurring mainly in the females were considered individual alterations, as these signs are often seen in experimental rats and were not dose-related. In the two highest dose groups, the general physical condition and behavior of male animals were considered to be normal throughout the entire observation period.

Body weights of animals in all treatment groups remained similar to those of animals in the control group throughout the one-year treatment period. No effect of the test article was noted on body weight development.

Mean food consumption in all treatment groups was slightly below that of control animals during the entire observation period, with several weeks reaching statistical significance. The differences were likely a result of the taste of the mixed diet, were not found to be dose related, and were still well within the historical background range for the animals.

Ophthalmoscopic exam failed to detect any abnormalities in any animals pre-treatment. At the end of the study, hyperemic conjunctiva and sanguineous adnexa were found in one female in the 600 mg/kg bw/day treatment group, while corneal opacity was noted in a single female in the 2000 mg/kg bw/day group. None of these changes were treatment-related.

All hematological parameters were similar to the control values for both males and females in the 1200 mg/kg bw/day and 2000 mg/kg bw/day treatment groups, and for females in the 600 mg/kg bw/day group, when assessed at week three (Table 1). In male rats of the 600 mg/kg bw/day group, slight decreases were seen in mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCH) as compared to controls. However, no statistically significant differences were noted in hematological parameters in any of the groups at the end of the third month or at the end of the sixth month. At the end of the study (month 12), male animals in the 600 mg/kg bw/day group had a higher percentage of neutrophils, and a lower percentage of lymphocytes, while the hematocrit and partial thromboplastin time values were lower than control values. Male animals in the 1200 mg/kg bw/day group all exhibited values similar to controls, while in females of this group, white blood cell counts were slightly higher and prothrombin time was slightly lower than control animals. Male and female animals in the 2000 mg/kg bw/day group exhibited slight elevations in white blood cell counts compared to controls, while females were found to also have a slightly lower percentage of eosinophils than control animals. On the whole, no toxicologically relevant differences in hematological parameters in any treated groups were found at the end of week 3, or at the end of the 3rd, 6th and 12th months. The statistical differences seen in some parameters at the end of the 12th month were not considered to be toxicologically meaningful since they were of a low magnitude or of singular occurrence. Furthermore, all of these changes were within historical control ranges for this strain of rat.

Table 1
Summary of selected hematology and clinical chemistry data[a] from the GanedenBC^{30}™ one-year repeated dose oral toxicity study in HsdBrlHan:Wistar rats.

Parameters	Group number (mg/kg bw/day: targeted–actual) Males				Group number (mg/kg bw/day: targeted–actual) Females			
	1 (0–0)	2 (600–585)	3 (1200–1147)	4 (2000–1951)	1 (0–0)	2 (600–761)	3 (1200–1467)	4 (2000–2525)
Hematology								
WBC ($\times 10^9$/L)								
Week 3	9.76 ± 1.47	9.00 ± 1.53	9.48 ± 1.62	10.65 ± 3.28	8.07 ± 1.78	7.52 ± 1.70	7.43 ± 1.50	7.18 ± 1.48
Month 3	8.36 ± 2.11	7.91 ± 1.65	8.63 ± 1.59	8.34 ± 2.28	4.80 ± 1.05	5.18 ± 1.43	4.78 ± 1.00	4.90 ± 1.17
Month 6	7.84 ± 1.78	7.48 ± 1.56	7.74 ± 1.55	8.22 ± 1.73	4.81 ± 1.13	4.94 ± 1.42	4.55 ± 1.13	4.75 ± 1.69
Month 12	5.67 ± 1.22	5.45 ± 1.02	6.38 ± 1.32	7.14 ± 1.45**	3.79 ± 0.43	4.04 ± 0.74	4.50 ± 1.07*	4.73 ± 1.36*
Neutrophils (%)								
Week 3	13.74 ± 2.35	13.02 ± 3.15	12.25 ± 4.02	13.19 ± 4.64	10.06 ± 2.85	12.46 ± 3.61	11.65 ± 4.34	11.47 ± 4.55
Month 3	16.49 ± 4.32	18.58 ± 6.45	14.69 ± 3.05	17.16 ± 5.73	13.74 ± 3.99	15.49 ± 4.20	13.35 ± 4.17	15.41 ± 6.25
Month 6	16.95 ± 2.70	18.06 ± 6.25	15.97 ± 4.68	17.63 ± 4.69	15.23 ± 4.89	17.27 ± 6.27	17.57 ± 6.23	16.87 ± 8.43
Month 12	23.04 ± 4.65	28.25 ± 6.32**	24.12 ± 8.58	25.99 ± 8.31	24.21 ± 4.89	30.35 ± 8.35**	26.81 ± 6.04	23.99 ± 7.80
Lymphocytes (%)								
Week 3	82.97 ± 2.76	83.67 ± 3.37	84.62 ± 4.33	83.93 ± 5.00	86.57 ± 3.26	83.90 ± 4.19	84.76 ± 5.69	85.65 ± 4.85
Month 3	79.09 ± 4.61	76.91 ± 6.54	80.96 ± 3.62	78.35 ± 6.60	81.90 ± 4.48	80.12 ± 4.60	82.11 ± 4.83	80.86 ± 6.71
Month 6	78.26 ± 3.26	76.82 ± 6.79	78.79 ± 5.83	76.98 ± 4.93	80.05 ± 5.13	78.06 ± 6.17	77.29 ± 6.24	78.41 ± 9.51
Month 12	72.12 ± 5.16	66.16 ± 6.47**	70.73 ± 9.29	69.00 ± 9.01	69.23 ± 5.29	62.91 ± 8.32**	66.75 ± 5.63	70.08 ± 8.57
Hematocrit (L/L)								
Week 3	0.46 ± 0.01	0.46 ± 0.01	0.46 ± 0.02	0.46 ± 0.02	0.46 ± 0.01	0.46 ± 0.02	0.46 ± 0.02	0.46 ± 0.01
Month 3	0.47 ± 0.02	0.66 ± 0.89	0.47 ± 0.03	0.47 ± 0.02	0.46 ± 0.01	0.46 ± 0.02	0.46 ± 0.02	0.46 ± 0.02
Month 6	0.47 ± 0.01	0.46 ± 0.02	0.47 ± 0.02	0.47 ± 0.02	0.46 ± 0.02	0.45 ± 0.02	0.46 ± 0.02	0.46 ± 0.02
Month 12	0.46 ± 0.02	0.44 ± 0.02*	0.46 ± 0.03	0.45 ± 0.03	0.44 ± 0.02	0.43 ± 0.03	0.44 ± 0.02	0.44 ± 0.02
MCV (fL)								
Week 3	52.46 ± 2.34	50.73 ± 1.94*	52.07 ± 2.19	51.89 ± 2.51	51.72 ± 2.79	51.61 ± 2.89	52.56 ± 2.87	51.74 ± 2.59
Month 3	49.65 ± 1.46	48.49 ± 2.41	49.65 ± 2.15	48.90 ± 2.44	51.42 ± 2.21	51.49 ± 2.00	52.19 ± 2.02	51.84 ± 1.92
Month 6	48.34 ± 1.57	46.97 ± 2.44	47.91 ± 2.02	47.84 ± 2.45	49.78 ± 2.34	50.14 ± 2.67	50.22 ± 2.00	50.29 ± 2.03
Month 12	48.37 ± 1.76	47.16 ± 2.59	48.64 ± 2.34	48.05 ± 2.87	50.51 ± 2.08	50.98 ± 2.78	50.75 ± 2.25	50.56 ± 1.85
MCH (pg)								
Week 3	18.27 ± 0.60	17.76 ± 0.61*	18.19 ± 0.65	18.02 ± 0.55	17.99 ± 0.82	18.01 ± 0.80	18.13 ± 0.71	18.00 ± 0.64
Month 3	17.33 ± 0.45	16.99 ± 0.69	17.36 ± 0.57	17.11 ± 0.63	17.73 ± 0.70	17.76 ± 0.66	17.92 ± 0.55	17.85 ± 0.52
Month 6	17.38 ± 0.43	16.95 ± 0.68	17.27 ± 0.54	17.26 ± 0.73	17.85 ± 0.67	18.00 ± 0.78	17.99 ± 0.63	18.00 ± 0.52
Month 12	17.68 ± 0.51	17.34 ± 0.77	17.79 ± 0.69	17.58 ± 0.84	18.33 ± 0.75	18.50 ± 0.87	18.45 ± 0.67	18.45 ± 0.59
APTT (sec)								
Week 3	20.05 ± 2.24	20.89 ± 1.61	21.13 ± 2.22	20.97 ± 2.22	20.56 ± 2.02	20.44 ± 1.96	19.71 ± 2.04	20.96 ± 2.03
Month 3	21.15 ± 2.59	21.26 ± 4.11	22.84 ± 3.05	21.38 ± 2.86	21.54 ± 2.22	21.73 ± 1.79	21.86 ± 1.70	22.66 ± 2.61
Month 6	22.00 ± 2.77	20.64 ± 2.83	21.39 ± 2.35	21.92 ± 3.08	22.99 ± 2.89	22.12 ± 1.58	22.39 ± 2.13	22.97 ± 1.83
Month 12	20.32 ± 2.38	18.43 ± 2.12*	19.18 ± 2.41	19.01 ± 2.52	18.93 ± 1.95	19.71 ± 1.83	19.44 ± 1.79	20.03 ± 1.25
PT (sec)								
Week 3	22.11 ± 1.93	23.00 ± 1.75	22.69 ± 2.79	22.34 ± 2.52	22.37 ± 2.02	21.45 ± 2.03	21.83 ± 2.26	22.99 ± 2.01
Month 3	21.55 ± 2.29	22.61 ± 4.92	22.88 ± 1.69	22.19 ± 2.15	22.19 ± 1.83	21.66 ± 1.86	21.73 ± 2.58	22.25 ± 2.24
Month 6	21.27 ± 3.42	20.69 ± 2.50	20.57 ± 2.57	21.40 ± 2.37	20.78 ± 2.04	20.34 ± 1.37	19.81 ± 1.70	20.76 ± 1.72
Month 12	25.26 ± 1.60	24.92 ± 1.80	24.07 ± 2.59	24.31 ± 2.14	22.12 ± 1.94	21.74 ± 2.24	20.47 ± 1.25**	21.38 ± 1.20
Chemistry								
Glucose (mmol/l)								
Week 3	5.99 ± 1.03	6.17 ± 1.05	5.92 ± 0.82	6.16 ± 0.95	5.56 ± 0.92	5.19 ± 0.94	5.63 ± 0.80	5.84 ± 0.85
Month 3	5.75 ± 0.92	5.69 ± 0.84	5.62 ± 0.76	5.43 ± 0.79	4.67 ± 0.72	4.61 ± 0.84	4.85 ± 0.49	4.98 ± 0.84
Month 6	7.12 ± 1.93	6.78 ± 0.79	6.98 ± 0.82	6.62 ± 0.96	5.86 ± 0.86	5.61 ± 0.84	5.96 ± 0.83	6.17 ± 1.12
Month 12	6.20 ± 0.60	5.66 ± 0.50**	5.79 ± 0.53*	5.32 ± 0.40**	5.13 ± 0.75	4.88 ± 0.70	4.99 ± 0.69	4.73 ± 0.57
T. Bil (μmol/l)								
Week 3	1.92 ± 0.31	1.90 ± 0.36	1.92 ± 0.38	1.86 ± 0.34	2.51 ± 0.50	2.47 ± 0.52	2.37 ± 0.53	2.58 ± 0.51
Month 3	3.17 ± 0.31	4.63 ± 6.16	3.20 ± 0.40	3.20 ± 0.56	3.54 ± 0.46	3.69 ± 0.34	3.72 ± 0.43	3.67 ± 0.61
Month 6	2.16 ± 0.34	2.15 ± 0.35	2.11 ± 0.47	2.19 ± 0.43	2.64 ± 0.65	2.77 ± 0.61	2.78 ± 0.66	3.00 ± 0.67
Month 12	1.88 ± 0.68	2.09 ± 0.44	2.25 ± 0.49	2.40 ± 0.63**	2.38 ± 1.14	2.68 ± 0.75	3.17 ± 0.99*	2.95 ± 1.04
Phos (mmol/l)								
Week 3	2.57 ± 0.19	2.40 ± 0.15**	2.47 ± 0.15	2.33 ± 0.19**	2.22 ± 0.28	2.07 ± 0.32	2.09 ± 0.25	2.07 ± 0.26
Month 3	2.15 ± 0.21	2.12 ± 0.23	2.05 ± 0.21	2.00 ± 0.15*	1.85 ± 0.30	1.76 ± 0.26	1.75 ± 0.37	1.66 ± 0.30
Month 6	1.85 ± 0.30	1.77 ± 0.21	1.82 ± 0.21	1.79 ± 0.21	1.60 ± 0.33	1.44 ± 0.28	1.41 ± 0.38	1.55 ± 0.35
Month 12	1.34 ± 0.20	1.42 ± 0.21	1.56 ± 0.20**	1.70 ± 0.20**	1.04 ± 0.19	1.27 ± 0.29**	1.48 ± 0.24**	1.60 ± 0.24**
Na^+ (mmol/l)								
Week 3	139.90 ± 1.21	140.15 ± 1.09	139.55 ± 1.05	139.60 ± 0.94	139.70 ± 1.49	139.40 ± 1.23	140.10 ± 1.17	140.20 ± 1.11
Month 3	141.75 ± 1.29	141.60 ± 1.14	141.75 ± 0.91	141.95 ± 0.60	140.75 ± 1.25	141.05 ± 1.15	141.25 ± 1.45	141.20 ± 1.67
Month 6	139.19 ± 1.30	139.43 ± 0.84	139.56 ± 1.19	140.08 ± 1.27	139.29 ± 2.02	138.67 ± 1.39	139.31 ± 1.46	139.49 ± 1.29
Month 12	142.14 ± 1.37	142.01 ± 1.64	141.94 ± 1.04	142.01 ± 0.80	141.71 ± 1.61	141.97 ± 1.65	141.25 ± 1.07	140.66 ± 1.60*
Cl^- (mmol/l)								
Week 3	100.43 ± 1.46	100.84 ± 1.59	100.20 ± 1.14	100.59 ± 1.39	100.48 ± 1.74	100.64 ± 1.47	101.21 ± 1.72	101.24 ± 1.28
Month 3	101.40 ± 1.00	101.84 ± 1.27	101.51 ± 1.04	102.15 ± 1.12	101.67 ± 1.68	101.85 ± 1.43	102.57 ± 1.94	102.55 ± 1.66

(*continued on next page*)

Table 1 (*continued*)

Parameters	Group number (mg/kg bw/day: targeted–actual) Males				Group number (mg/kg bw/day: targeted–actual) Females			
	1 (0–0)	2 (600–585)	3 (1200–1147)	4 (2000–1951)	1 (0–0)	2 (600–761)	3 (1200–1467)	4 (2000–2525)
Month 6	101.08 ± 1.08	101.74 ± 0.70*	101.85 ± 0.90*	101.85 ± 1.13*	101.14 ± 1.80	100.70 ± 1.27	101.76 ± 1.69	101.19 ± 1.62
Month 12	104.02 ± 0.94	104.24 ± 1.01	103.64 ± 1.06	103.90 ± 1.09	103.54 ± 1.61	102.64 ± 2.22	103.31 ± 1.65	102.34 ± 1.51
AST (U/l)								
Week 3	99.92 ± 14.70	97.01 ± 16.63	99.92 ± 11.83	98.15 ± 13.91	102.69 ± 13.93	93.81 ± 6.80*	91.05 ± 8.50**	91.37 ± 11.66**
Month 3	101.68 ± 15.80	101.49 ± 13.10	98.46 ± 12.04	98.57 ± 16.86	109.47 ± 18.55	99.45 ± 14.35*	99.51 ± 11.55*	100.31 ± 11.49*
Month 6	116.00 ± 17.36	116.63 ± 21.88	114.83 ± 27.37	111.75 ± 25.28	110.60 ± 13.14	110.14 ± 16.41	114.52 ± 43.71	106.79 ± 26.02
Month 12	116.82 ± 25.81	115.38 ± 25.93	105.54 ± 20.95	114.09 ± 31.95	136.59 ± 40.90	129.79 ± 61.59	119.32 ± 39.74	120.03 ± 71.95**
ALT (U/l)								
Week 3	47.83 ± 10.54	47.96 ± 12.22	48.40 ± 8.72	47.95 ± 9.92	45.11 ± 8.59	37.54 ± 5.25**	37.63 ± 5.05**	37.54 ± 7.18**
Month 3	42.79 ± 9.40	44.59 ± 9.39	45.83 ± 10.76	44.02 ± 7.40	43.62 ± 7.30	41.25 ± 8.75	39.84 ± 5.47	46.53 ± 8.96
Month 6	62.01 ± 12.72	63.21 ± 16.57	65.88 ± 18.50	67.61 ± 17.28	59.97 ± 10.14	60.01 ± 11.50	59.18 ± 19.66	59.90 ± 19.04
Month 12	73.85 ± 26.76	70.63 ± 17.34	68.29 ± 20.73	69.60 ± 24.30	75.60 ± 16.26	77.30 ± 54.83	71.09 ± 29.26	73.85 ± 45.96
GGT (U/l)								
Week 3	1.75 ± 0.56	1.72 ± 0.38	1.77 ± 0.55	2.10 ± 0.50*	2.36 ± 0.66	1.96 ± 0.56	2.26 ± 0.56	2.46 ± 0.69
Month 3	1.45 ± 0.65	1.83 ± 0.79	2.04 ± 0.75*	2.06 ± 0.68*	2.12 ± 0.69	2.03 ± 0.72	1.94 ± 0.82	2.18 ± 0.77
Month 6	1.64 ± 0.63	2.02 ± 0.76	2.14 ± 0.69*	2.57 ± 0.70**	1.96 ± 0.78	1.70 ± 0.84	2.04 ± 0.64	2.08 ± 0.38
Month 12	1.33 ± 0.91	1.71 ± 0.96	1.27 ± 0.76	1.40 ± 0.94	1.23 ± 0.89	1.03 ± 0.86	1.30 ± 0.81	1.39 ± 1.04
Albumin (g/l)								
Week 3	33.18 ± 0.95	32.99 ± 0.74	33.21 ± 1.03	33.12 ± 0.88	34.81 ± 1.31	34.97 ± 1.28	34.90 ± 0.92	35.52 ± 1.09
Month 3	33.63 ± 0.86	33.21 ± 0.84	33.32 ± 0.79	33.18 ± 0.96	34.90 ± 1.84	35.60 ± 1.15	35.17 ± 1.27	35.76 ± 1.61
Month 6	33.90 ± 1.08	33.37 ± 0.81	33.62 ± 0.87	33.53 ± 1.31	36.49 ± 1.75	36.29 ± 1.14	36.08 ± 1.21	37.19 ± 1.81
Month 12	34.01 ± 0.77	33.39 ± 0.79*	33.37 ± 0.62*	33.83 ± 0.89	37.17 ± 1.49	36.85 ± 1.57	36.99 ± 1.21	37.27 ± 1.46
Protein (g/l)								
Week 3	58.46 ± 2.38	57.93 ± 2.04	58.02 ± 2.31	58.08 ± 2.24	61.17 ± 3.02	61.58 ± 2.34	61.71 ± 2.19	62.86 ± 3.02
Month 3	60.43 ± 2.28	60.95 ± 1.78	60.91 ± 2.03	60.06 ± 2.50	62.47 ± 3.49	64.49 ± 2.60	64.34 ± 2.62	65.36 ± 3.67**
Month 6	61.62 ± 2.89	61.06 ± 2.60	61.56 ± 1.74	61.01 ± 2.83	65.61 ± 3.98	66.05 ± 2.73	65.55 ± 2.38	67.67 ± 3.22
Month 12	62.43 ± 2.81	60.77 ± 1.89	61.53 ± 1.41	61.57 ± 2.30	66.88 ± 3.48	66.46 ± 3.65	67.31 ± 2.33	68.59 ± 2.95
A/G ratio								
Week 3	1.33 ± 0.09	1.34 ± 0.07	1.35 ± 0.08	1.33 ± 0.07	1.32 ± 0.08	1.31 ± 0.06	1.32 ± 0.09	1.29 ± 0.09
Month 3	1.25 ± 0.08	1.20 ± 0.08	1.22 ± 0.09	1.25 ± 0.11	1.27 ± 0.09	1.24 ± 0.08	1.25 ± 0.10	1.22 ± 0.08
Month 6	1.23 ± 0.08	1.21 ± 0.08	1.21 ± 0.07	1.23 ± 0.09	1.26 ± 0.09	1.22 ± 0.08	1.23 ± 0.08	1.22 ± 0.08
Month 12	1.20 ± 0.09	1.22 ± 0.06	1.19 ± 0.06	1.22 ± 0.06	1.26 ± 0.08	1.25 ± 0.08	1.22 ± 0.07	1.20 ± 0.09*

Values are mean ± SD.

* $p < 0.05$.

** $p < 0.01$.

[a] Data that is not shown was not statistically different between groups.

Clinical chemistry analyses revealed no clear test article related alterations (Table 1). Slight increases in gamma glutamate (GGT) activity in male animals at week 3 in the 2000 mg/kg bw/day group and at the 3rd and 6th months in the 1200 mg/kg and 2000 mg/kg bw/day groups disappeared at the end of the treatment period and remained within normal ranges throughout the study. Elevated phosphorus concentrations in all female treatment groups and in the 1200 mg/kg and 2000 mg/kg bw/day male groups at the end of the study were within historical control ranges. No histopathological changes were seen to substantiate their relevance. There were other sporadic statistical differences seen; however, none were considered to be of biological significance since they were within normal historical ranges for this strain of rat and no dose-response relationship was noted.

Urinalysis results were unremarkable for any of the treatment groups when compared to control values, when assessed pre-treatment, and at the end of the 3rd, 6th and 12th months. All values remained within the normal range throughout the study.

Necropsy results at the end of the study did not reveal any macroscopic changes that could be attributed to administration of GanedenBC^{30}™. In animals euthanized during the course of the study period, moribund conditions were caused by individual alterations. Pale liver and decreased testicular size occurred with similar incidence in control and treated males. These findings are also common in untreated rats and thus had no toxicological relevance. Findings in female genital organs included ovarian cysts, yellowish to dark red formations, thickened uterine wall and horns, congestion and bleeding, and were observed in a very small number of animals in the control and treated groups; however, these are species-specific changes and also occur in untreated animals of this species of rat with similar age.

Organ weights assessed at the end of the treatment period were similar to the control values in all treatment groups. A slight statistically significant difference compared to control values was seen in mean liver weight of male rats in the 600 mg/kg bw/day group; however, this result was without any biological significance because there was no dose response and no correlating histopathology.

Histological examination did not reveal any test article-related or toxic lesions in the investigated organs. Several findings with similar incidence and severity occurred in the control and treated animals (Table 2), but were considered incidental, and were in accordance with historical control data. These included alveolar histocytosis, mineral deposits, and pituitary adenomas. Furthermore, no morphological evidence of acute or subacute injury to the alimentary tract was observed, while examinations of the cardiovascular system, immune system, hematopoietic system, skeleton, muscular system and the central and peripheral nervous system were negative.

3.2. One-generation reproductive toxicity study in rats

This study was undertaken in order to obtain general information regarding the effects of GanedenBC^{30}™ administration on

Table 2
Summary of histopathology findings from the GanedenBC^{30}™ one-year repeated dose oral toxicity study in HsdBrlHan:Wistar rats. The table contains only the list of organs with findings; however all organs and tissues stated in the study protocol were examined. Data is presented as the number of animals with findings/number of animals examined.

	Group number (mg/kg bw/day: targeted–actual) Males				Group number (mg/kg bw/day: targeted–actual) Females			
	1(0–0) $n = 20$	2 (600–585) $n = 2$	3 (1200–1147) $n = 1$	4 (2000–1951) $n = 20$	1 (0–0) $n = 20$	2 (600–761) $n = 5$	3 (1200–1467) $n = 5$	4 (2000–2525) $n = 20$
Lungs								
Emphysema	3/20	–	–	4/20	3/20	–	–	1/20
Alveolar Histiocytosis	6/20	–	–	3/20	4/20	–	–	1/20
Granuloma	0/20	–	–	0/20	1/20	–	–	0/20
Thymus								
Cyst	0/20	–	–	0/20	1/20	–	–	0/20
Skin								
Exudative dermatitis	0/20	–	–	0/20	1/20[b]	–	–	1/20
Kidney								
Lympho-histiocytic infiltration	4/20	–	–	1/20	0/20	–	0/1	0/1
Pyelitis	1/20	–	–	1/20	1/20	–	0/1	0/1
Fibrosis	2/20	–	–	1/20	0/20	–	0/1	0/1
Mineral deposits	0/20	–	–	0/20	1/20	–	0/1	5/20
Cyst	0/20	–	–	0/20	0/20	–	1/1	0/1
Liver								
Normal	20/20	1/1[a]	1/1[a]	20/20	20/20	–	–	20/20
Sarcoma (metastasis)	0/20	–	–	0/20	0/20	–	–	1/20**
Pituitary								
Adenoma	0/19	1/1	–	0/17	3/20	1/1	2/2	5/17
Spleen								
Sarcoma (metastasis)	0/20	–	–	0/20	0/20	–	–	1/20**
Testis								
Decreased intensity of spermatogenesis	1/20	–	–	1/20	–	–	–	–
Ovary								
Cyst	–	–	–	–	2/20	–	–	0/20
Lack of corpora lutea	–	–	–	–	1/20	–	–	0/20
Uterus								
Sarcoma	–	–	–	–	0/20	–	–	1/1**
Autolysis of organs	–	–	–	–	0/20	–	1/1[c]	0/20
Dilation	–	–	–	–	3/20	2/5	0/3	2/20
Congestion	–	–	–	–	0/20	0/5	0/3	1/20
Hematoma	–	–	–	–	0/20	1/5	0/3	0/20
Endometritis	–	–	–	–	0/20	0/5	1/3	0/20
Estrus phase	–	–	–	–	1/20	4/5	2/3	0/20
Myoma	–	–	–	–	1/20	1/5	0/3	0/20

– = No data.
[a] No abnormal findings correlating with necropsy observation.
[b] Moribund animal (1 female in control group, 1 female in 2000 mg/kg bw/day group).
[c] Dead animal (1 female in 1200 mg/kg bw/day group).

male and female reproductive performance in HsdBrlHan rats of Wistar origin.

Analysis of the dietary intake of the rats and mean dose levels of GanedenBC^{30}™ in the various dose groups revealed the following: 600 mg/kg bw/day target dose group (males received a calculated mean daily intake of 715 mg/kg bw/day, females received 1079 mg/kg bw/day); 1200 mg/kg bw/day target dose group (males: 1348 mg/kg bw/day, females: 2082 mg/kg bw/day); 2000 mg/kg bw/day target dose group (males: 2372 mg/kg bw/day, females: 3558 mg/kg bw/day). A further breakdown of the intake of GanednBC^{30}™ in females of the parental generation revealed the following mean intakes in the different treatment groups during the pre-mating, gestation and lactation periods: 600 mg/kg bw/day target group (908 mg/kg bw/day during pre-mating period, 775 mg/kg bw/day during gestation, 1951 mg/kg bw/day during lactation); 1200 mg/kg bw/day target group (1769 mg/kg bw/day, 1593 mg/kg bw/day, and 3700 mg/kg bw/day); 2000 mg/kg bw/day target group (2983 mg/kg bw/day, 2596 mg/kg bw/day, and 6438 mg/kg bw/day).

Of the forty male and eighty female animals of the parental generation that composed the control and treatment groups, no mortalities occurred during the entire observation period. Of the females, 17/20 became pregnant in the control group, whereas 15/20, 18/20 and 19/20 became pregnant in the 600 mg/kg, 1200 mg/kg and 2000 mg/kg bw/day treatment groups, respectively (Table 3). Of the pregnant animals, 17/17 delivered live born pups in the control group, whereas 14/15, 18/18 and 19/19 animals in the 600 mg/kg, 1200 mg/kg, and 2000 mg/kg bw/day groups, respectively, delivered live-born offspring.

Of the non-pregnant females in all groups (Table 3), follow-up revealed that all but one female from the control group was sperm-positive by vaginal smear. As included in results presented below, necropsy showed hydrometra in 1/3 non-pregnant females from the control group and 2/5 in the 600 mg/kg bw/day group. No other alterations were found by necropsy. Histopathology revealed uterine dilation in 2/5 non-pregnant females in the 600 mg/kg bw/day group, 1/2 in the 1200 mg/kg bw/day group and 1/1 in the 2000 mg/kg bw/day group. No other alterations were found by histopathology examination.

No clinical symptoms were noted for any groups of male animals and the physical condition and behavior was considered to be normal throughout the study. Two female animals were found

Table 3
Summary of selected P(parents) data, delivery data and clinical observation of pups from the one-generation reproduction toxicity study on GanedenBC^{30}™.

	Group number (mg/kg bw/day targeted) Males				Group number (mg/kg bw/day targeted) Females			
	1 (0)	2 (600)	3 (1200)	4 (2000)	1 (0)	2 (600)	3 (1200)	4 (2000)
# of treated animals	10	10	10	10	20	20	20	20
# of animals that died	0	0	0	0	0	0	0	0
# of paired animals	10	10	10	10	20	20	20	20
# of mated animals	10	10	10	10	–	–	–	–
# of fertile animals	10	10	10	10	–	–	–	–
# of sperm positive animals	–	–	–	–	19	20	20	20
# of pregnant females	–	–	–	–	17	15	18	19
# of females with live pups	–	–	–	–	17	14	18	19
# of females not delivered	–	–	–	–	0	1	0	0
Duration of pregnancy (days)±SD	–	–	–	–	22.21 ± 0.54	22.16 ± 0.27	22.25 ± 0.36	22.26 ± 0.48
# of implantations	–	–	–	–	194	184	219	222
# of total births	–	–	–	–	179	164	184	206
No milk in stomach	–	–	–	–	4	4	1	3
Cold	–	–	–	–	1	4	3	1
Pale	–	–	–	–	0	0	1	0
Cyanotic	–	–	–	–	0	2	0	0
# of viable pups	–	–	–	–	177	160	182	206
On postnatal day 7	–	–	–	–	174	156	179	200
On postnatal day 14	–	–	–	–	174	149*	175	200
On postnatal day 21	–	–	–	–	174	148**	174	200
# of dead pups days 0–21	–	–	–	–	3	12**	8	6
On postnatal day 0–7	–	–	–	–	3	4	3	6
On postnatal day 7–14	–	–	–	–	0	7**	4*	0
On postnatal day 14–21	–	–	–	–	0	1	1	0
# of stillborns	–	–	–	–	2	4	2	0
# of extrauterine mortality	–	–	–	–	0	0	0	2
# of intrauterine mortality	–	–	–	–	17	24	37*	16
Live birth index (%)	–	–	–	–	99	98	99	100

* $p < 0.05$.
** $p < 0.01$.

to have scarring. One animal was a part of the control group, while the second was a part of the 1200 mg/kg bw/day treatment group. All other female animals remained in normal physical condition, and had normal behavior, throughout the study period.

Body weights remained similar to control animals for males and females in all treatment groups throughout the study period. Furthermore, no differences in body weight or weight gain were noted between dams of the control and treatment groups.

No differences were seen in food consumption in males of all three treatment groups during the pre-mating and mating periods, or in females of all treatment groups during the pre-mating, gestation and lactation periods, when compared to controls.

No effects of the treatment were noted on the estrous cycle of any females; however, the estrous cycle was irregular in most animals of all groups for unknown reasons. Due to the low number of females with a regular cycle across all groups, correct evaluation of the cycle was not feasible. During the course of mating, the cycles became normal and no disturbances were noted in the mating process.

The mean duration of pregnancy was similar in all groups and no differences were seen in the percentage of dams that delivered, the percentage with viable fetuses and in live birth index. No effect of treatment was found in the number of implantations, number of total births, number of live-born, or stillborn pups. No stillborn pups were seen in the 2000 mg/kg bw/day treatment group.

No treatment-related effects were seen on reproductive performance of male or female animals, as the number and percentage of mated and fertile male animals, as well as the copulatory and fertility indices, were not affected. The number and percentage of non-pregnant females was highest in the 600 mg/kg bw/day treatment group and the percentage of pregnant animals was less than the control group. The percentage of pregnancies with live-born offspring in this group were less than the control values, while the number of pregnancies not delivered exceeded control values; however, the pre-coital interval was similar in all groups.

Gross pathological examination did not reveal any treatment-related alterations. Pulmonary hemorrhages (1/10 animals in the 600 mg/kg, 2/10 in the 1200 mg/kg, and 1/10 in the 2000 mg/kg groups) and alveolar dilation (3/10, 5/10, 3/10 and 3/10 animals in the control and three treatment groups, respectively) were noted in some male animals across all groups, but were attributed to the exsanguination process and had no toxicological relevance. Hydrometra was noted in three females in the 600 mg/kg bw/day group and one female animal in the control group, but is a frequent observation in experimental rats and has no toxicological significance.

Measurement of organ weights did not demonstrate any treatment-related effects. In males, there were slight differences from controls in the weights of the brain (600 mg/kg group) and testes (600 mg/kg and 1200 mg/kg groups) (Table 4). The weights of the adrenal glands were slightly below control values, while the weights of the kidneys were slightly higher (1200 mg/kg and 2000 mg/kg groups). In females, the weights of the adrenal glands (2000 mg/kg group) and ovaries (600 mg/kg group) slightly exceeded control values. However, all values were within historical control ranges. The differences in testes weight were not dose-related, as there were no such findings in the highest dose group.

Histopathological examination showed that the investigated reproductive organs in male animals of all groups were normal. In females, the ovaries had normal structure, characteristic for the species, age and phase of the active sexual cycle. The uterus, cervix and vagina all had normal structural characteristics. Some animals in the control and treatment groups showed dilatation of the uterine tubes, which is a normal occurrence in this strain of rat. In the

Table 4
Summary of organ weight data from one-generation reproduction toxicity study on GanedenBC^{30}™.

	Group number (mg/kg bw/day targeted) Males				Group number (mg/kg bw/day targeted) Females			
	1 (0)	2(600)	3 (1200)	4 (2000)	1 (0)	2 (600)	3 (1200)	4 (2000)
Mean organ weights (g ± SD)								
Body weight	406.6 ± 44.17	412.2 ± 46.87	380.1 ± 39.03	410.8 ± 37.68	270.2 ± 22.08	273.5 ± 31.36	276.7 ± 23.68	269.7 ± 21.41
Brain	2.29 ± 0.14	2.16 ± 0.10*	2.18 ± 0.12	2.26 ± 0.13	1.84 ± 0.09	1.84 ± 0.11	1.83 ± 0.13	1.87 ± 0.10
Thymus	0.36 ± 0.04	0.36 ± 0.11	0.33 ± 0.09	0.34 ± 0.06	0.23 ± 0.25	0.16 ± 0.08	0.15 ± 0.08	0.15 ± 0.07
Spleen	0.83 ± 0.15	0.84 ± 0.09	0.79 ± 0.08	0.82 ± 0.08	0.66 ± 0.10	0.65 ± 0.11	0.63 ± 0.12	0.62 ± 0.12
Liver	12.78 ± 1.23	12.82 ± 1.49	11.75 ± 1.15	13.12 ± 1.54	12.15 ± 1.43	12.21 ± 2.53	12.10 ± 2.03	12.00 ± 1.63
Kidneys	2.52 ± 0.27	2.49 ± 0.27	2.43 ± 0.23	2.89 ± 0.50*	1.87 ± 0.16	1.89 ± 0.31	1.97 ± 0.27	2.01 ± 0.29
Adrenals	0.083 ± 0.009	0.083 ± 0.009	0.072 ± 0.011*	0.073 ± 0.009*	0.090 ± 0.018	0.085 ± 0.013	0.096 ± 0.017	0.103 ± 0.019*
Testes	3.54 ± 0.19	3.30 ± 0.35*	3.25 ± 0.14*	3.52 ± 0.27	–	–	–	–
Epididymides	1.60 ± 0.16	1.52 ± 0.19	1.48 ± 0.09	1.65 ± 0.19	–	–	–	–
Ovaries	–	–	–	–	0.116 ± 0.020	0.138 ± 0.023*	0.133 ± 0.027	0.122 ± 0.026
Uterus	–	–	–	–	0.42 ± 0.11	0.45 ± 0.14	0.41 ± 0.08	0.45 ± 0.12
Organ weight relative to body weight (% ± SD)								
Brain	0.565 ± 0.031	0.527 ± 0.047	0.576 ± 0.045	0.553 ± 0.057	0.683 ± 0.054	0.679 ± 0.073	0.663 ± 0.039	0.694 ± 0.051
Thymus	0.089 ± 0.003	0.090 ± 0.039	0.086 ± 0.020	0.083 ± 0.016	0.088 ± 0.097	0.060 ± 0.036	0.055 ± 0.029	0.057 ± 0.031
Spleen	0.204 ± 0.032	0.206 ± 0.022	0.209 ± 0.021	0.200 ± 0.021	0.243 ± 0.032	0.238 ± 0.037	0.227 ± 0.043	0.231 ± 0.048
Liver	3.149 ± 0.126	3.113 ± 0.175	3.103 ± 0.260	3.189 ± 0.144	4.501 ± 0.432	4.445 ± 0.709	4.373 ± 0.600	4.461 ± 0.602
Kidneys	0.621 ± 0.036	0.605 ± 0.045	0.643 ± 0.054	0.700 ± 0.074**	0.696 ± 0.058	0.688 ± 0.063	0.710 ± 0.052	0.748 ± 0.102
Adrenals	0.0205 ± 0.0029	0.0204 ± 0.0032	0.0188 ± 0.0019	0.0181 ± 0.0032	0.0334 ± 0.0056	0.0311 ± 0.0040	0.0349 ± 0.0064	0.0381 ± 0.0071*
Testes	0.878 ± 0.091	0.806 ± 0.096	0.860 ± 0.074	0.860 ± 0.048	–	–	–	–
Epididymides	0.396 ± 0.053	0.371 ± 0.040	0.392 ± 0.034	0.403 ± 0.038	–	–	–	–
Ovaries	–	–	–	–	0.0434 ± 0.0082	0.0507 ± 0.0088	0.0483 ± 0.0094	0.0453 ± 0.0094
Uterus	–	–	–	–	0.156 ± 0.044	0.167 ± 0.062	0.150 ± 0.033	0.167 ± 0.046
Organ weight relative to brain weight (% ± SD)								
Body weight	17757.9 ± 1027.92	19105.4 ± 1769.26	17443.6 ± 1332.63	18245.2 ± 1770.97	14728 ± 1116.4	14880 ± 1483.6	15,125 ± 915.9	14,477 ± 1096.1
Thymus	15.79 ± 1.21	16.93 ± 6.22	14.92 ± 3.57	15.03 ± 2.22	12.73 ± 13.82	8.58 ± 4.33	8.17 ± 4.19	8.16 ± 4.14
Spleen	36.24 ± 5.78	39.12 ± 2.99	36.35 ± 3.37	36.45 ± 4.96	35.83 ± 5.23	35.16 ± 5.12	34.28 ± 6.55	33.32 ± 6.06
Liver	558.69 ± 31.40	593.79 ± 52.09	539.31 ± 36.28	583.07 ± 73.74	661.67 ± 71.97	663.30 ± 123.83	662.95 ± 111.16	643.84 ± 83.87
Kidneys	110.09 ± 6.86	115.33 ± 10.41	111.62 ± 6.63	128.53 ± 23.85	102.23 ± 9.42	102.37 ± 13.59	107.40 ± 10.90	107.97 ± 14.44
Adrenals	3.62 ± 0.43	3.85 ± 0.34	3.29 ± 0.40	3.26 ± 0.46	4.93 ± 0.96	4.61 ± 0.59	5.28 ± 0.96	5.51 ± 0.97
Testes	155.10 ± 10.59	152.78 ± 12.58	149.41 ± 9.06	156.55 ± 13.54	–	–	–	–
Epididymides	70.14 ± 7.92	70.39 ± 6.39	68.15 ± 4.08	73.54 ± 10.50	–	–	–	–
Ovaries	–	–	–	–	6.37 ± 1.19	7.50 ± 1.22**	7.30 ± 1.47	6.52 ± 1.34
Uterus	–	–	–	–	22.83 ± 5.86	24.56 ± 7.60	22.57 ± 4.51	23.98 ± 5.94

Values are mean ± SD.
– = No data.
* $p < 0.05$.
** $p < 0.01$.

sexually active animals the cortex contained primary, secondary and tertiary follicles and corpora lutea, indicating the active maturation of oocytes and ovulation.

In terms of the offspring, mortality was low across all treatment groups and control between postnatal days 0 and 21. However, there was a statistically significant slight increase in mortality observed for the 600 mg/kg treatment group compared to the control. The mean number of live pups was similar across all groups. The treatment groups showed no effects on the ratio of genders in the litters.

Clinical observation revealed no treatment-related effects in the offspring. Some common signs (including no milk in the stomach, pale, cold and cyanotic pups) were seen with similar incidence in the litters of all groups.

No treatment-related effects were seen with body weight gain and development, and the number of animals with negative responses on the surface-righting reflex and suckling ability assessments were comparable in all groups.

Furthermore, no treatment-related effects were noted on gross pathological examination in any of the offspring. Two stillborn pups from the control group were subjected to necropsy, which revealed autolyzed organs (1/2 pups), hydrops fetalis (1/2) and opened ductus Botalli (2/2). Four stillborn and one dead pup were subjected to necropsy in the 600 mg/kg treatment group. A patent ductus Botalli was seen in all stillborn pups in this dose group, while abdominal organs were autolyzed in the dead pup. In the 1200 mg/kg treatment group, two stillborn pups were found to have autolyzed organs, patent ductus Botalli and airless lungs, while in the 2000 mg/kg group, two live born pups were autopsied on day 0. No macroscopic findings were seen in one pup, while in the other, no milk was found in the stomach and necrotic lesions were found in the liver. None of these findings were deemed to have toxicological significance or attributable to administration of GanedenBC^{30}™.

4. Conclusion

The study was conducted as a continuation of the previously published safety assessment of GanedenBC^{30}™ (Endres et al., 2009), a commercially available probiotic strain of *B. coagulans*.

A one-year chronic oral toxicity study combined with a one-generation reproduction study was conducted to further investigate safety with long-term chronic consumption. The study reported herein adds to the body of evidence in support of chronic consumption of GanedenBC^{30}™ by humans. The conclusion from the one-year study is that GanedenBC^{30}™ caused no signs of toxicity in male or female HsdBrlHan: Wistar rats after one year of diet-mixed administration. The NOEL was 1948 mg/kg bw/day for the males and 2525 mg/kg bw/day for the females – the highest dose tested. The conclusion from the reproduction toxicity study is that GanedenBC^{30}™ caused no signs of toxicity on the parental generation (male or female) of the same strain of rat during the course of

the study with diet-mixed administration. The NOEL for the male rats is 2372 mg/kg bw/day and 3558 mg/kg bw/day for the females of the parental group. The NOEL for the reproductive performance for the males is 2372 mg/kg bw/day and 3558 mg/kg bw/day for females. The NOEL for the F1 offspring is 3558 mg/kg bw/day. In conclusion, to calculate the acceptable daily intake (ADI), the lowest NOEL from the study of 1948 mg/kg was used and a 100-fold safety factor was applied. Since the test article concentration was 6.88×10^{10} CFUs/g, using 70 kg as the mass for an average human, it is determined that GanedenBC^{30}™ is safe for chronic consumption at an accumulative daily intake of 9.38×10^{10} CFUs per day.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Safety assessment of a proprietary preparation of a novel Probiotic, *Bacillus coagulans*, as a food ingredient

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ABSTRACT

It has been demonstrated that some strains of *Bacillus coagulans* can survive extremes of heat, acidity of the stomach, and bile acids, to which commonly consumed probiotics are susceptible. A toxicological safety assessment was performed on a proprietary preparation of *B. coagulans* – GanedenBC^{30}™ – a novel probiotic. Seven toxicologic studies were conducted and included: *in vitro* bacterial reverse mutation assay; *in vitro* chromosomal aberration assay; micronucleus assay in mice; acute and 90 day subchronic repeated oral toxicity studies were conducted in Wistar Crl:(WI) BR rats; acute eye and skin irritation studies were conducted in rabbits.

The results of this toxicological safety assessment indicate that GanedenBC^{30}™ *B. coagulans* does not demonstrate mutagenic, clastogenic, or genotoxic effects. Furthermore, the results of the acute and 90-day subchronic oral toxicity studies in rats resulted in the conclusion of a NOAEL greater than 1000 mg/kg per day. Since the concentration of the cell mass used in the 90-day study was 1.36×10^{11} CFUs/g, this corresponds to 95.2×10^{11} CFUs for a 70 kg human and since the suggested human dose is in the range of 100×10^{6} to 3×10^{9} CFUs, this gives a safety factor ranging from 3173 to 95,200 times. Based upon scientific procedures and supported by history of use, GanedenBC^{30}™ is considered safe for chronic human consumption.

1. Introduction

Probiotics are defined as viable organisms (generally bacteria or yeast) that have demonstrated beneficial effects on the health of a host (Lee, 1999). While the concept of utilizing probiotics for human health has been around for over 100 years (De Vecchi and Drago, 2006), recently a great deal of interest has been focused on the importance of probiotic bacteria in treating or preventing specific disorders such as irritable bowel syndrome, eczema, allergies, *Helicobacter pylori* infection and sequelae, as well as for support of intestinal and immunological health (Tappenden and Deutsch, 2007; Quigley, 2007; Rastall et al., 2005; Hyronimus et al., 2000; Spiller, 2008; McFarland and Dublin, 2008; Lesbros-Pantoflickova et al., 2007; Ouwehand, 2007).

Probiotic bacteria such as the *Lactobacillus* species are very sensitive to normal physiological conditions such as the very low pH of the stomach and bile salts when consumed. In addition, the viability of these bacteria is affected by manufacturing methods as well as storage and shipping conditions (Bezkorovainy, 2001; Ljungh and Wadstrom, 2006; Graff et al., 2008). On the contrary, some strains of *Bacillus coagulans* are able to survive the extremes of heat, acidity of the stomach and bile acids – although in general, the strains of this species are quite heterogeneous (Patel et al., 2006; De Clerck et al., 2004; De Vecchi and Drago, 2006; Hyronimus et al., 2000; Katsutoshi et al., 2003). Strains with these qualities have an increased chance of survival through the gastrointestinal tract, thus allowing for transient population of the small

Abbreviations: A/G, albumin to globulin ratio; Alb, albumin; ALKP, alkaline phosphatase; ALT, alanine transaminase; ANOVA, analysis of variance; APTT, activated partial thromboplastin time; AST, aspartate transaminase; ATCC, American Type Tissue Collection; BC30 or GBI-30-SF, GanedenBC^{30}™; Bw, body weight; CFU, colony forming unit; Creat, creatinine; DMSO, dimethyl sulfoxide; EEC, European Economic Community; EC, European Community; ELISA, enzyme-linked immunosorbent assay; EU, European Union; FBS, fetal bovine serum; GGT, gamma-glutamyltransferase; GRAS, generally recognized as safe; Hct, hematocrit; Hgb, hemoglobin; MC, methyl cellulose; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; NOAEL, no observed adverse effect level; OECD, Organization for Economic Cooperation and Development; Plt, platelet; PT, prothrombin time; RBC, red blood cell; RDW, red blood cell distribution width; Retic, reticulocyte; S.D., standard deviation; T Bili, total bilirubin; T chol, total cholesterol; T Prot, total protein; UV, ultraviolet; v/v, volume per volume; WBC, white blood cell.

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and large intestines by *B. coagulans* (Adami and Cavazzoni, 1999). *B. coagulans*, when taken orally, has also shown beneficial effects on the intestinal environment, stool frequency and characteristics, and dermal attributes in animals and humans (Adami and Cavazzoni, 1999; Donskey et al., 2001; Katsutoshi et al., 2003).

The majority of probiotics studied and sold in the market today are classified as lactic acid producing organisms (De Vecchi and Drago, 2006; Lee, 1999), including many *Lactobacillus* species. Until 1974 *B. coagulans* was classified as *Lactobacillus sporogenes*. Bergey's Manual (Buchanan and Gibbons, 1974; Bergey's Manual, 1974) reclassified this bacterium as *B. coagulans* because, although it shares taxonomic characteristics with the other *Lactobacillus* species such as producing lactic acid, none of the latter are spore-forming (Sanders et al., 2001; Gandhi, 1994). As 16S RNA ribotyping, in addition to fatty acid analysis, became available and routine for identification and classification of various bacteria, it has become clear that *B. coagulans* is the correct and well-accepted taxonomic classification.

The reported history of *B. coagulans* dates back to 1915, when an outbreak of coagulation in evaporated milk was described in the Iowa Agricultural Experiment Station (Sarles and Hammer, 1932). In 1932, *L. sporogenes* was first isolated and described by Horowitz-Wlassowa and Nowotelnow (Gandhi, 1994). The observers noted that the milk was curdled, slightly bitter, and with flavor of a cheesy nature that was not at all unpleasant.

More recently, in 1978 a patent was issued for a method (USPTO No. 4,110,477) for improving the flavor and shelflife of natto (a traditional Japanese food made from fermented soybeans) by the addition of *B. coagulans* along with its close relative *Bacillus natto (Bacillus subtilis)*, which conventionally has been used to manufacture this commonly consumed food.

While many strains of *B. coagulans* have been widely consumed around the world for decades at the least, to the best of the authors' knowledge no comprehensive toxicologic assessment for *B. coagulans* has been published in the public domain. Unlike other non-pathogenic species of bacteria, *B. coagulans* is not generally considered a natural part of the gastrointestinal tract flora. Because it lacks the ability to adhere to the intestinal epithelium, it is completely eliminated in four to five days unless chronic administration is maintained (Donskey et al., 2001). Therefore, we have conducted a thorough assessment to evaluate the safety of chronic oral consumption of GanedenBC^{30}™. The purpose of this constellation of research is to present the safety assessment of a proprietary strain of *B. coagulans* known as GanedenBC^{30}™ and includes the following *in vitro* and *in vivo* toxicology studies: Ames mutagenicity, chromosomal aberration, mouse micronucleus test, acute eye irritation and acute skin irritation in the rabbit, acute oral toxicity in the rat, as well as a 90-day subchronic oral toxicity in the rat.

2. Materials and methods

2.1. Test product

The test product, GanedenBC^{30}™, supplied by Ganeden Biotech, Inc. (Mayfield Heights, OH, USA) is sold as a dietary ingredient for use in functional foods and dietary supplements. The organism is a gram-positive spore-forming rod that is aerobic to microaerophilic in nature. Its size is 0.9 μm × 3.0 μm × 5.0 μm. GanedenBC^{30}™ is manufactured as a pure cell mass consisting solely of *B. coagulans*. The pure cell mass is spray-dried with maltodextrin to achieve the desired concentration of 15×10^9 CFU/g for the finished product. For the purpose of these toxicologic studies, pure, uncut GanedenBC^{30}™ was used. The concentration varies slightly from batch to batch and therefore is reported for each study.

2.2. Bacterial reverse mutation (Ames) study

Five strains of bacteria, including four strains of *S. typhimurium* (TA98, TA100, TA1535, TA1537) and one strain of *E. coli* (WP2 [uvrA]) were obtained from Xenometrix GmbH (Allschwil, Switzerland). This study is well known and has been previously described (Ames et al., 1975; Maron and Ames, 1983). The test article, obtained at a concentration of 4.5×10^{10} CFU/g, was mixed with sterile water not more than 30 min prior to the start of the experiment. The study was performed both with and without an S9 activation system. The Aroclor™ 1254-induced rat liver S9 was purchased from Trinova Biochem GmbH (Giessen, Germany). A cytotoxicity assessment was performed on test doses of 5–5000 μg/plate to determine the appropriate dose range for the assay. Doses used in the study were: 10, 50, 100, 500, and 5000 μg/plate. Experiments were performed in triplicate.

Positive controls in the experiment without S9 activation included 2-nitrofluorene (CAS#607-57-8) for strain TA98, sodium-azide (CAS#26628-22-8) for strains TA100 and TA1535, 9-aminoacridine (CAS#52417-22-8) for strain TA1537 and methyl-methansulfonate (CAS#66-27-3) for strain WP2. Positive controls in the experiment with S9 activation included benzo(a)pyrene (CAS#50-32-8) for strains TA98 and TA1537, and 2-aminoanthracene (CAS#613-13-8) for strains TA100, TA1535 and WP2. All strains were tested for spontaneous revertant colonies using distilled water as a negative control.

Frozen stock cultures were grown overnight at 37 ± 2 °C to a density of 10^9 cells/ml. Sterile culture tubes were filled with 0.1 ml of test article culture or control, 0.5 ml of S9/cofactor mix or 0.5 ml phosphate buffer (pH 7.4), and 2.0 ml top agar. The mixture was spread onto minimal glucose plates and incubated at 37 °C ± 2 °C for 72 ± 4 h. The number of revertant colonies per plate was determined by hand counting. Criteria for a valid assay included sensitivity of TA98, TA100, TA1535, TA1537 and WP2 to UV light, sensitivity of *S. typhimurium* strains to crystal violet, resistance of strains TA98 and TA100 to ampicillin, reversion rates within historical ranges, and a threefold increase in revertant colonies with exposure to positive controls. A response was considered positive if there was over three times the background average number of revertant colonies on a plate (two times for strain TA100), or if there was a dose-related increase in colonies. If these criteria were not met, the test article was considered non-mutagenic.

Statistical regression analysis was not necessary based on the results. Because the definitive assay yielded negative results, an independent repeat assay was performed, with an increase in S9 in the S9/cofactor mix from 4% to 10%. The experiments were conducted according to Organization for Economic Cooperation and Development (OECD) Guidelines for the Testing of Chemicals: Bacterial Reverse Mutation Test (Guideline 471, as adopted July 21, 1997).

2.3. Micronucleus assay in mice

Male mice of strain BALB/dByJNarl, aged 7–8 weeks were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Body weights at the start of the study were 21.9–25.9 grams. Animals assigned randomly to five groups of five, and were housed with five animals per cage. Conditions included 50 ± 20% relative humidity, 21 ± 2 °C temperature, and a 12-h light–dark cycle. Animals had free access to Laboratory Autoclavable Rodent Diet 5010 (PMI® Nutrition International, Inc., MO, USA). Drinking water was available *ad libitum*.

Animals were weighed and observed for signs of illness or other abnormalities at the start of the study. Randomization was generated with Microsoft Excel, 2003 SP2. Water for injection was utilized as the negative control, and was used as the vehicle to prepare the test article to desired concentration on the same day as administration. The test article was obtained at a concentration of 1.93×10^{11} CFUs/g. Mitomycin C (Taiwan Biotech Co. Ltd., Taiwan) served as the positive control. Doses were administered by oral route, except for the positive and negative controls which were administered only once by intraperitoneal injection on the last test article administration day.

Doses of test article included 500, 1000 and 2000 mg/kg bw/day given for three days. Body weights were measured on days 1–5. Blood samples were obtained by tail trimming approximately 48 h after the last administration. Samples were smeared on acridine orange-coated slides, and 2000 reticulocytes were scored under a fluorescence microscope for the presence of micronuclei and the slides were blind coded. The proportion of reticulocytes to total erythrocytes was an indicator to evaluate bone marrow toxicity. Percentages were determined by flow cytometry and were based on analysis of 50,000 erythrocytes.

Criteria for a valid test included negative control data comparable to historical control data, and positive control data of significantly increased levels of micronucleated reticulocytes compared to the negative control ($p \leqslant 0.05$, *t*-test). Data was analyzed using one-way analysis of variance (ANOVA) (SigmaStat, V3.11, 2004). A *p* value of $\leqslant 0.05$ was used as criterion for statistical significance. If statistically significant, the data were analyzed for a dose–response relationship. A repetition of the experiment was performed if positive findings were not dose dependant.

2.4. In vitro chromosomal aberration assay

Chinese hamster ovary cells (CHO-K1) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) as repository number CCL-61. The cell line was tested by ELISA (Böhringer, Mannheim) and found to be free of mycoplasma contamination. Cells used in the assay were within five passages from the frozen stock to assure karyotypic stability.

Cells were maintained as monolayers in McCoy's 5A medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS), 0.22% sodium bicarbonate, 2 mM L-glutamine, and 1% penicillin–streptomycin solution, at 7.0–7.2 pH in a humidified incubator at 37 °C and 5% CO_2 in air. Post-mitochondrial fraction (S9)

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of the liver homogenate from Sprague-Dawley rats induced with Aroclor 1254 (Moltox, Inc. USA) was used for metabolic activation. The final concentration of S9 was 1% (v/v).

A dose range finding test was done according to OECD guidelines, which suggests the greatest concentration of the test article tested should produce greater than 50% cytotoxicity, if possible, with a maximum concentration of 5000 μg/ml for relatively non-cytotoxic substances. The test article was obtained at a concentration of 1.93×10^{11} CFUs/g and was prepared at 5 mg/ml in medium and was further diluted with medium to the final concentration desired. Five concentrations were utilized in the chromosomal aberration assay, including 312.5, 625, 1250, 2500 and 5000 μg/ml. One micromolar mitomycin C (Roche, USA) served as the positive control in the schemes without S9 activation, while 40 μM cyclophosphamide (Sigma, USA) served as the positive control in schemes with S9 activation. Both positive controls were prepared in DMSO, with a final concentration of 0.5% in culture medium. Culture medium alone served as the negative control.

Three treatment schemes were utilized, including incubation of the test article with cells for 3 h both with and without an S9 metabolic activation system (Schemes I and II, respectively), and incubation for 20 h without S9 (Scheme III). Due to only two analyzable concentrations in treatment scheme III (312.5 and 625 μg/ml), this scheme was repeated with 78.125, 156.25, 312.5 and 1250 μg/ml concentrations. Colcemid was added to all cell culture medium to a final concentration of 0.1 μg/ml for the final 2 h of incubation prior to harvesting cells.

Cells were evaluated for cytotoxicity and chromosomal integrity by cell counting on fixed slides that were blind coded. Two cultures were scored for each dose, and 100 metaphases were observed for each culture. Chromosomal and chromatid aberrations were scored, and percents of structurally aberrant cells were calculated, excluding cells with chromosome or chromatid gaps.

If more than 3% aberrant cells were observed, the percent was analyzed by a one-tail binomial test and compared pair-wisely to the negative control. If a concentration gave a significant result, a trend test was performed to determine the existence and extent of dose–responsiveness.

2.5. Acute oral toxicity study in rats

Wistar Crl:(WI) BR rats were obtained from Toxi-Coop Kkt. (Budapest, Hungary). Rats were housed five per cage at a temperature of 22 ± 3 °C, a relative humidity of 30–70%, and a 12 h light–dark cycle.

Animals received Ssniff® SM R/M-Z+H complete diet for rats, produced by Ssniff Spezialdiäten GmbH (Soest, Germany). Tap water was available *ad libitum*. Randomization and statistical analysis was performed with SPSS PC+ software (SPSS Inc., Chicago, IL, USA). Body weights at randomization were 190–200 g for males, and 171–180 g for females. Each group consisted of 5 male and 5 female rats.

Detailed clinical observations were made prior to exposure of the animals to the test article. The test article was obtained at a concentration of 1.04×10^{11} CFUs/g, and was diluted in 1% methylcellulose (Dow Chemical TEVA Gyógyszergyár ZRt., Hungary) 30 min prior to administration. A single oral dose of 5000 mg/kg/bw of test article was administered to the treatment group and MC solution of 1% was administered to the control group by oral gavage. Following administration, the animals were observed for clinical signs for four continuous hours, and then twice daily (once daily on weekends) for 14 days.

Observations were focused on skin, fur, eyes, mucous membranes, autonomic activity, circulatory and central nervous system, somatomotor activity, behavior pattern, tremors, convulsions, salivation, stool consistency, lethargy, sleep, changes in gait, posture and response to handling. Animals were weighed on the day of treatment, and on days 2, 8, and 15. A gross pathological organ examination (and histopathological examination if necessary) was performed after the animals were sacrificed with diethyl ether (Reanal, Budapest, Hungary) on the 15th day.

The study was performed according to the United States FDA Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food Redbook II Draft Guidance, Acute Oral Toxicity Tests (1993), as well as the OECD Guideline for the Testing of Chemicals No. 423; Acute Oral Toxicity – Acute Toxic Class Method, adopted December 17, 2001.

2.6. Subchronic 13-week oral toxicity study in rats

Wistar Crl:(WI) BR rats were obtained from Toxi-Coop Kkt. (Budapest, Hungary). The animals were housed five per cage, at a temperature of 22 ± 3 °C, a relative humidity of 30–70%, and a 12-h light–dark cycle.

Animals received Ssniff® SM R/M-Z+H complete diet for rats (Ssniff Spezialdiäten GmbH, Soest, Germany). Tap water was available *ad libitum*. The drinking water was analyzed once after the study and was determined to be free of contaminants. An equal number of animals from each weight and sex group were randomized to the two experimental groups. Body weights of the animals at randomization were 192–218 g for males, and 159–181 g for females. Each group consisted of 20 rats as 10 males and 10 females.

The test article was obtained at a concentration of 1.36×10^{11} CFUs/g, and weighed daily and suspended in 1% methylcellulose (Dow Chemical, TEVA Gyógyszergyár ZRt., Hungary) in distilled water. Concentration and homogeneity of five parallel samples of the test suspensions and controls were checked by gravimetry during weeks 1, 4, 8 and the last week. Doses of 0, 100, 300 and 1000 mg/kg bw/day in a volume of 10 ml/kg was given daily by oral gavage for 90 (males) or 91 (females) days. Weekly adjustments were made for body weight changes.

Clinical observations were made once daily following treatment at approximately the same time each day. Detailed clinical observations were made on all animals outside the cage using a standard method both before the first exposure, and once a week thereafter. The focus of the observations was as described in the acute oral toxicity study above. All animals were weighed on the day of randomization, on day one, once weekly thereafter, and on the day of autopsy. Food was weighed weekly, and the average food consumption per animal was calculated. Water consumption was measured over a 24-h period weekly, and average water consumption per animal was calculated.

On the 13th week of administration, sensory reactivity to different types of stimuli (auditory, visual and proprioceptive) was measured, as well as general physical condition and behavior of animals. A modified Irwin test was performed (Irwin, 1968). Hematology and clinical chemistry results were evaluated at the termination of the study. Animals were fasted for 16 h prior to blood collection. Animals were sacrificed with diethyl ether (Reanal, Budapest, Telepes Street 53, Hungary), and blood samples were collected by heart puncture.

Gross pathological examination was performed on all organs after sacrificing the animals, including organ weight and appearance. A histopathological examination was performed on the control group and the highest dose group, and on the middle dose groups when appropriate.

Statistical analysis was performed for body weights, food consumption, hematology, clinical chemistry and organ weights. Randomization and statistical analysis were performed with SPSS PC+ software (SPSS Inc., Chicago, IL USA). Barlett's homogeneity of variance between groups was determined, and where there was no significant heterogeneity detected, a one-way ANOVA was performed. If statistically significant results were obtained, Dunnett's test was used to assess the inter-group differences. If positive results were detected, inter-group comparisons were performed using Mann-Whitney *U*-test.

The study was performed according to the United States FDA Toxicological Principles for the Safety Assessment of Food Ingredients Redbook 2000, IV.C.3a; Short-term Toxicity Studies with Rodents (2003), as well as the OECD Guideline for the Testing of Chemicals No. 408; Repeated Dose 90-Day Oral Toxicity Study in Rodents, adopted September 21, 1998.

2.7. Acute eye irritation study in rabbits

Male New Zealand White rabbits (age 11 weeks) were obtained from Tetrabbit Kft. (2173 Kartal, Császár út 135, Hungary). Both eyes of animals were examined 24 h prior to the start of the study. Animals showing eye irritation, ocular defects or pre-existing corneal injury were not used in the experiment. The animals weighed 2521–3000 g at the beginning of the study, and 2780–3143 g at the end of the study. Animals were housed individually in metal cages at 20 ± 3 °C, relative humidity of 30–70% and a 12 h light–dark cycle. Animals were fed PURINA Base-Lap gr. diet for rabbits *ad libitum* from Agribands Europe, (H-5300 Karcag, Madarasi road, Hungary). Tap water was routinely analyzed for contaminants and was available *ad libitum*.

Test article was used at a concentration of 1.93×10^{11} CFU/g. The dosage for the study was 0.1 g of the undiluted cell mass. Three healthy male animals were selected, and the dose was placed into the conjunctival sac of the left eye of a single rabbit. After determination that the pain reaction was very low and that anesthesia was not required with application of the test article, the dose was added to the eyes of the remaining two animals, with the untreated right eyes serving as the control. Eyes were not washed after the application.

Eyes were examined at 1, 24, 48 and 72 h after the treatment for severity, nature and duration of reactions. The duration of the observation period was sufficient to determine the reversibility or irreversibility of any changes. Any clinical signs of toxicity or signs of ill-health of the animals were recorded during the study. Eye irritation scores were evaluated according to the Draize (1977) and OECD 405 (April 24, 2002) scoring systems. The study was performed in accordance with OECD Guidelines for Testing of Chemicals No. 405; Acute Eye Irritation/Corrosion, adopted April 24, 2002.

2.8. Acute skin irritation study in rabbits

Male New Zealand White rabbits (age 11 weeks) were obtained from Tetrabbit Kft. (2173 Kartal, Császár út 135, Hungary). Housing, feeding and drinking were the same as described above for the eye irritation study. Animals weighed 2525–2624 g at the beginning of the study, and 2712–2834 g at the end of the study.

Test article was used at a concentration of 1.93×10^{11} CFUs/g. An undiluted dose of 0.5 g was moistened sufficiently with water to ensure good contact with the skin, and a patch test was applied to approximately 6 cm^2 of intact skin on three animals, starting with just one animal to ensure no corrosive or severe irritation. Sterile gauze pads and adhesive hypoallergenic plaster kept the test article in place. The trunks of the animals were wrapped in plastic wrap for 4 h, which was the duration of the test item exposure. After 4 h, the test item was removed by washing the animals with body temperature water. Untreated skin on each animal served as the negative control.

Animals were examined for erythema and edema at 1, 24, 48 and 72 h after the test article removal. The test article was evaluated according to the Draize (1959)

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method (OECD 404, 2002) for any irritant effect. The study was performed in accordance with OECD Guidelines for Testing of Chemicals No. 404; Acute Dermal Irritation/Corrosion, adopted April 24, 2002.

3. Results

3.1. Bacterial reverse mutation (Ames) study

The bacterial reverse mutation assay was performed to evaluate whether Ganeden *B. coagulans* cell mass has mutagenic properties. All criteria for a valid bacterial reverse mutation (Ames) study as described in the materials and methods section were met. The utility of the AMES study for a biological test article such as *B. coagulans*, may be questionable. The study was performed both as a typical part of a toxicologic safety assessment for a natural product as well as to investigate for any mutagenic properties that the bacteria or its fermentate may have. While the negative results of the study were encouraging, when considered alone the data may be less meaningful and therefore additional studies such as the chromosome aberration assay and mouse micronucleus study were employed to further investigate genotoxicity and clastogenicity.

There were no revertants exceeding three times the background average either with or without the S9 metabolic activation system. In addition, no dose-dependent increase in revertants was observed. In conclusion, the results of this study showed that the *B. coagulans* cell mass, GanedenBC^{30}™ had no mutagenic effect for any strain used in this test. Furthermore, the results of the repeat assay confirmed the results of the definitive assay.

3.2. Micronucleus assay in mice

The micronucleus test was conducted to investigate for the formation of micronuclei containing chromosome fragments or whole chromosomes, which are indicative of cytogenetic damage. There were no differences in body weight between the treatment groups compared to the control group and no signs of toxicity were noted in clinical observations following administration of the test article at doses of 500, 1000 and 2000 mg/kg bw/day. Animals in the positive control group showed a significant increase in the frequency of micronuclei compared to the negative controls. None of the treatment groups were positive for statistically significant induction of micronuclei in reticulocytes, and the ratio of reticulocytes to total erythrocytes in these groups showed no significant decrease compared to the negative control group. The average reticulocyte to total erythrocytes ratio in the negative control group was 3.87%. The treatment groups were 3.69%, 3.65% and 3.69% in the 500, 1000 and 2000 mg/kg bw/day groups respectively. The positive control caused a 32.8% decrease in the ratio. This study indicates that Ganeden BC^{30}™ did not cause signs of toxicity in the bone marrow of the mice in the range of the doses tested.

The incidence of micronucleated reticulocytes in the peripheral blood per 1000 reticulocytes was 1.8 ± 0.8 in the negative control group, which was within the historical reference range. The positive control group had a mean frequency of 31.2 ± 5.5, which was a statistically significant increase compared with the negative control group. The test article dose groups had 1.3 ± 1.2, 2.2 ± 1.0 and 0.9 ± 0.4 micronucleated reticulocytes per 1000 reticulocytes, at the test dose levels of 500, 1000, and 2000 mg/kg bw/d respectively. These values were not statistically significant, and thus did not demonstrate any signs of toxicity with administration of Ganeden BC^{30}™ in the mouse peripheral blood micronucleus assay.

3.3. In vitro chromosomal aberration assay

The purpose of performing the chromosome aberration assay in cultured mammalian cells is to investigate for any potential the test article may have for causing structural damage to either chromosomes or chromatids.

Cells in the negative control group had 20 ± 2 chromosomes upon karyotypic analysis. The percentage of chromosomal aberrations measured in the negative control groups was zero. None of the dose levels of Ganeden BC^{30}™ tested produced any statistically significant increase in aberrant cells, while the positive control groups did induce a significant increase when compared with the negative controls as expected. Therefore, under the conditions of the assay, Ganeden BC^{30}™ produced a negative response for induction of structural chromosomal aberrations both with and without the metabolic activation system in Chinese hamster ovary cells.

3.4. Acute oral toxicity study in rats

A 14-day oral toxicity study in rats was performed to investigate the test article for acute toxicity. The results of such studies may provide preliminary toxicity data that is useful in determining appropriate dose levels for future repeated-dose oral toxicity studies as well as determining possible target organs that should be closely examined in such toxicity studies of a longer duration.

A single oral dose of 5000 mg/kg bw produced no treatment-related signs in any of the animals. Neither weight-loss nor changes in body weight resulted with the treatment compared with the control group. All of the organs examined in both the male and female dose groups were free from any gross pathological changes and thus, per OECD Guideline No. 423, histopathological examination was not performed. There was no evidence of any toxicity in the acute toxicity study as the results were unremarkable.

3.5. Subchronic 13-week oral toxicity study in rats

A 13-week repeated-dose toxicity study was performed in rats to determine a NOAEL for defined toxicological endpoints and is used to establish a safe chronic oral dose for humans. Ganeden BC^{30}™ was administered orally by gavage at doses of 100, 300 and 1000 mg/kg bw/day for 90 consecutive days. There were no deaths and no treatment-related signs were observed throughout the 13-week treatment period in any of the groups. Appearance and behavior of the animals were similar for all groups in the study.

The mean body weight of the males in the 100 mg/kg group was below that of the controls on day 50, and from days 71 to 90 in the study. The difference was 6–7% lower than the control groups, but was not considered related to the test article because of a lack of a dose response. The mean body weight of the males in the 1000 mg/kg group was lower than the control groups, in this case, from days 22–90. No statistically significant differences in body weight were noted in the male or female rats in the 300 mg/kg group, when compared to the control. Similar effects were not observed in the female rats in any of the treatment groups. A summary of total body weights and body weight gains can be found in Table 1.

Average daily food consumption was similar in all groups except for a slightly lower mean value ($p < 0.05$) with the males in the highest dose group during week 8, and a slightly higher mean value ($p < 0.01$) with the females in the highest dose group during week 6. Water consumption for all of the male dose groups was similar when compared with the controls. Statistically significant lower water consumption ($p < 0.05$) was noted in the females in the 100 mg/kg group during days 57–58 and 88–89, and in the females in the 300 mg/kg group ($p < 0.05$) on days 57–58, 85–86 and 88–89. Females in the 1000 mg/kg dose group also had decreased water consumption, but only during days 57–58 ($p < 0.01$) and 88–89 ($p < 0.05$).

Some statistically significant differences were observed from the results of the hematology and clinical chemistry parameters

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Table 1
Summary of body weight (BW) and weekly body weight grain (BWG) data in the rat subchronic 13-week oral toxicity study.

		1	8	15	22	29	36	43	50
Females									
Control	BW	171.8 ± 5.37	200.6 ± 8.9	219.7 ± 13.34	238.8 ± 15.96	247.2 ± 23.64	257.5 ± 23.03	264.2 ± 20.41	278.1 ± 24.18
	BWG		28.8 ± 6.78	19.10 ± 6.54	14.10 ± 7.99	13.40 ± 11.96	10.30 ± 7.41	6.70 ± 7.30	13.90 ± 7.62
100 mg/kg/day	BW	172.8 ± 7.77	203.1 ± 7.55	232.2 ± 11.99	234.80 ± 20.95	251.5 ± 21.88	259.1 ± 18.76	271.3 ± 21.26	282.7 ± 26.17
	BWG		30.3 ± 8.31	20.10 ± 6.97	11.60 ± 11.57	16.70 ± 9.93	7.60 ± 8.13	12.20 ± 5.45	11.40 ± 8.04
300 mg/kg/day	BW	173.2 ± 7.67	205.0 ± 7.96	222.80 ± 5.49	235.6 ± 9.00	252.3 ± 11.2	263.3 ± 10.24	271.3 ± 11.87	281.4 ± 15.78
	BWG		31.8 ± 3.71	17.80 ± 10.41	12.80 ± 9.93	16.70 ± 9.32	11.00 ± 6.43	8.00 ± 6.41	10.10 ± 8.91
1000 mg/kg/day	BW	172.5 ± 5.40	200.7 ± 10.19	222.50 ± 11.10	238.5 ± 12.15	252.3 ± 17.08	262.3 ± 18.41	273.1 ± 20.06	282.4 ± 20.46
	BWG		28.2 ± 7.48	21.80 ± 8.99	16.00 ± 7.27	13.80 ± 9.43	10.00 ± 6.46	10.80 ± 7.51	9.30 ± 9.44

		57	64	71	78	85	90/91	Sum (1–91)
Control	BW	283.2 ± 27.87	285 ± 26.03	289.2 ± 24.23	296.6 ± 27.02	297.7 ± 30.36	300.5 ± 28.04	
	BWG	5.10 ± 6.38	1.80 ± 7.50	4.20 ± 8.15	7.40 ± 6.06	1.10 ± 7.03	2.80 ± 5.96	128.70 ± 27.01
100 mg/kg/day	BW	287.1 ± 25.92	288 ± 22.2	296 ± 26.09	302.1 ± 29.5	304.1 ± 30.78	307.5 ± 29.26	
	BWG	4.40 ± 8.45	0.90 ± 6.74	8.00 ± 6.60	6.10 ± 4.86	2.00 ± 7.85	3.40 ± 7.07	134.70 ± 28.88
300vmg/kg/day	BW	286.4 ± 18.55	292.3 ± 15.08	295.4 ± 14.72	301.1 ± 16.15	301.7 ± 13.94	306.4 ± 14.71	
	BWG	5.00 ± 9.02	5.90 ± 7.05	3.10 ± 4.77	5.70 ± 5.12	0.60 ± 6.22	4.70 ± 6.27	133.20 ± 19.36
1000 mg/kg/day	BW	288.7 ± 23.76	293.9 ± 21.66	301.9 ± 22.09	306.8 ± 21.80	306.7 ± 26.57	313.2 ± 23.50	
	BWG	6.30 ± 7.23	5.20 ± 6.09	8.00 ± 4.69	4.90 ± 6.24	−0.10 ± 7.87	6.50 ± 8.50	140.70 ± 23.59

Males		1	8	15	22	29	36	43	50
Control	BW	210.8 ± 6.94	275.1 ± 11.18	329.3 ± 17.93	362.8 ± 21.53	396.7 ± 26.16	424.5 ± 27.00	447.1 ± 31.08	473.2 ± 34.83
	BWG		64.30 ± 6.00	54.20 ± 9.35	33.50 ± 8.64	33.90 ± 5.97	27.80 ± 5.09	22.60 ± 4.97	26.10 ± 5.07
100 mg/kg/day	BW	211.3 ± 6.02	272.2 ± 12.22	320.6 ± 18.47	354.3 ± 21.38	381.2 ± 24.22	404.3 ± 26.59	424.5 ± 33.60	445.1 ± 35.29*
	BWG		60.90 ± 7.02	48.40 ± 8.10	33.70 ± 4.69	26.90 ± 5.51**	23.10 ± 3.41*	20.20 ± 8.13	20.60 ± 3.31*
300 mg/kg/day	BW	211.9 ± 5.32	272.1 ± 6.87	325.4 ± 11.15	362.5 ± 15.79	388.3 ± 17.36	417.9 ± 20.73	439.3 ± 22.21	464.1 ± 24.01
	BWG		60.20 ± 3.79	53.30 ± 6.57	37.10 ± 6.28	25.80 ± 5.45**	29.60 ± 5.27	21.40 ± 3.72	24.80 ± 3.55
1000 mg/kg/day	BW	210.0 ± 6.55	265.6 ± 10.23	312.6 ± 15.43	344.0 ± 10.32*	369.5 ± 10.56**	393.0 ± 10.17**	411.8 ± 11.29**	431.6 ± 13.83**
	BWG		55.60 ± 7.17**	47.00 ± 7.13	31.40 ± 8.14	25.50 ± 3.81**	23.50 ± 3.72*	18.80 ± 5.16	19.80 ± 5.81**

		57	64	71	78	85	90/91	Sum (1–91)
Control	BW	482.2 ± 37.05	488.5 ± 36.56	511.2 ± 40.02	527.5 ± 40.76	539.9 ± 42.24	543.9 ± 42.05	
	BWG	9.00 ± 6.09	6.30 ± 4.11	22.70 ± 7.48	16.30 ± 5.38	12.40 ± 3.66	4.00 ± 4.74	333.10 ± 40.08
100 mg/kg/day	BW	457.1 ± 38.79	463.9 ± 39.24	476.1 ± 38.96*	492.6 ± 45.33*	501.7 ± 45.45*	505.4 ± 42.80*	
	BWG	12.00 ± 4.35	6.80 ± 6.30	12.20 ± 5.71**	16.50 ± 7.47	9.10 ± 4.95	3.70 ± 3.89	294.10 ± 39.08
300 mg/kg/day	BW	483.2 ± 26.48	493.2 ± 26.74	508.9 ± 27.73	521.0 ± 29.59	534.3 ± 31.93	541.3 ± 35.09	
	BWG	19.10 ± 4.41**	10.00 ± 4.24	15.70 ± 4.47*	12.10 ± 4.38	13.30 ± 4.55	7.00 ± 4.45	329.40 ± 31.60
1000 mg/kg/day	BW	440.3 ± 12.01*,	451.4 ± 13.12**	461.8 ± 15.51**	475.3 ± 20.16**	481.9 ± 19.58**	487.7 ± 21.47**	
	BWG	8.70 ± 2.98	11.10 ± 3.57*	10.40 ± 5.06**	13.50 ± 8.15	6.60 ± 6.60*	5.80 ± 4.24	277.70 ± 21.50

Values are mean ± S.D.
* $p < 0.05$.
** $p < 0.01$.

tested. However, since they fell into the historically normal range for the laboratory it was concluded that no clinically relevant test item related hematological or clinical chemistry changes were observed in either the male or female rats receiving GanedenBC^{30}™ at 100, 300, or 1000 mg/kg/day (Tables 2–4).

Gross pathological evaluation at the end of the study revealed several cases of pinprick-sized hemorrhages and pale, pillow-like raised areas in the lungs in all groups, which were likely caused by exsanguination of the animals. No treatment-related histopathological findings were noted upon examination of the animals at the end of the study.

Absolute organ weights differed only in the brains of the males in the 1000 mg/kg group, the liver in the 100 and 1000 mg/kg groups, and the testes in the 100 mg/kg group. However, the differences were considered to be the consequence of the lower mean body weights of these groups. Importantly, the relative organ weights compared to body weights did not differ for these organs (Table 5).

The relative kidney weight was lower than the control in the males in the 300 mg/kg group, and higher in the males in the 1000 mg/kg group (Table 5). The changes were not considered to be of biological significance or related to the test article, most importantly because they were not corroborated with any histological findings. The relative weight of the adrenal glands was lower than the control group for the females in the 300 mg/kg group, but was due to individual variation and not considered related to administration of the test article because of a lack of a dose response.

In the 90-day subchronic oral toxicity study, no toxicologically significant differences between the treated groups (100, 300 and 1000 mg/kg bw/day) and the controls were observed with respect to food consumption, water consumption, sensory reactivity, general and behavioral conditions, hematological and clinical chemistry evaluations. GanedenBC^{30}™ caused neither treatment-related macroscopic or microscopic signs nor changes in the organ weights of the male and female rats at 100, 300 and 1000 mg/kg/day after the 13-week treatment period. The test item was well tolerated.

Since there were no signs of toxicity noted with respect to gross or histopathological examinations, nor with hematology, clinical chemistry, or organ weights for the 1000 mg/kg dose group, the differences in the mean body weight of the males described above is not considered related to the test article, but rather a result of biological variation. Hence the NOAEL for both males and females is considered to be >1000 mg/kg body weight per day, which was the highest dose tested.

3.6. Acute eye irritation study in rabbits

Ganeden BC^{30}™ cell mass applied to the mucosa of the eyes resulted in slight to moderate conjunctival irritant effect within 1 h that was fully reversible in 72 h. There were no negative signs observed in either the cornea or the iris. According to EC criteria for classification and labeling requirements for dangerous substances and preparations, the test article is not required to be classified,

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Table 2
Summary of hematological data in the rat subchronic 13-week oral toxicity study.

Females	RBC (10^{12}/L)	Hgb (g/dL)	Hct (%)	MCV(fL)	MCH (pg)	MCHC (g/dL)	RDW (%)
Control	7.98 ± 0.33	15.03 ± 0.55	41.55 ± 1.57	52.08 ± 1.80	18.81 ± 0.61	36.15 ± 0.65	11.32 ± 0.56
100 mg/kg/day	7.85 ± 0.31	15.02 ± 0.27	4170 ± 068	53.19 ± 2.27	19.18 ± 1.05	36.01 ± 0.75	11.64 ± 0.62
300 mg/kg/day	7.65 ± 0.49	14.53 ± 0.56*	40.12 ± 1.60*	52.54 ± 2.45	19.04 ± 0.83	36.22 ± 0.71	12.47 ± 2.20
1000 mg/kg/day	7.91 ± 0.53	15.06 ± 0.55	41.38 ± 135	52.42 ± 2.22	19.09 ± 0.88	36.39 ± 0.50	11.52 ± 0.44
Females	PLT (10^9/L)	MPV (fL)	APTT (s)	PT (s)	Retic. (%)	WBC (10^9/L)	
Control	1034.90 ± 92.06	7.74 ± 0.58	18.25 ± 0.79	23.65 ± 1.00	2.22 ± 0.42	1.42 ± 0.32	
100 mg/kg/day	1017.70 ± 110.59	7.21 ± 0.38*	18.59 ± 0.54	24.06 ± 1.06	2.41 ± 0.43	1.83 ± 0.79	
300 mg/kg/day	1023.00 ± 213.31	7.23 ± 0.35*	18.39 ± 0.50	24.10 ± 1.28	2.97 ± 2.20	1.68 ± 0.53	
1000 mg/kg/day	996.90 ± 161.60	7.26 ± 0.43*	18.32 ± 1.05	23.79 ± 0.76	2.26 ± 0.54	2.33 ± 1.60	
Males	RBC (10^{12}/L)	Hgb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)
Control	8.69 ± 0.41	15.66 ± 0.61	43.85 ± 1.92	50.46 ± 1.11	18.02 ± 0.37	35.70 ± 0.31	12.51 ± 0.46
100 mg/kg/day	8.60 ± 0.45	15.48 ± 0.58	42.97 ± 1.62	50.01 ± 1.73	18.04 ± 0.69	36.10 ± 0.45	12.06 ± 0.48*
300 mg/kg/day	8.56 ± 0.36	15.74 ± 0.74	43.61 ± 2.04	50.97 ± 1.22	18.40 ± 0.48	36.08 ± 0.42	12.46 ± 0.28
1000 mg/kg/day	8.73 ± 0.40	15.53 ± 0.61	43.60 ± 1.64	49.97 ± 1.67	17.80 ± 0.61	35.62 ± 0.58	12.53 ± 0.55
Males	PLT (10^9/L)	MPV (fL)	APTT (s)	PT (s)	Retic. (%)	WBC (10^9/L)	
Control	1030.90 ± 136.70	7.69 ± 0.91	17.65 ± 0.62	25.56 ± 0.89	1.93 ± 0.16	3.40 ± 0.95	
100 mg/kg/day	957.20 ± 72.51	7.70 ±0.82	17.61 ± 0.79	25.20 ± 0.89	1.87 ± 0.30	4.01±1.47	
300 mg/kg/day	989.00 ± 106.87	7.70 ± 0.48	18.19 ± 0.75	25.48 ± 1.23	2.21 ± 0.21*	3.20 ± 1.00	
1000 mg/kg/day	1104.30 ± 161.76	7.77 ± 0.61	17.63 ± 1.50	26.53 ± 0.89*	1.86 ± 0.30	3.48 ± 1.65	

Values are mean ± S.D.
* $p < 0.05$.

Table 3
Summary of male serum biochemical data in the rat subchronic 13-week oral toxicity study.

Males	Glucose (mmol/l)	Urea (mmol/l)	Creat. (umol/l)	Na^+ (mmol/l)	K^+ (mmol/l)	Cl^- (mmol/L)	Ca^{++} (mmol/l)	P (mmol/l)	T. Chol. (mmol/l)
Control	6.94 ± 0.95	6.80 ± 0.94	54.62 ± 6.58	145.61 ± 1.01	5.28 ± 0.49	105.19 ± 1.49	2.47 ± 0.05	2.42 ± 0.25	1.46 ± 0.26
100 mg/kg/day	6.81 ± 1.22	7.67 ± 1.28	58.85 ± 9.79	145.20 ± 0.51	5.06 ± 0.45	104.66 ± 1.45	2.41 ± 0.06*	2.39 ± 0.18	1.58 ± 0.15
300 mg/kg/day	6.85 ± 1.44	6.98 ± 0.62	54.69 ± 4.46	144.81 ± 0.71	4.99 ± 0.46	104.7 ± 1.45	2.43 ± 0.06	2.34 ± 0.17	1.60 ± 0.36
1000 mg/kg/day	6.74 ± 0.90	6.81 ± 0.63	54.81 ± 4.13	145.19 ± 1.07	5.35 ± 0.35	104.20 ± 1.16	2.40 ± 0.08*	2.360 ± 0.17	1.68 ± 0.28
Males	T. Prot. (g/L)	Alb (g/L)	AST (U/L0)	ALT (U/L)	ALKP (U/L)	GGT (U/L)	T. Bil. (umol/L)	A/G	
Control	55.84 ± 1.58	29.79 ± 1.09	119.20 ± 13.74	53.00 ± 4.32	90.90 ± 13.47	6.00 ± 0.00	3.49 ± 0.21	1.16 ± 0.07	
100 mg/kg/day	54.69 ± 2.12	28.68 ± 2.09	128.40 ± 19.40	54.60 ± 9.37	87.60 ± 12.57	6.00 ± 0.00	3.04 ± 0.36	1.10 ± 0.08*	
300 mg/kg/day	53.95 ± 2.02*	28.08 ± 1.43*	120.70 ± 16.67	54.80 ± 3.29	83.50 ± 11.25	6.00 ± 0.00	3.47 ± 0.63	1.08 ± 0.04*	
1000 mg/kg/day	55.98 ± 1.69	28.96 ± 0.97	134.70 ± 32.44	55.80 ± 4.18	90.50 ± 16.57	6.00 ± 0.00	3.32 ± 0.72	1.08 ± 0.06*	

Values are mean ± S.D.
* $p < 0.05$.
** $p < 0.01$.

Table 4
Summary of female serum biochemical data in the rat subchronic 13-week oral toxicity study.

Females	Glucose (mmol/l)	Urea (mmol/l)	Creat. (umol/l)	Na^+ (mmol/l)	K^+(mmol/l)	Cl^- (mmol/l)	Ca^{++}(mmol/l)	P (mmol/l)	T. Chol. (mmol/l)
Control	6.55 ± 0.86	6.87 ± 0.80	57.83 ± 7.35	144.78 ± 1.29	4.64 ± 0.38	105.05 ± 1.69	2.49 ± 0.10	1.96 ± 0.24	1.83 ± 0.37
100 mg/kg/day	7.44 ± 0.89	7.21 ± 1.73	56.13 ± 7.49	143.79 ± 1.49	4.40 ± 0.44	103.65 ± 2.60	2.43 ± 0.08	1.74 ± 0.39	1.69 ± 0.34
300 mg/kg/day	6.72 ± 0.52	7.06 ± 1.34	53.95 ± 4.10	144.58 ± 1.74	4.40 ± 0.32	105.55 ± 1.33	2.43 ± 0.06	1.85 ± 0.33	1.49 ± 0.26
1000 mg/kg/day	7.06 ± 0.69	7.58 ± 1.16	57.77 ± 7.27	144.85 ± 1.42	4.44 ± 0.37	105.70 ± 1.96	2.43 ± 0.07	2.03 ± 0.25	1.77 ± 0.47
Females	T. Prot. (g/l)	Alb (g/l)	AST (U/L0)	ALT (U/L)	ALKP (U/L)	GGT (U/L)	T. Bil. (Mmol/l)	A/G	
Control	54.63 ± 3.50	31.06 ± 3.07	115.50 ± 19.28	46.90 ± 5.82	55.80 ± 11.04	6.00 ± 0.00	4.05 ± 0.66	1.34 ± 0.15	
100 mg/kg/day	54.78 ± 2.81	31.02 ± 1.92	120.60 ± 46.93	50.20 ± 11.35	52.80 ± 5.07	6.00 ± 0.00	3.80 ± 0.37	1.31 ± 0.12	
300 mg/kg/day	53.60 ± 2.11	28.97 ± 1.95	100.80 ± 17.05	49.80 ± 4.71	56.30 ± 10.56	6.00 ± 0.00	3.55 ± 0.46	1.19 ± 0.10**	
1000 mg/kg/day	52.80 ± 1.60	28.97 ± 1.23*	111.30 ± 22.19	45.50 ± 4.40	57.20 ± 9.04	6.00 ± 0.00	3.47 ± 0.62*	1.22 ± 0.06*	

Values are mean ± S.D.
* $p < 0.05$.
** $p < 0.01$.

nor is it obligatory to label the test article with regard to eye irritation.

3.7. Acute skin irritation study in rabbits

GanedenBC^{30}™ cell mass applied to the skin resulted in very slight erythema, but no edema at 1 h after removal of the patch. At 24 h, the animals no longer had any signs of erythema. According to EC directive 2001/59/EEC the test article is not classified as irritating to the skin. The observed clinical sign of very slight erythema on the treated skin surface was concluded as fully reversible.

4. Discussion

New species and strains of probiotic bacteria are becoming commercially available as dietary ingredients in both dietary supplements and functional foods. It is especially important that

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Table 5
Summary of mean organ weights (g) and organ weight relative to body weight (%) in the rat subchronic 13-week oral toxicity study.

		Control	100 mg/kg/day	300 mg/kg/day	1000 mg/kg/day
Males					
Body weight	g	522.20 ± 38.94	497.00 ± 66.06	523.70 ± 30.98	470.20 ± 20.87**
Brain (g)	g	2.23 ± 0.08	2.23 ± 0.14	2.20 ± 0.06	2.14 ± 0.06*
	%	0.430 ± 0.036	0.452 ± 0.048	0.422 ± 0.030	0.456 ± 0.024
Liver	g	12.40 ± 1.44	11.29 ± 1.23*	12.35 ± 0.93	11.19 ± 0.78*
	%	2.371 ± 0.146	2.283 ± 0.193	2.358 ± 0.106	2.379 ± 0.110
Heart	g	1.38 ± 0.16	1.27 ± 0.11	1.35 ± 0.06	1.29 ± 0.09
	%	0.265 ± 0.021	0.258 ± 0.027	0.257 ± 0.013	0.275 ± 0.017
Spleen	g	1.13 ± 0.21	0.97 ± 0.18	1.04 ± 0.11	1.01 ± 0.14
	%	0.216 ± 0.034	0.196 ± 0.024	0.199 ± 0.020	0.215 ± 0.026
Kidneys	g	3.02 ± 0.34	2.81 ± 0.17	2.82 ± 0.20	2.93 ± 0.20
	%	0.576 ± 0.029	0.569 ± 0.051	0.538 ± 0.035*	0.623 ± 0.038*
Thymus	g	0.45 ± 0.08	0.35 ± 0.11	0.41 ± 0.49	0.36 ± 0.12
	%	0.086 ± 0.014	0.070 ± 0.017	0.078 ± 0.018	0.075 ± 0.024
Testes	g	4.10 ± 0.48	3.68 ± 0.32*	3.76 ± 0.21	3.78 ± 0.36
	%	0.786 ± 0.082	0.748 ± 0.086	0.720 ± 0.051	0.805 ± 0.070
Epididymides	g	1.57 ± 0.15	1.46 ± 0.11	1.50 ± 0.18	1.59 ± 0.24
	%	0.301 ± 0.033	0.297 ± 0.038	0.288 ± 0.041	0.337 ± 0.047
Adrenals	g	0.065 ± 0.009	0.061 ± 0.008	0.065 ± 0.011	0.064 ± 0.006
	%	0.0126 ± 0.0021	0.0124 ± 0.0019	0.0125 ± 0.0023	0.0137 ± 0.0013
Females					
Body weight	g	284.70 ± 27.35	294.40 ± 28.78	292.60 ± 16.02	296.40 ± 22.97
Brain	g	2.01 ± 0.06	2.03 ± 0.10	1.99 ± 0.09	2.03 ± 0.08
	%	0.711 ± 0.065	0.695 ± 0.065	0.681 ± 0.039	0.688 ± 0.055
Liver	g	6.84 ± 0.91	7.52 ± 0.85	7.36 ± 1.35	7.24 ± 0.73
	%	2.394 ± 0.118	2.556 ± .0209	2.506 ± 0.379	2.441 ± 0.145
Heart	g	0.87 ± 0.08	0.91 ± 0.14	0.88 ± 0.09	0.89 ± 0.06
	%	0.308 ± 0.024	0.310 ± 0.042	0.303 ± 0.032	0.299 ± 0.019
Spleen	g	0.67 ± 0.08	0.75 ± 0.14	0.77 ± 0.17	0.73 ± 0.10
	%	0.236 ± 0.027	0.256 ± 0.048	0.260 ± 0.050	0.246 ± 0.029
Kidneys	g	1.77 ± 0.18	1.85 ± 0.17	1.81 ± 0.16	1.90 ± 0.18
	%	0.623 ± 0.038	0.634 ± 0.077	0.617 ± 0.045	0.639 ± 0.035
Thymus	g	0.27 ± 0.05	0.31 ± 0.06	0.27 ± 0.07	0.32 ± 0.07
	%	0.096 ± 0.018	0.104 ± 0.022	0.092 ± 0.024	0.108 ± 0.021
Uterus	g	0.80 ± 0.23	0.85 ± 0.23	0.78 ± 0.26	0.91 ± 0.34
	%	0.287 ± 0.097	0.294 ± 0.106	0.269 ± 0.094	0.312 ± 0.124
Ovaries	g	0.144 ± 0.030	0.147 ± 0.010	0.147 ± 0.015	0.142 ± 0.030
	%	0.0509 ± 0.0113	0.0505 ± 0.0074	0.0504 ± 0.0065	0.0480 ± 0.0095
Adrenals	g	0.094 ± 0.011	0.094 ± 0.012	0.085 ± 0.011	0.091 ± 0.012
	%	0.0333 ± 0.0041	0.0321 ± 0.0031	0.0289 ± 0.0034*	0.0307 ± 0.0039

Values are mean ± S.D.
* $p < 0.05$.
** $p < 0.01$.

novel probiotics are properly tested for safety and efficacy. The focus of this paper is to present a comprehensive toxicologic assessment to support the safety for chronic consumption of the novel probiotic GanedenBC^{30}™. An acute oral toxicity and a 13-week subchronic oral toxicity study were conducted in Wistar rats. In addition, *in vitro* studies were conducted to evaluate mutagenicity, genotoxicity, and clastogenicity. There was no evidence for any mutagenic or genotoxic effect of GanedenBC^{30}™ in either the AMES assay or the *in vitro* chromosomal aberration study. Negative results were also concluded for the micronucleus assay in mice, indicating that under the test conditions GanedenBC^{30}™ does not produce cytogenetic damage. Furthermore, GanedenBC^{30}™ did not produce any biologically significant skin or eye irritation.

5. Conclusion

In conclusion, the studies described in this paper were conducted as a comprehensive safety assessment of GanedenBC^{30}™, a commercially available probiotic strain of *B. coagulans*. As part of a pre-clinical safety evaluation program, several tests have been performed. GanedenBC^{30}™ demonstrated no evidence to suggest mutagenicity or genotoxicity in a number of commonly utilized genetic toxicity assays. No treatment-related mortality, morbidity or clinical symptoms resulted from an acute oral toxicity study using a single dose of 5000 mg/kg. In a subchronic oral toxicity study, GanedenBC^{30}™ in daily doses of 100, 300 and 1000 mg/kg bw/day for 90 days was well tolerated and did not cause either lethality or toxic clinical symptoms in either male or female rats. The NOAEL derived from the results of the 90-day study is 1000 mg/kg. Since the concentration of the *B. coagulans* used was 1.36×10^{11} CFUs/g, this corresponds to 1.36×10^{11} CFUs/kg. For an average 70 kg human being, this corresponds to 95.2×10^{11} CFUs. Because the suggested human dose is in the range of 100×10^{6} to 3×10^{9} CFUs, this gives a safety factor ranging from 3173 to 95,200 times. Based upon scientific procedures and supported by history of use, GanedenBC^{30}™ is considered safe for chronic human consumption.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgement

Thanks to Ganeden Biotech, Inc., for supplying GanedenBC^{30}™ for this study.

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Public Health Service

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JUL 3 1 2012

Re: GRAS Notice No. GRN 000399

Dear Dr. Endres:

The Food and Drug Administration (FDA) is responding to the notice, dated August 11, 2011, that you submitted on behalf of Ganeden Biotech, Inc. (Ganeden) in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on August 19, 2011, filed it on August 23, 2011, and designated it as GRAS Notice No. GRN 000399.

The subject of the notice is a preparation of *Bacillus coagulans* strain GBI-30, 6086 spores (*B. coagulans* spore preparation). The notice informs FDA of the view of Ganeden that *B. coagulans* spore preparation is GRAS, through scientific procedures, for use as an ingredient in baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes;[1] sugar; and sweet sauces, toppings, and syrups at a maximum level of approximately 2×10^9 colony forming units per serving (cfu/serv).

In its notice, Ganeden includes a statement from a panel of individuals (Ganeden's GRAS panel) who evaluated the data and information that are the basis for Ganeden's GRAS determination. Ganeden considers the members of its GRAS panel to be qualified by scientific training and experience to evaluate the safety of substances added to food. Ganeden's GRAS panel evaluated the method of manufacture, specifications, exposure, and safety data and information for *B. coagulans* spore preparation. Based on this review, Ganeden's GRAS panel concluded that *B. coagulans* spore preparation produced in accordance with current good manufacturing practices and meeting established specifications is GRAS under the conditions of its intended use.

Ganeden discusses the history of *B. coagulans* and the identification and characteristic properties of *B. coagulans* strain GBI-30, 6086 as follows: *B. coagulans* was first described in 1915 at the Iowa Agricultural Experiment Station associated with the coagulation of evaporated milk. *B. coagulans* strain GBI-30, 6086 was identified by genetic comparison with a known *B. coagulans*

[1] Ganeden states that *B. coagulans* spore preparation is not intended for use in any product that would require additional review by the United States Department of Agriculture.

strain. Published information describes *B. coagulans* strain GBI-30, 6086 as a Gram-positive, sporeforming, L+ lactic acid producing bacterium.

Ganeden describes the manufacture of *B. coagulans* spore preparation. All ingredients, fermentation tanks, and culture media are heat sterilized prior to inoculation with *B. coagulans* GBI-30, 6086 vegetative cells. The fermentation medium contains soy- and milk-derived ingredients. The strain is fermented under pH- and temperature-controlled, aseptic conditions. The pH is controlled by adding ammonium hydroxide or sodium hydroxide. The flow of nutrients to the vegetative cells is discontinued during fermentation to induce spore formation. After fermentation, the spores undergo centrifugation and freeze- or spray-drying before packaging. Ganeden states that all materials used in the manufacturing process are food-grade and meet appropriate regulations.

Ganeden provides specifications for appearance and moisture for *B. coagulans* spore preparation. Limits for microbiological contaminants include yeast, total coliforms, *Escherichia coli,* Staphylococci, *Salmonella,* and *Pseudomonas aeruginosa.* Also included are limits for heavy metal contaminants: arsenic ($\leq$ 2 milligrams per kilogram (mg/kg)), cadmium ($\leq$ 2 mg/kg), lead ($\leq$ 5 mg/kg), and mercury ($\leq$ 2 mg/kg).

Ganeden states that *B. coagulans* spore preparation is comprised of nearly 100% spores, which survive harsher conditions than vegetative cells.[2] Ganeden states that the relative proportions of spores and vegetative cells are consistent among batches. A study shows that *B. coagulans* spore preparation is stable for three months under storage at 40°C and 75% relative humidity.

Ganeden states that *B. coagulans* spore preparation is currently added to the intended foods at levels of 10^8 to 2 x 10^9 cfu/serv. A publication from The United States Department of Agriculture Center for Nutrition Policy and Promotion (October 2000) states that males aged 51 and older consume the largest number of servings of food a day at 18.2 servings per day. Using this estimate of the number of servings per day at a level of 2 x 10^9 cfu/serv, Ganeden estimates the daily intake of *B. coagulans* spores at 36.4 x 10^9 cfu per day (cfu/day).

Ganeden discusses published and unpublished information supporting the safety of *B. coagulans* strain GBI-30, 6086. Published information describes *B. coagulans* strain GBI-30, 6086 as a nonpathogenic and nontoxigenic bacterium. In addition, an unpublished *in vitro* test shows that *B. coagulans* strain GBI-30, 6086 is sensitive to many clinically-used antibiotics, and that there is no evidence that the strain transmits antibiotic resistance to other bacterial species. Unpublished genetic sequencing and bioinformatic analyses show that *B. coagulans* strain GBI-30, 6086 does not contain genes homologous to those encoding known protein toxins.

Ganeden also discusses a published *in vitro* study, published studies conducted in animals, and published studies conducted in humans to support the safety of *B. coagulans* spore preparation. An *in vitro* study conducted using a continuous culture fermentation system designed to mimic conditions in the human ascending colon provides a basis to conclude that *B. coagulans* strain GBI-30, 6086 spores germinate and persist for at least 72 hours after discontinuing administration of spores into the system. The study authors state that *B. coagulans* strain GBI-30, 6086 spores did not alter the human fecal microbiota present in the fermentation system. An *in vivo* study in piglets conducted using biomass comprised of about 50% spores of *B. coagulans* strain CNCM I-1061 shows that the strain is transient, persisting for up to seven days in the piglet gastrointestinal

[2] Ganeden states that *B. coagulans* spores survive manufacturing conditions and the acidic conditions of the stomach.

tract. Ganeden concludes that taken together, these data show that *B. coagulans* strain GBI-30, 6086 spores are transient in the human gastrointestinal tract.

Ganeden discusses published studies conducted to assess the potential toxicity of *B. coagulans* spore preparation. *In vitro* studies show that *B. coagulans* spore preparation is not mutagenic or genotoxic. Studies conducted using rats show that *B. coagulans* spore preparation does not induce acute, subchronic, chronic, or reproductive toxicity following consumption of up to 2000 mg/kg body weight per day (equivalent to approximately 2.6×10^{13} cfu per person per day) spores.

Ganeden cites published human studies designed to investigate effects of *B. coagulans* spore preparation on patients with certain health conditions. In these studies, *B. coagulans* spore preparation was administered as a medical food or dietary supplement. Parameters assessed in these studies included arthritis activity, erythrocyte sedimentation rate, C-reactive protein levels, blood levels of cytokines and activated T lymphocytes, irritable bowel syndrome symptoms, gastrointestinal symptoms, and severity of dyspepsia. No adverse events were observed in these studies after the consumption of up to 2×10^9 cfu/day spores. Ganeden also notes that more than one billion servings of *B. coagulans* spore preparation have been consumed since 2003 at levels up to 2×10^9 cfu/serving, and that there have been no reports of adverse events.

Based on: 1) published and unpublished information demonstrating the safety of *B. coagulans* strain GBI-30, 6086; 2) published animal studies supporting the safety of *B. coagulans* spore preparation; 3) published human studies in which no adverse events were observed following consumption of *B. coagulans* spore preparation, and 4) the history of safe use of *B. coagulans* spore preparation, Ganeden concludes that *B. coagulans* spore preparation is GRAS for the intended uses in foods.

Standards of Identity

In the notice, Ganeden states its intention to use *B. coagulans* spore preparation in several food categories, including foods for which standards of identity exist, located in Title 21 of the Code of Federal Regulations. We note that an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity.

Potential Labeling Issues

In describing the human studies conducted using *B. coagulans* spore preparation, Ganeden includes studies that describe potential health benefits. These studies raise a potential issue under the labeling provisions of the Federal Food, Drug, and Cosmetic Act (FD&C Act). Under section 403(a) of the FD&C Act, a food is misbranded if its labeling is false or misleading in any particular. Section 403(r) of the FD&C Act lays out the statutory framework for the use of labeling claims that characterize the level of a nutrient in a food or that characterize the relationship of a nutrient to a disease or health-related condition. If products that contain *B. coagulans* spore preparation bear any claims on the label or in labeling, such claims are the purview of the Office of Nutrition, Labeling, and Dietary Supplements (ONLDS) in the Center for Food Safety and Applied Nutrition. The Office of Food Additive Safety neither consulted with ONLDS on this labeling issue nor evaluated the information in the notice to determine whether it would support any claims made about *B. coagulans* spore preparation on the label or in labeling.

Allergen Labeling

The Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) amends the FD&C Act to require that the label of a food that is or contains an ingredient that bears or contains a "major food allergen" declare the presence of the allergen (section 403(w)). FALCPA defines a "major food allergen" as one of eight foods or food groups (i.e., milk, eggs, fish, Crustacean shellfish, tree nuts, peanuts, wheat, and soybeans) or a food ingredient that contains protein derived from one of those foods. Ganeden states that soy- and milk-derived ingredients used during the manufacturing process for *B. coagulans* spore preparation will be appropriately labeled in the finished products. Issues associated with labeling food are the responsibility of ONLDS. However, issues associated with FALCPA should be directed to the Food Allergen Coordinator in FDA's Center for Food Safety and Applied Nutrition (Food Allergen Coordinator, Office of Food Additive Safety, HFS-200, 5100 Paint Branch Parkway, College Park, Maryland 20740).

Section 301(ll) of the FD&C Act

The Food and Drug Administration Amendments Act of 2007, which was signed into law on September 27, 2007, amends the FD&C Act to, among other things, add section 301(ll). Section 301(ll) of the FD&C Act prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FD&C Act, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In its review of Ganeden's notice that *B. coagulans* spore preparation is GRAS for the intended uses, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing *B. coagulans* spore preparation. Accordingly, this response should not be construed to be a statement that foods that contain *B. coagulans* spore preparation, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by Ganeden, as well as other information available to FDA, the agency has no questions at this time regarding Ganeden's conclusion that *B. coagulans* spore preparation is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of *B. coagulans* spore preparation. As always, it is the continuing responsibility of Ganeden to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000399, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying at www.fda.gov/grasnoticeinventory.

Sincerely,

Dennis M. Keefe, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

Gut Pathogens

RESEARCH Open Access

Bacillus Coagulans GBI-30 (BC30) improves indices of *Clostridium difficile*-Induced colitis in mice

Leo R Fitzpatrick[1*], Jeffrey S Small[1], Wallace H Greene[2], Kelly D Karpa[1] and David Keller[3]

Abstract

Background: Probiotics have beneficial effects in rodent models of *Clostridium difficile* (*C. diffiicle*)-induced colitis. The spore forming probiotic strain *Bacillus Coagulans* GBI-30, 6086 (BC30) has demonstrated anti-inflammatory and immune-modulating effects *in vitro*. Our goal was to determine if BC30 improved *C. difficile*-induced colitis in mice. Starting on study day 0, female C57BL/6 mice were dosed by oro-gastric gavage for 15 days with vehicle (saline) or BC30 (2×10^9 CFU per day). Mice in the *C. difficile* groups received an antibiotic mixture (study days 5 to 8 in the drinking water), and clindamycin (10 mg/kg, i.p., on study day 10). The *C. difficile* strain VPI 10463 was given by gavage at 10^4 CFU to induce colitis on day 11. On day 16, stools and colons were collected for further analyses.

Results: All mice treated with BC30 survived on study day 13, while two mice treated with vehicle did not survive. On day 12, a significant difference ($p = 0.0002$) in the percentage of mice with normal stools (66.7%) was found in the BC30/*C. difficile* group, as compared to the vehicle/*C. diffcile* group (13.0%). On study day 16, 23.8% of mice treated with BC30 had normal stools, while this value was 0% with vehicle treatment (p value = 0.0187). On this day, the stool consistency score for the BC30/*C. difficile* group (1.1 ± 0.2) was significantly lower ($p < 0.05$) than for the vehicle/*C. difficile* cohort (1.9 ± 0.2). BC30 modestly attenuated the colonic pathology (crypt damage, edema, leukocyte influx) that was present following *C. difficile infection*. Colonic MIP-2 chemokine contents (pg/2 cm colon) were: 10.2 ± 0.5 (vehicle/no *C. difficile*), 24.6 ± 9.5 (vehicle/*C. difficile*) and 16.3 ± 4.3 (BC30/*C. difficile*).

Conclusion: The probiotic BC30 improved some parameters of *C. difficile*-induced colitis in mice. BC30 prolonged the survival of *C. diffiicle* infected mice. Particularly, this probiotic improved the stool consistency of mice, in this infectious colitis model.

Keywords: *Clostridium difficile*, probiotics, colitis, mice

Background

Clostridium Difficile (*C. difficile*) infection can cause nosocomial-related diarrhea [1]. The spectrum of *C. difficile*-associated disease (CDAD) ranges from mild antibiotic associated diarrhea to severe (or even life threatening) pseudomembranous colitis [1]. CDAD is caused by the actions of two exotoxins (toxin A and toxin B), which are produced by pathogenic strains of *C. difficile* [2,3].

Previous data suggests that toxin A can activate the nuclear factor-kappa B (NF-κB) signal transduction system in monocytes and colonic epithelial cells [4,5]. This activation of NF-κB leads to secretion of a key pro-inflammatory chemokine (IL-8) and subsequently to neutrophil influx into the colonic tissue [4,5]. Neutrophils play a key role in the pathogenesis of CDAD, both in humans and in mice [6].

CDAD is often treated successfully with standard antibiotics such as vancomycin, or metronidazole [7,8]. However, recurrence occurs in many patients [6,8]. Some clinical studies have focused on combined treatment with vancomycin and probiotics such as *Saccharomyces boulardii* for recurrent disease [8-11]. Therefore, initial treatment regimens with probiotics, or their use for prevention of recurrent disease, may be attractive as part of the overall therapeutic strategy for CDAD [8-11].

Probiotics are live microorganisms which, when ingested, can confer health benefits [12]. Typically, probiotics include various strains of *Lactobacillus* and/or *Bifidobacteria* species. They exist as either single entities or as combination products (e.g., VSL #3) [13,14]. Other known

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probiotics include certain non-pathogenic *Escherichia coli* (*E. coli*) strains like Nissle 1917 and M-17 [13,15]. Mechanisms explaining the potential role of probiotics as anti-colitis therapies have been reviewed in detail elsewhere [13]. Recently, our laboratory has shown that a non-pathogenic strain of *E. coli* can inhibit colitis in mice by immunomodulating the NF-κB signal transduction system and inhibiting associated pro-inflammatory cytokines [15]. Of note, a recent paper found that *Lactobacillus acidophilus* was effective for treating CDAD in mice [16].

Of direct relevance to this study, the novel spore forming probiotic strain GanedenBC^{30} (*Bacillus coagulans* GBI-30, 6086) is relatively resistant to extreme temperatures, as well as stomach acidity, digestive enzymes and bile salts [17]. *Bacillus Coagulans GBI-30* (BC30) has been used for human consumption to ameliorate symptoms in various gastrointestinal disorders, as well as an immunomodulating agent in *ex-vivo* viral challenge, and *in vivo* human immunodeficiency virus research [17,18].

Preliminary research to articulate its mechanisms of action demonstrated anti-inflammatory and immunomodulating effects *in vitro* [19]. Therefore, as a logical extension to this *in vitro* evaluation, we evaluated the effectiveness of BC30 for inhibiting *C. difficile* induced colitis in mice. Since NF-κB activation and chemokine secretion also play important roles in the pathogenesis of CDAD, we also evaluated the effects of BC30 on this critical transcription factor, as well as MIP-2, in this murine CDAD model [4-7].

Results

BC30 prolongs mouse survival after the administration of *C. difficile*

Figure 1 shows an overview of the key events associated with the *C. diff*icile induced colitis model that was used for this study. As shown in Figure 2, all mice treated with BC30 (100%) survived on study day 13, while 2 mice treated with vehicle did not survive on that day (92.3% survival). By day 14, 21/23 mice survived in the BC30/*C. difficile* treatment group, while 23/26 mice survived in the Vehicle/*C. difficile* treatment group. As expected, all mice (6/6) that did not receive *C. difficile* survived for the duration of the study. Therefore, the cumulative survival rates in the study were: 100% (Vehicle/no *C. difficile*), 88.5% (Vehicle/*C. difficile*) and 91.3% (BC30/*C. difficile*). However, there was not a statistically significant difference in the survival rate between any of the treatment groups.

Despite the delay in mortality rate in the BC30 treatment group, there were similar body weight profiles in both *C. difficile* treatment groups. Mice in these treatment groups lost weight (by approximately 10%) between days 11 and 14, and then subsequently began to gain weight. There were no differences in the body weights (grams) on day 16: 19.5 ± 0.4 (Vehicle/*C. difficile*) and 18.8 ± 0.4 (BC30/*C. difficile*). In contrast, mice that did not receive *C. difficile* gained weight during the study period (body weight on day 16 = 20.6 ± 0.5 grams).

BC30 treatment significantly improves the stool consistency in *C. difficile* infected mice

Figure 3 illustrates that 13% of mice in the *Vehicle/C. difficile* treatment group had normal stools on day 12; while 67% of the probiotic (BC30) treated mice still had normal stools. On days 13 and 14, altered stool consistency was seen in both *C. difficile* treatment groups. However, evidence of a normal stool was seen in 24% of BC30 treated mice on day 16, but no vehicle treated animals (0%) showed a normal stool consistency on that day. Statistical significance ($p < 0.05$) between the vehicle and BC30 treatment groups was found on study days 12 and 16.

Moreover, as shown in Figure 4, the mean stool consistency score (day 16) was significantly lower in BC30/*C. difficile* treated mice (1.1 ± 0.2) than in corresponding Vehicle/*C. difficile* treated animals (1.9 ± 0.2).

BC30 slightly attenuates indices of *C. difficile*-induced colonic pathology

The distal colonic weight was significantly increased in mice treated with vehicle plus *C. difficile*. However, treatment with the probiotic (BC30) only slightly normalized the colonic weight. Values (mg/cm colon) on day 16 were: 17.4 ± 0.8 (Vehicle/no *C. difficile*), 26.3 ± 0.7 (Vehicle/*C. difficile*) and 25.4 ± 0.6 (BC30/*C. difficile*). A statistically significant difference ($p < 0.05$) was found between the Vehicle/no *C. difficile and* Vehicle/*C. difficile* treatment groups, but not between the two *C. difficile* treated groups of mice.

Representative histology pictures are shown in Figure 5. *C. difficile* infection caused altered colonic histopathology. Specifically, crypt damage, submucosal edema and the influx of inflammatory cells in the lamina propria and submucosa were evident in these mice (panel B). Overall, BC30 treatment resulted in a modest attenuation of the colonic histological pathology observed in Vehicle/*C. difficile* treated mice (panel C). The overall colonic histology scores on study day 16 were: 2.55 ± 0.39 (Vehicle/No *C. difficile*), 5.19 ± 0.22 (Vehicle/*C. difificile*) and 4.96 ± 0.34 (BC30/*C. difficile*). A statistically significant difference ($p < 0.05$) was found between the Vehicle/no *C. difficile and* Vehicle/*C. difficile* treatment groups, but not between the two *C. difficile* treated groups of mice.

BC30 attenuates colonic NF-kB p65 binding and chemokine content in *C. diffiicle* infected mice

As shown in Figure 6, there were reductions in the colonic nuclear binding of p65 and the colonic MIP-2 content respectively, when mice were treated with BC30. The mean absorbance values for p65 binding were:

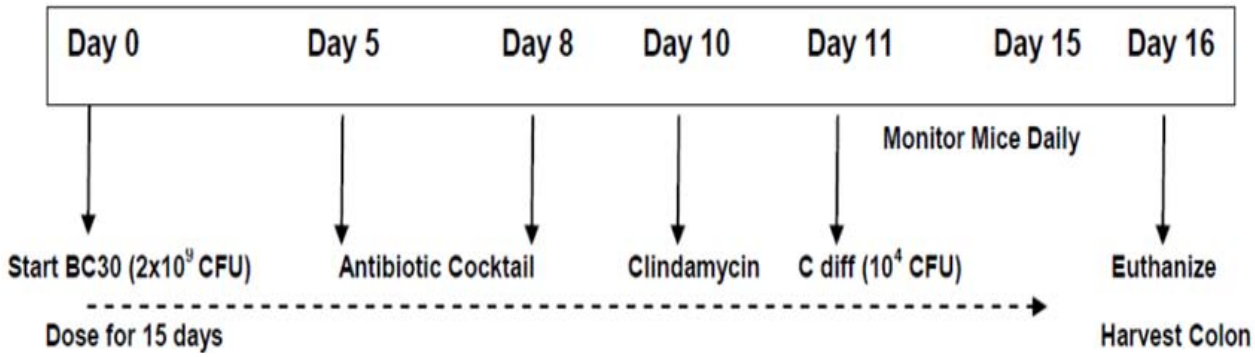

Figure 1 Study Overview. This figure shows an overview of the key events associated with the *Clostridium difficile* induced colitis model that was used for this study. Female C57BL/6 mice were dosed during study days 0 through 15 with 2×10^9 CFU of BC30, or vehicle (0.9% saline). On study days 5 through 8, mice were given an antibiotic coktail in the drinking water as described in the Methods section. On day 10, clindamycin was administered i.p. at a dose of 10 mg/kg. On day 11, mice received either *Clostridium difficile* (designated as C diff) [1×10^4 CFU of VPI 10463], or vehicle, by oro-gastric gavage. Body weight and stool consistency data were collected daily on study days 11 through 16. On study day 16, mice were euthanized and the colons were removed for measuring morphometric, biochemical and histological indices of colitis.

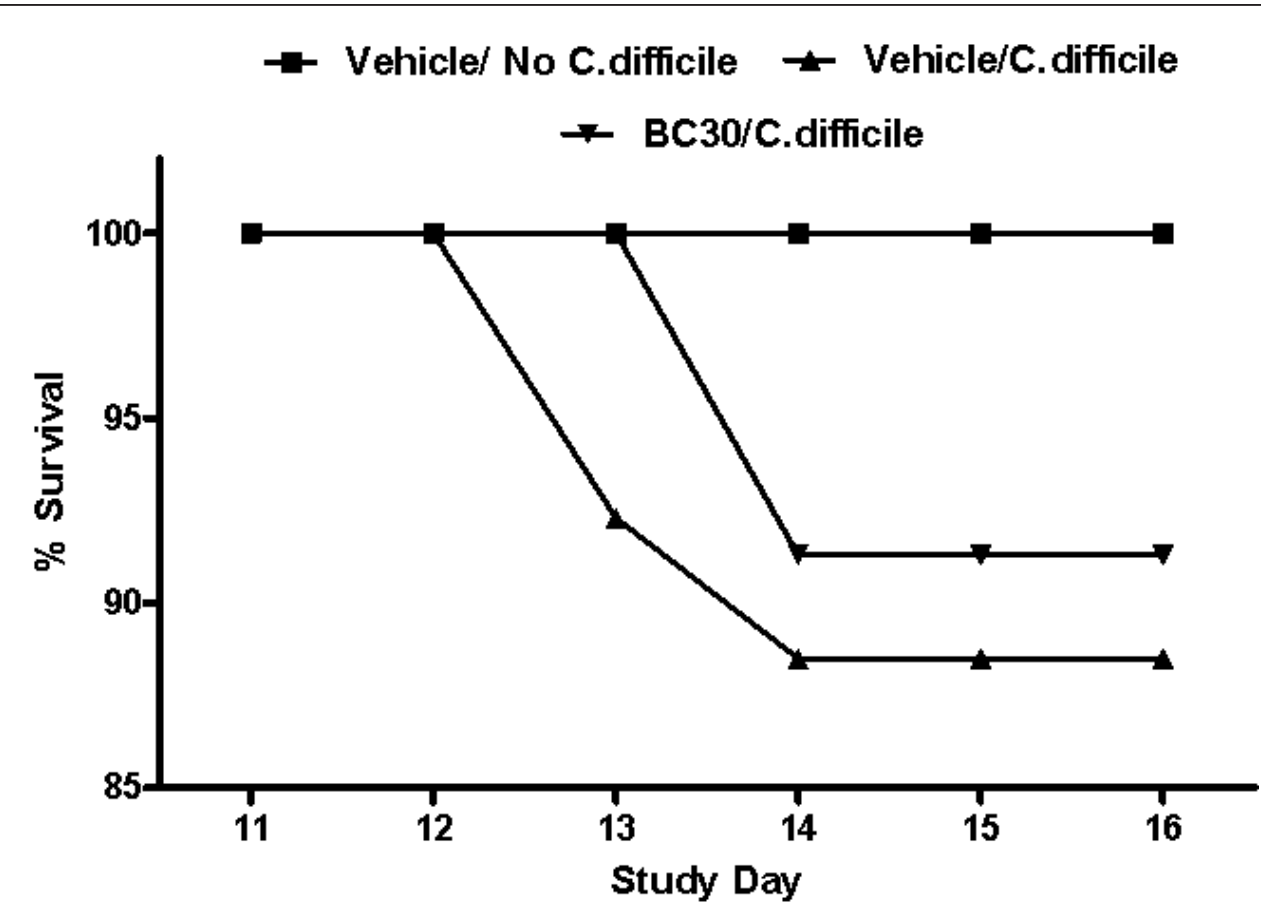

Figure 2 Mouse survival data. All mice (6/6) that did not receive *C. difficile* survived for the duration of the study. As shown, 100% of mice treated with BC30 survived on study day 13, while 2 mice treated with vehicle did not survive on that day (92.3% survival). By day 14, 21/23 mice survived in the BC30/*C. difficile* treatment group, while 23/26 mice survived in the vehicle/*C. difficile* treatment group. Therefore, the cumulative survival rates in the study were: 100% (Vehicle/no *C. difficile*), 88.5% (Vehicle/*C. difficile*) and 91.3% (BC30/*C. difficile*). However, there was not a statistically significant difference in the survival rate between any of the treatment groups.

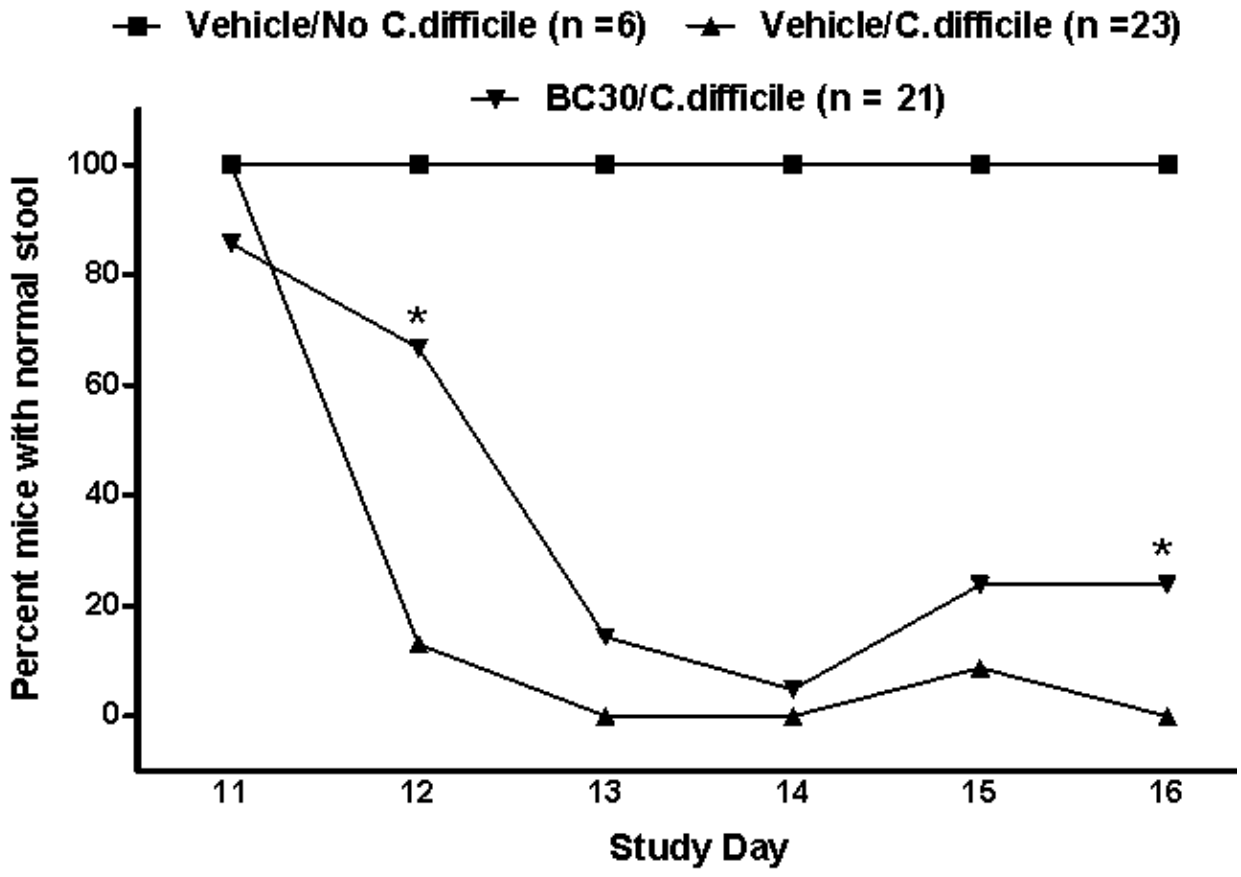

Figure 3 Stool consistency data. All mice that did not receive C. difficile had normal stools throughout the study. In contrast, only 13% of mice in the *Vehicle/C. difficile* treatment group had normal stools on day 12, while 67% of the BC30 treated mice still had normal stools. On days 13 and 14, altered stool consistency was seen in both *C. difficile* treatment groups. However, evidence of a normal stool was seen in 24% of BC30 treated mice on day 16, but no vehicle treated animals (0%) showed a normal stool consistency on that day. Statistical significance (* $p < 0.05$) between the vehicle and BC30 treatment groups was found on study days 12 and 16.

0.022 ± 0.006 (Vehicle/no *C. difficile*), 0.048 ± 0.004 (Vehicle/*C. difficile*) and 0.039 ± 0.004 (BC30/*C. difficile)*. Specifically, as shown in Figure 6A, the nuclear binding of NF-κB p65 was increased 2.2 fold in the colons of Vehicle/*C. difficile* treated mice, but only 1.8 fold in probiotic treated animals. Similarly, the colonic MIP-2 content was significantly increased in vehicle treated mice, but the increase was less dramatic in BC30 treated mice (Figure 6B). On study day 16, the MIP-2 values (pg/2 cm colon) were: 10.2 ± 0.5 (Vehicle/no *C. difficile*), 24.6 ± 9.5 (Vehicle/*C. difficile*) and 16.3 ± 4.3 (BC30/*C. difficile)*. Due to the variability in the colonic MIP-2 values within the Vehicle/*C. difficile* treatment group, a statistically significant reduction in the colonic MIP-2 content was not found with BC30 treatment.

Discussion

Recently, Chen et al. described a murine model of CDAD that could be used for testing the efficacy of applicable pharmacological agents (antibiotics, probiotics) [6]. Infection of female C57BL/6 mice with 10^3 to 10^4 CFU of *C. dififcile* was associated with significant weight loss, diarrhea and mortality [6]. In a similar fashion, we also showed that the infection of vehicle treated mice resulted in transient weight loss, altered stool consistency and some evidence of mortality (11.5%).

In contrast to vehicle treatment, mice treated with the probiotic (BC30) had a delayed onset in mortality (no deaths until day 14), as well as a slightly reduced overall mortality rate (8.7%), when compared to vehicle treated animals (Figure 2). In a similar fashion, mice treated with BC30 had a delayed onset in the appearance of altered stool consistency (Figure 3). Specifically, on day 12, 87% of vehicle treated mice had evidence of loose stools or diarrhea. In contrast, only 33% of BC30 treated animals had evidence of altered stool consistency ($p < 0.05$ vs. vehicle). Moreover, on the final study day (day 16), BC30 treated mice still had a higher incidence of normal stools

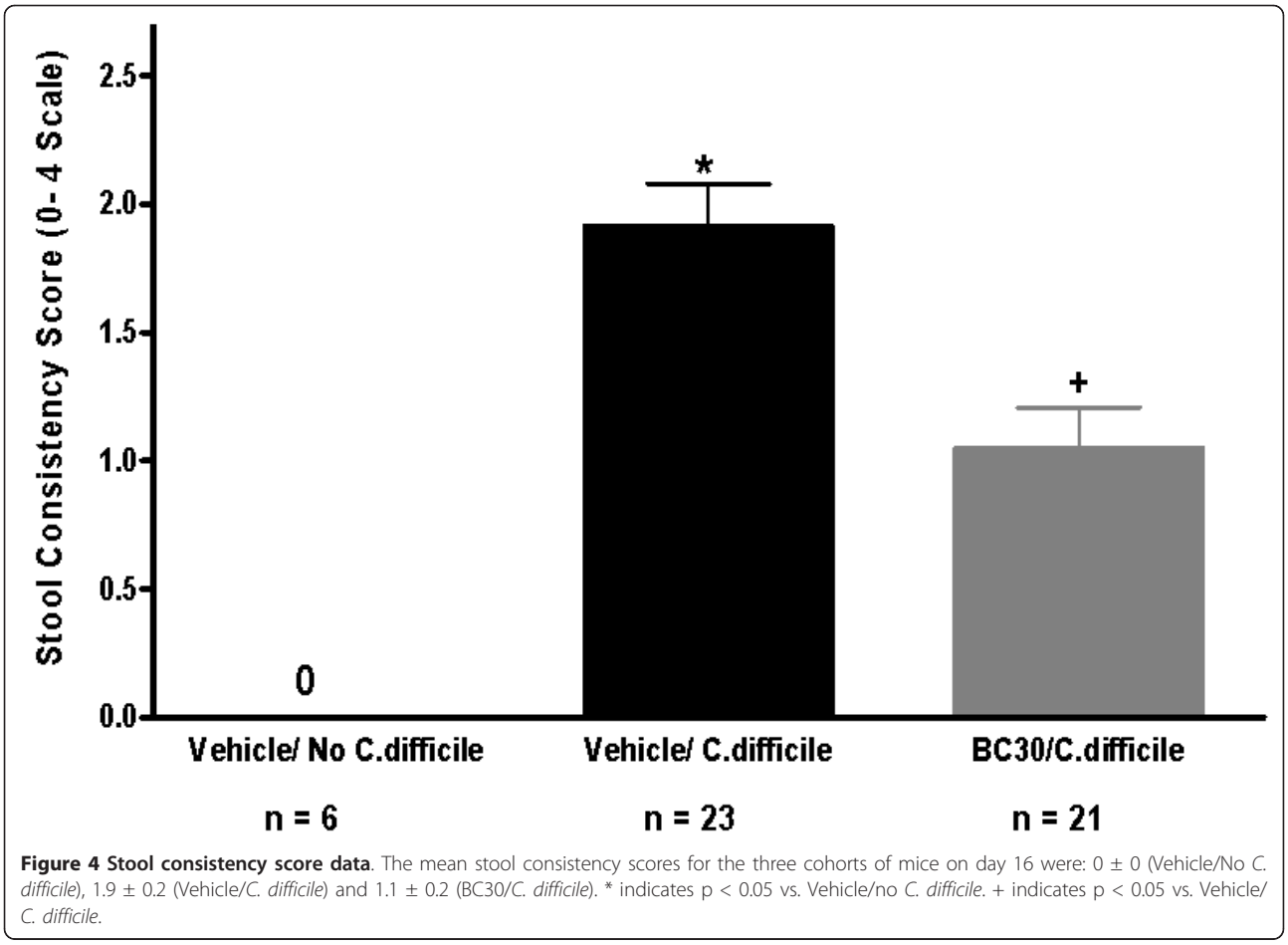

Figure 4 Stool consistency score data. The mean stool consistency scores for the three cohorts of mice on day 16 were: 0 ± 0 (Vehicle/No *C. difficile*), 1.9 ± 0.2 (Vehicle/*C. difficile*) and 1.1 ± 0.2 (BC30/*C. difficile*). * indicates $p < 0.05$ vs. Vehicle/no *C. difficile*. + indicates $p < 0.05$ vs. Vehicle/ *C. difficile*.

(Figure 3), as well as a significantly lower stool consistency score (Figure 4). These results demonstrate evidence of improved stool consistency in *C. difficile* infected mice that were pre-treated with BC30.

Murine CDAD is associated with a specific colonic histopathology that includes crypt damage, submucosal edema and the influx of inflammatory cells [6]. These pathological changes were also evident in our Vehicle/*C. diifficle* treated cohort of mice (panel B, Figure 5). In contrast, mice treated with BC30 showed some evidence of attenuated colonic histopathology, including decreased leukocyte influx into the colon (panel C, Figure 5). However, the overall comparisons of mean colonic histology scores were not statistically different on day 16.

Data from other studies suggests that toxin A secreted by *C. difficile* can activate the NF-κB signal transduction system in monocytes and colonic epithelial cells [4,5]. This activation of NF-κB leads to the secretion of a key pro-inflammatory chemokine (IL-8) and subsequently neutrophil influx into the colonic tissue [4,5]. Interestingly, BC-30 can significantly inhibit the IL-8 directed migration of human neutrophils *in vitro* [19]. Based on these results, we measured the effects of BC30 on the nuclear binding of NF-κB p65, as well the murine chemokine (MIP-2) content in the colons of *C. difficile* infected mice. Probiotic treatment resulted in reductions of both colonic p65, as well as the MIP-2 content (Figure 6). However, statistical significance was not achieved compared to values in vehicle treated mice.

Nevertheless, these effects of BC30 on NF-κB mediated pathological processes (Figure 6) may contribute to the observed improvement in stool consistency observed in the probiotic-treated mice. For example, NF-κB activation is involved in the up-regulation of Fas-ligand, which subsequently leads to colonocyte apoptosis [20]. Colonocyte apoptosis could diminish the barrier function of the colonic mucosa, and contribute to the altered stool consistency associated with CDAD [20]. By reducing the colonic activation of NF-κB (Figure 6), it is possible that BC-30 treatment improved the barrier function of the colonic mucosa (Figure 5C), thereby improving stool consistency (Figures 2 and 3). Possibly, the improvement in stool consistency was unrelated to direct effects on colonization of *C. difficile*, or an alteration in the production of toxins A and B, because all infected mice showed evidence of infection and exotoxin production by ELISA

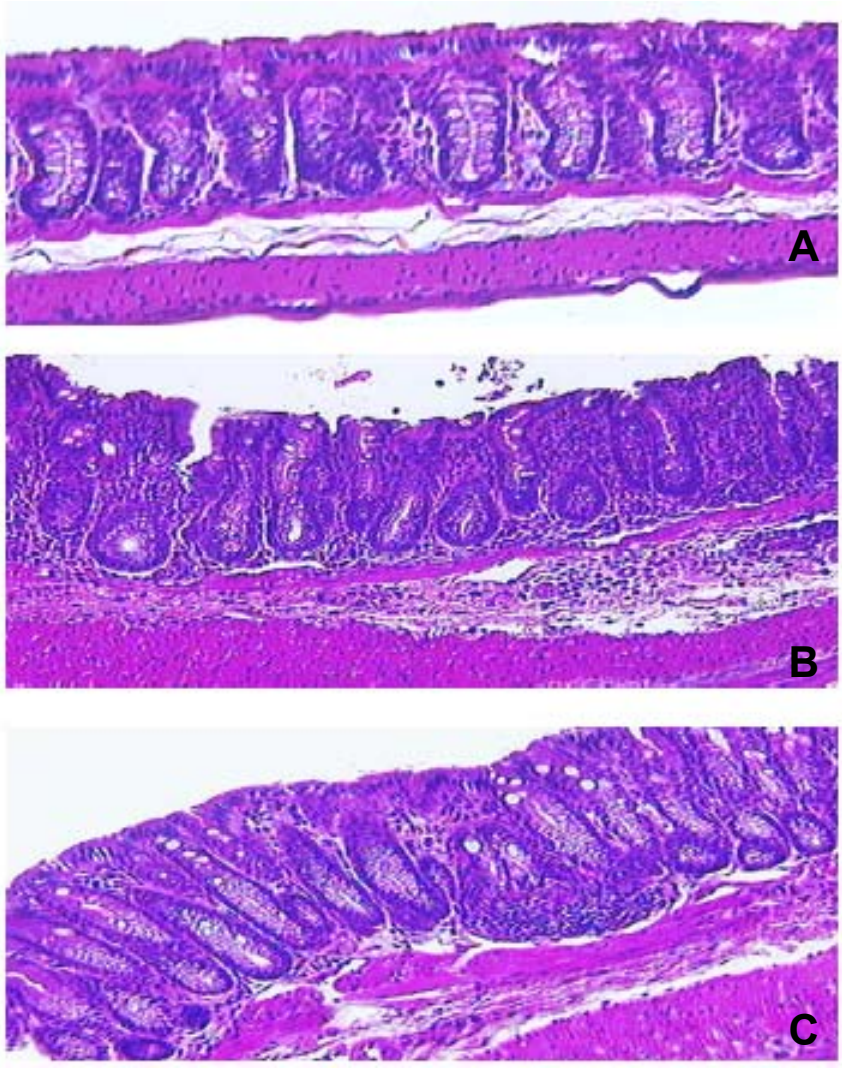

Figure 5 Representative colonic histology pictures (day 16). The pictures are from hematoxylin and eosin (H&E) stained colonic specimens, at a magnification of 200-fold. Panel A shows a normal histological appearance within the colon of a mouse that was not infected with *C. difficile*. Panel B is from the colon of a Vehicle/*C. difficile* treated mouse. There is evidence of crypt damage, submucosal edema and the influx of inflammatory cells in the lamina propria and sub-mucosa. Panel C is from the colon of a BC30/*C. difficile* treated animal. Although, colonic pathology is still present, it is less prominent than in panel B.

(data not shown). Nevertheless, the ELISA kit utilized in this study does not quantify either the numbers of *C. difficile* in the colon, or the actual amounts of toxin production. Therefore, it is also possible that BC30 attenuated the level of *C. difficile* colonization and/or production of toxins in the colon. Future studies are needed to better understand the mechanisms, by which BC30 favorably impact stool consistency, as we observed in this study.

Of importance to this study, it is probable that the use of antibiotics in this murine CDAD model (during study days 5 to 10) resulted in anti-microbial effects that altered the levels of BC30 in the colon (David Keller, personal communication). Therefore, future studies with this murine model of CDAD should focus on effects of BC30 on the recurrence of *C. difficile* following treatment with vancomycin [6]. Using this recurrence paradigm, the unwanted anti-microbial effects of antibiotics will not negatively impact the presence of BC-30 in the mouse colon. Finally, it would also be interesting to test other *Bacillus coagulans* strains in this type of experimental paradigm.

Conclusions

The probiotic BC30 improved some parameters of *C. difficile*-induced colitis in mice. BC30 prolonged the survival of *C. diffiicle* infected mice. Particularly, this probiotic

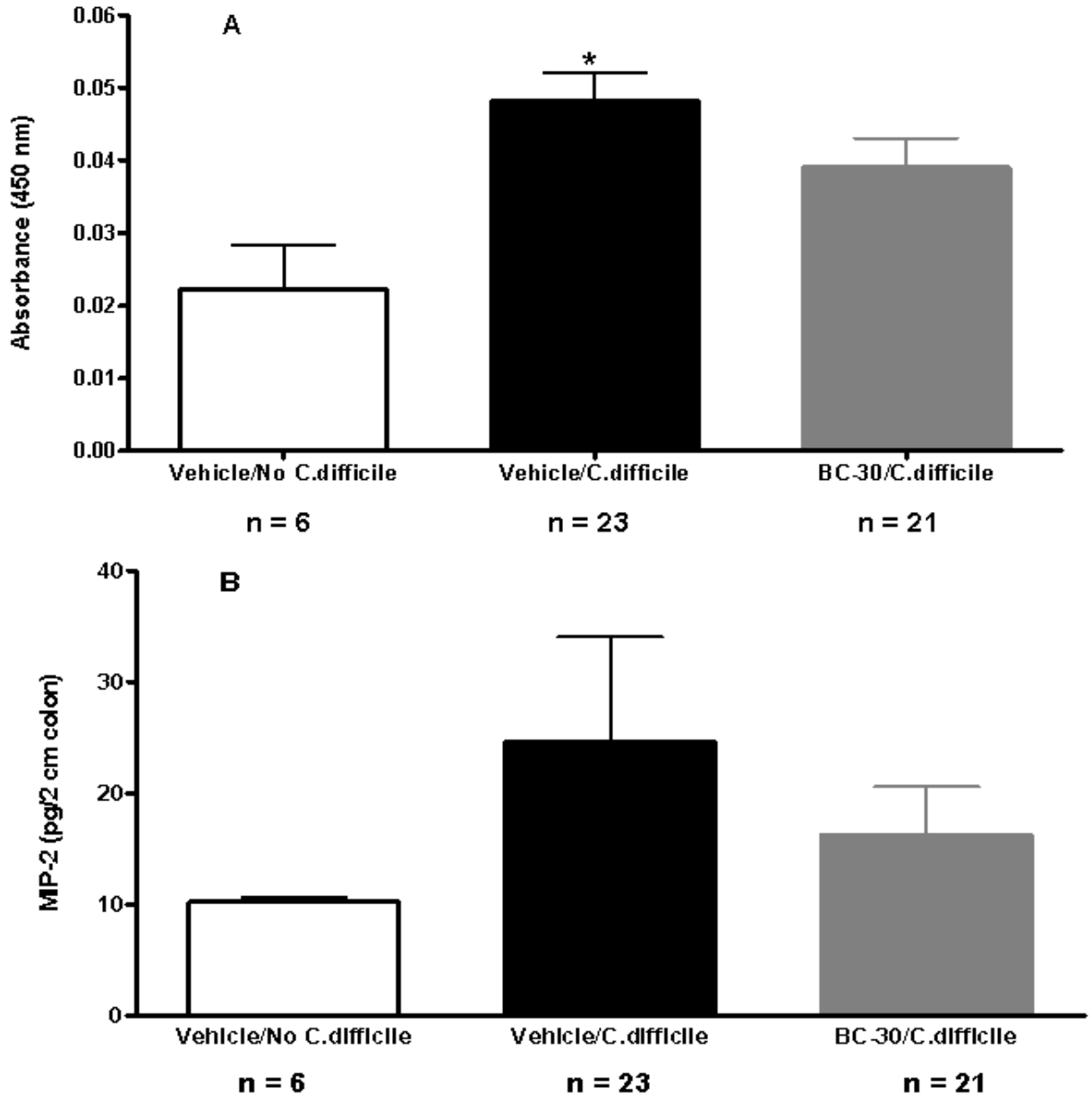

Figure 6 Panel A shows the colonic NF-κB p65 data. The mean absorbance values for p65 binding were: 0.022 ± 0.006 (Vehicle/no *C. difficile*), 0.048 ± 0.004 (Vehicle/*C. difficile*) and 0.039 ± 0.004 (BC30/*C. difficile)*. The * symbol indicates $p < 0.05$ vs. Vehicle/no *C. difficile*. **Panel B** shows the colonic MIP-2 chemokine data. The MIP-2 values (pg/2 cm colon) were: 10.2 ± 0.5 (Vehicle/no *C. difficile*), 24.6 ± 9.5 (Vehicle/*C. difficile*) and 16.3 ± 4.3 (BC30/*C. difficile)*.

improved the stool consistency of mice, in this infectious colitis model. Our results support the concept that probiotics like BC30 may find a niche for the treatment of CDAD.

Methods

Bacillus Coagulans GBI-30, 6086 (BC30)

BC30 was obtained from Ganeden Biotech Inc. (Mayfield Heights, OH).

Murine *Clostridium difficile*-Induced Colitis

We followed the protocol developed by Chen et al., with slight modifications [6]. BC30 (2×10^9 CFU per day), or vehicle (0.9% saline), was dosed by oro-gastric gavage from study day 0 until study day 15. Both body weight and stool consistency data were collected daily on study days 11 through 16. Stool samples from all mice were scored based on the consistency of the fecal sample, as shown here: 0 = normal, 1 = loose stool,

2 = loose/some diarrhea, 3 = diarrhea and 4 = severe watery diarrhea.

On day 16, we confirmed the presence of *Clostridium difficile* and associated toxins (A and B) with a Wampole™ CD quick check complete kit from Inverness Medical (Princeton, NJ). On this study day, mice were euthanized; and the distal colon was collected for evaluating morphometric (colon weight), histological and biochemical parameters. An overview of the study design is shown in Figure 1. This protocol was approved by the Internal Animal Care and Use Committee (IACUC) at the Penn State College of Medicine.

Colonic Histology Evaluation

Using coded slides from the distal colon, four areas from each slide were scored on a three-point severity scale: 0 = Normal, 1 = Mild, 2 = Moderate, 3 = Severe, for three different parameters. These three parameters were epithelial damage, mucosal/submucosal edema and leukocyte infiltration. Therefore, the total score for each slide (i.e., mouse) was between 0 and 9.

Colonic NF-κB p65 Assay

We utilized a TransAM™ NF-κB p65 assay kit from Active Motif (Carlsbad, CA). This assay measures the nuclear binding of p65 to a consensus NF-κB binding site. For the assay, we used 20 μg of protein from colonic nuclear extracts. The results are expressed as the absorbance at 450 nm, as described previously by our laboratory [15].

Colonic MIP-2 Content

MIP-2 (macrophage inflammatory protein-2) is a functional murine homolog of the human chemokine, IL-8. The colonic MIP-2 content was measured with an ELISA kit from R&D systems (Minneapolis, MN). Results are expressed as pg/2 cm colon.

Statistical Analyses

Statistical analyses were done with a GraphPad Prism® software program (San Diego, CA). All data are expressed as the mean ± SEM. Differences in mouse survival, as well as the percentages of mice with normal stools, were determined with the Fisher's exact test. Stool consistency scores and colonic histology scores were evaluated with the Kruskal-Wallace test, followed by Dunn's test to compare individual treatment groups. All other parameters were evaluated by ANOVA, followed by the Newman Keuls test for individual treatment groups. A p value of < 0.05 was considered to be statistically significant for all parameters.

List of Abbreviations

BC30: *Bacillus Coagulans GBI-30*; NF-κB: Nuclear Factor-kappa B; CDAD: *Clostridium difficile*-associated disease; MIP-2: macrophage inflammatory protein-2.

Acknowledgements

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Authors' contributions

LRF contributed to the technical and intellectual aspects of the manuscript. WHG, KDK and DK contributed to the intellectual aspects of the paper. JSS contributed to the technical aspect of the manuscript. All the authors read and approved the manuscript.

Competing interests

None of the authors have any conflict of interest disclosures to make regarding this manuscript, with the exception of Dr. David Keller. Dr. Keller is a paid employee of Ganeden Biotech Inc.

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Bacillus coagulans GBI-30, 6086 limits the recurrence of *Clostridium difficile*-Induced colitis following vancomycin withdrawal in mice

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Abstract

Background: Recently, we found that the probiotic strain *Bacillus coagulans* GBI-30, 6086 (GanedenBC^{30}) improved indices of *Clostridium difficile (C. difficile)*-induced colitis in mice (Fitzpatrick et al., *Gut Pathogens*, 2011). Our goal was to determine if BC30 could also prevent the recurrence of *C. difficile*-induced colitis in mice, following initial treatment with vancomycin. During study days 0 through 5, mice were treated with antibiotics. On day 6, the *C. difficile* strain VPI 10463 was given by oro-gastric gavage at ≈ 5x10^4 CFU to induce colitis. Mice were treated on study days 6 to 10 with vancomycin (50 mg/kg) (vanco) or vehicle (saline) by gavage. On days 10 to16, mice were dosed by gavage with saline vehicle or BC30 (2 x 10^9 CFU per day). Mice were monitored for mortality, weight loss and diarrhea. On study days 14, 16 and 17, stools and colons were collected for analyzing other parameters of colitis.

Results: The mean stool consistency score in Vehicle/C.difficile/Vanco mice increased from 0.4 (day 10) to a range of 1.1 to 1.4 (days 14 to 17), indicating the recurrence of colitis. On days 13 through 17, the stool consistency scores for the vancomycin/BC30 mice were significantly lower ($p < 0.05$) than for the vancomycin/vehicle cohort of animals. On day 17, 88.9% of mice treated with BC30 had normal stools, while this value was 0% with vehicle treatment (p value = 0.0004). Colonic myeloperoxidase (Units/2 cm colon) was significantly ($p < 0.05$) reduced from 4.3 ± 0.7 (Vehicle/C.difficile/Vanco) to 2.6 ± 0.2 (BC30/C. Difficle/Vanco). The colonic histology score and Keratinocyte derived-chemokine level in the colon were also lower in BC30 treated mice.

Summary: In BC30-treated mice, there was evidence of better stool consistency, as well as improved biochemical and histological indices of colitis, following initial treatment of animals with vancomycin.

Conclusion: BC30 limited the recurrence of *CD*-induced colitis following vancomycin withdrawal in mice.

Keywords: *Clostridium difficile*, GanedenBC30, Probiotics, Colitis, Mice

Background

Clostridium difficile (*C. difficile*) infection (CDI) is a very common cause of health-care associated diarrhea and colitis [1]. Moreover, CDI is associated with significant morbidity, as well as increased health care costs [2]. The spectrum of *C. difficile* associated disease (CDAD) ranges from mild antibiotic associated diarrhea to severe and life threatening pseudomembranous colitis [3]. CDAD is caused by the actions of two toxins (toxin A and toxin B), which are produced by pathogenic strains of *C. difficile* [4,5]. Toxin A results in the activation of three transcription factors (NF- kB, AP1 and CREB). NF-kB (nuclear factor-kappa B) is involved in chemokine production, and also plays a role in colonocyte apoptosis [6,7]. AP-1 (activator protein-1) plays a role in IL-8 production in response to stimulation of colonocytes with toxin A [8]. CREB (Cyclic-AMP Response Binding Protein) is critical for the production of prostaglandin E_2 via inducible cyclooxygenase-2 (COX-2) [9]. This prostaglandin plays an important role in the fluid secretion and diarrhea associated with CDAD.

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CDAD is often treated successfully with standard antibiotics such as vancomycin (vanco) or metronidazole [10,11]. However, recurrence occurs in at least 20% of patients [11]. Some clinical studies have focused on combined treatment with vancomycin and probiotics such as *Saccharomyces boulardii* for the treatment of recurrence [12-15]. Therefore, the use of probiotics, for prevention of recurrent disease, may be attractive as part of the overall therapeutic strategy for CDAD [12-15].

Bacillus coagulans GBI-30, 6086 (GanedenBC^{30}) is a spore-forming probiotic strain that is resistant to extreme temperatures and survives in the gut environment [16]. BC30 was shown to have anti-inflammatory and immunomodulatory effects *in vitro* and *in vivo* [17,18]. Previously, we reported that BC30 improved various parameters of C. difficile-induced colitis in mice [18]. Additionally, BC30 prolonged the survival time in C. difficile-infected mice [18]. While the initial research focused on primary treatment of C. diifficile, this study reached the ability to prevent re-occurrences of C. *Difficile* infection following withdrawal of Vancomycin.

Recently, other investigators have described the recurrence of CDAD following vancomycin withdrawal in mice [10,19]. Overall, recurrence is associated with some evidence of disease (weight loss, diarrhea), as well as typical histological evidence of CDAD [10,19]. With knowledge of this previous scientific information, the goal of our study was to determine if BC30 could prevent recurrence of *CD*-induced colitis following vancomycin withdrawal in mice.

Results

Effects of BC30 on mouse survival and body weight, as well as the presence of *C. difficile* infection and toxins

Figure 1 shows an overview of the key events associated with the *C. difficile* recurrence model that we used for this study. Cumulative survival rates in the study were: 100% (Vehicle/No *C. difficile*), 87.5% (Vehicle/*C. difficile*/No Vanco), 100% (Vehicle/*C. difficile*/Vanco) and 100% (BC30/*C. difficile*/Vanco). No statistically significant differences were found for mouse survival.

The incidence rates of C. difficile infection from study days 14, 16 and 17 were: 0% (0/6, Vehicle/No *C. difficile*), 100% (7/7, Vehicle/*C. difficile*/No Vanco), 97% (28/29, Vehicle/*C. difficile*/Vanco) and 89% (25/28, BC30/*C. difficile*/Vanco) [Figure 2A]. The percentages of toxin A/B positive stools from these study days were: 0% (Vehicle/No *C. difficile*), 57% (Vehicle/*C. difficile*/No Vanco), 41% (Vehicle/*C. difficile*/Vanco) and 64% (BC30/*C. difficile*/Vanco). However, the semi-quantitative determination of toxin A/B levels (n = 6–13 per treatment group) showed increased absorbance readings (1.429 ± 0.456) from the stools of *Vehicle/C. difficile*/Vanco treated mice, as compared to absorbance readings (1.128 ± 0.410) from stools of BC30/*C. difficile*/Vanco treated animals [Figure 2B].

The mean body weights (grams) of mice on study day 6 were: 20.7 ± 0.5 (Vehicle/No *C. difficile*), 21.7 ± 0.6 (Vehicle/*C. difficile*/No Vanco), 21.8 ± 0.3 (Vehicle/*C. difficile*/Vanco) and 21.9 ± 0.3 (BC30/*C. difficile*/Vanco). Of note, surviving Vehicle/*C. difficile*/No Vanco treated mice did transiently lose an average of 1.1 grams between study days 7 and 9. On study day 17, the mean body weights (grams) of remaining mice (n = 2 to 9 per treatment group) were: 20.5 ± 0.5 (Vehicle/No *C. difficile*), 21.5 ± 0.7 (Vehicle/*C. difficile*/No Vanco), 22.4 ± 0.6 (Vehicle/*C. difficile*/Vanco) and 22.1 ± 0.5 (BC30/*C. difficile*/Vanco). There were no statistically significant differences in net body weight gains during the study (days 6 to 17).

BC30 treatment significantly improved the stool consistency in C. difficile infected mice

Figure 3 illustrates the effects of BC30 treatment on stool consistencies in *C.difficile* treated mice. The mean stool consistency score in Vehicle/*C. difficile*/Vanco treated mice

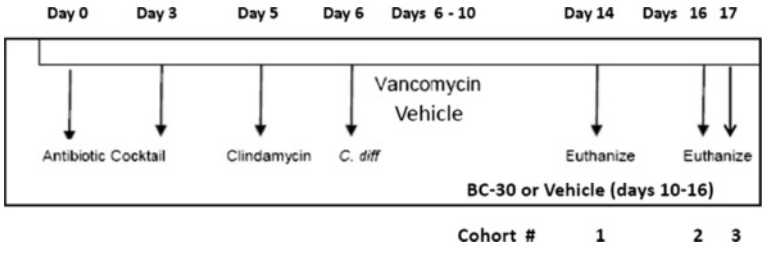

Figure 1 Study overview. The key events associated with the *Clostridium difficile* induced colitis mouse model are shown. On study days 0 through 3, C57BL/6 mice received an antibiotic mixture of kanamycin, gentamicin, colistin, metronidazole and Vanco in the drinking water, followed by clindamycin (10 mg/kg, i.p., on day 5). On day 6, the C. *difficile* strain VPI 10463 was given by oro-gastric gavage at ≈ $5x10^4$ CFU to induce colitis. Mice were treated on study days 6 to 10 with Vanco (50 mg/kg) or vehicle (saline) by gavage. On days 10 to 16, mice were dosed by gavage with vehicle (50% maltodextrin/saline, n=29) or BC30 (2 x 10^9 CFU per day, n=28). One negative control group of mice (n=6) was dosed with vehicle, but did not receive *C. difficile*, while a positive control group (initial n of 8) received *C. difficile* but not Vanco. Mice were monitored daily (days 6 to 17) for mortality, weight loss and stool consistency. On study days 14, 16 and 17, stools and colons were collected for further analyses.

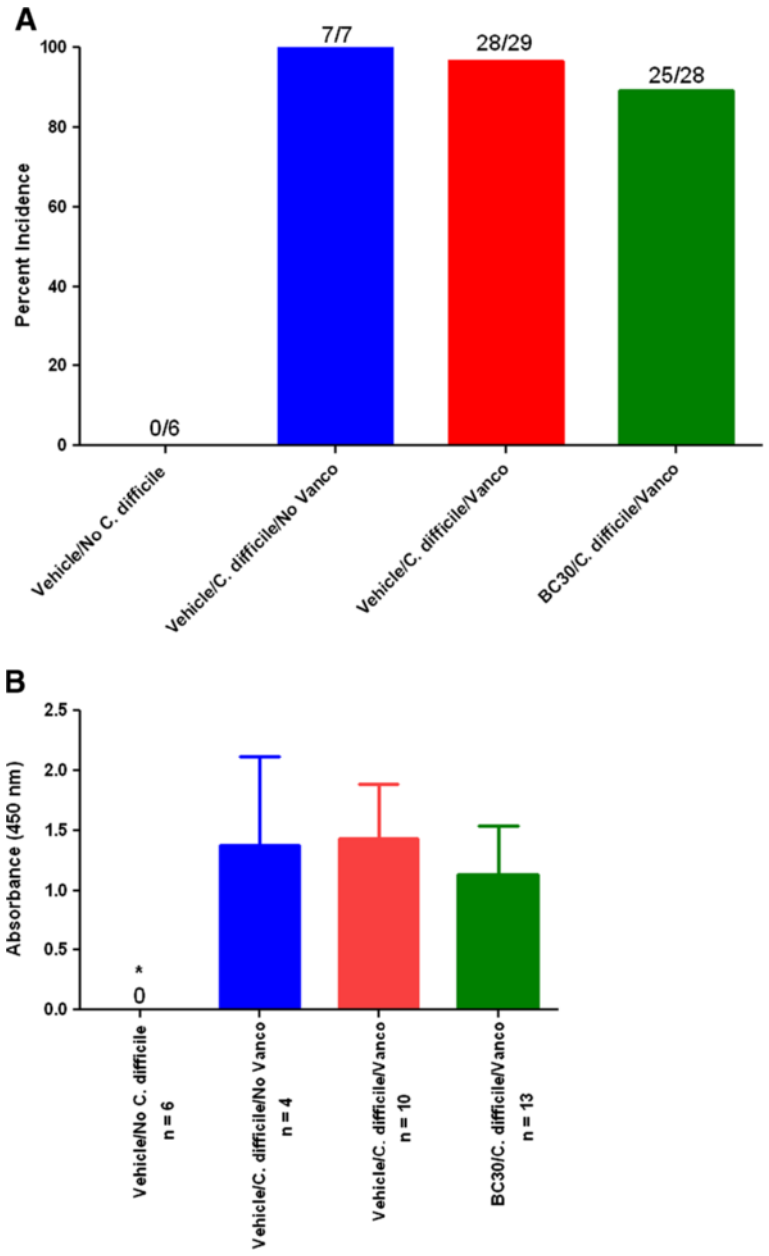

Figure 2 Infection and toxin data. A) The percentages of animals positive for *C. difficile* in the stool were determined by ELISA on study day 14, 16 and 17. * indicates p< 0.05 vs. all other *C. difficile* infection groups. **B**) Toxin A/B levels were determined in a semi-quantitative fashion with an appropriate ELISA kit, as described in the *Methods* section. The values in the graph represent absorbance readings at 450 nm. * indicates p< 0.05 vs. all other *C. difficile* infection groups.

(red symbols and lines) increased from 0.4 (day 10) to a range of 1.1 to 1.4 for days 14 to 17. This increase in stool consistency score indicates the recurrence of colitis. In contrast, during this time period, there was virtually no increase in the mean stool consistency score of BC30/*C. difficile*/Vanco treated mice (green symbols and lines). The stool consistency scores were significantly lower in this cohort of animals ($p < 0.05$ vs. Vehicle/*C. difficile*/Vanco treatment) on study days 13 through 17 [Figure 3A].

In Figure 3B, a significant difference ($p<0.05$) in the percentage of mice with normal stools was evident in the BC30/*C. difficile*/Vanco group, as compared to the Vehicle/*C. difficile*/Vanco group, on days 14 to 17. On day 17, 88.9% of mice treated with BC30 had normal stools compared to 0% of mice with normal stools in the Vehicle treated animals ($p=0.0004$ vs. Vehicle).

Stool sizes (lengths, with higher numbers indicative or more normal stools) in mm (n = 2 to 18 per group)

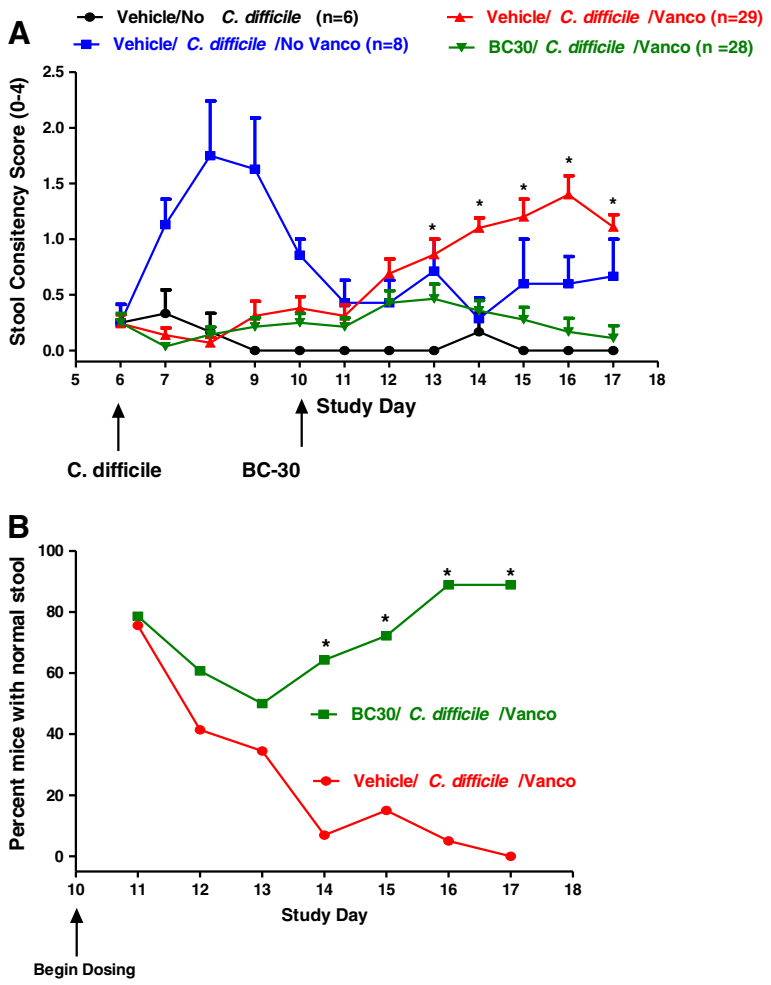

Figure 3 Stool consistency data. A) Mice were randomized on study day 6 to one of four treatment groups. All mice in the negative control group (n=6) that did not receive *C. difficile* (black symbols, lines) generally had normal stools throughout the study. Stool consistency scores were higher in the group of mice (blue symbols, bars) that were treated with Vehicle/*C. difficile*/No Vanco. In these animals, disease was prominently present on days 7 to 9. For the other two experimental groups, mice received Vehicle/*C. difficile*/Vanco and either Vehicle (red symbols, lines) to induce disease recurrence (study days 11 to 17), or BC30 at a dose of 2 x 10^9 CFU per day (green symbols, lines). * indicates $p < 0.05$ vs. BC30/*C. difficile*/Vanco treatment group on study days 13 through 17. **B**) The percentages of mice with normal stools in the Vehicle/*C. difficile*/Vanco (red symbols, lines) and BC30/*C. difficile*/Vanco (green symbols/lines) treatment groups is shown in this panel. Data are shown for study days 10 through 17. On days 14 through 17, significant differences (* $p < 0.05$) were found in the percentages of mice with normal stools in the Vehicle/BC30/Vanco group as compared to the Vehicle/C. difficile/Vanco group. On day 17, 88.9% of mice treated with BC30 had normal stools while this value was 0% with vehicle treatment.

were: 6.9 ± 0.6 (Vehicle/No *C. difficile*), 5.7 ± 0.6 (Vehicle/*C. difficile*/No Vanco), 5.9 ± 0.6 (Vehicle/*C. difficile*/ Vanco) and 7.4 ± 0.4 (BC30/*C. difficile*/Vanco). However, there were no statistically significant differences in stool sizes between treatment groups.

BC30 treatment improved biochemical and histological indices of recurrent CDAD in mice

Colonic myeloperoxidase (MPO) was measured with colonic samples from study days 14, 16, and 17. As shown in Figure 4, MPO (Units/2 cm colon) was significantly reduced ($p < 0.05$) from 4.3 +/−0.7 (Vehicle/*C. difficile*/Vanco treatment) to 2.6 +/−0.2 (BC30/*C. difficile*/Vanco treatment).

Representative colonic histology pictures are shown in Figure 5. *C. difficile* infection, without subsequent Vanco administration, caused altered colonic histopathology. Specifically, some crypt damage as well as modest submucosal edema and moderate influx of inflammatory cells into the lamina propria and sub-mucosa were evident in the colon of this mouse (panel B). In a somewhat similar fashion, Vehicle/*C. difficile*/Vanco treated mice had clear evidence of histological pathology, including significant sub-mucosal edema (panel C). Overall, BC30 treatment (panel D) resulted in a significant improvement of the

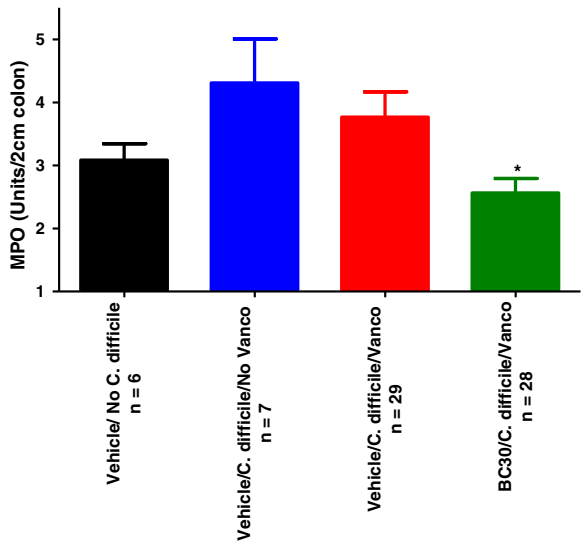

Figure 4 Colonic myeloperoxidase. Measurements of colonic myeloperoxidase (MPO) levels for all mice are shown as Units per 2 cm of colon. Colonic MPO was significantly ($p < 0.05$) reduced from 4.3 ± 0.7 (Vehicle/Vanco, red bar) to 2.6 ± 0.2 (BC30/Vanco, green bar).

altered colonic histological pathology, which was observed in the Vehicle/*C. difficile* cohort of animals (panel C). The mean colonic histology scores were: 3.12 ± 0.35 (Vehicle/No *C. difficile*), 4.49 ± 0.32 (Vehicle/*C. difificile*/*No Vanco*), 5.19 ± 0.15 (Vehicle/*C. difificile*/*Vanco*) and 4.29 ± 0.20 (BC30/*C. difficile*/Vanco). Of note, there was a significant reduction ($p< 0.05$) in the mean histology score of BC30/*C. difficile*/Vanco treated mice, as compared to Vehicle/*C. difificile*/Vanco treated animals (Figure 5E).

The KC (keratinocyte derived chemokine) results (pg/2 cm colon) for all cohorts of mice were: 18.6 ± 1.2 (Vehicle/No *C. difficile*), 26.1 ± 4.3 (Vehicle/*C. difficile*/*No Vanco*), 20.8 ± 2.8 (Vehicle/*C. difificile*/*Vanco*) and 18.6 ± 1.9 (BC30/*C. difficile*/Vanco). Generally, colonic KC levels were higher in both *C. difficile*/No Vanco and *C. difficile*/Vanco treated mice. In contrast, the BC30/*C. difficile*/Vanco treatment group had a colonic KC content that was equivalent to mice that were not infected with *C. difficile*. However, there were no statistically significant differences between any of the treatment groups.

Some representative colonic COX-2 immunohistochemistry pictures are shown in Figure 6. Interestingly, immunostaining for COX-2 was evident primarily in the colonic epithelial cells from a mouse that was not infected with *C. difficile* (panel A). In the colon of a Vehicle/*C. difficile*/Vanco treated animal there was prominent brown COX-2 staining in colonocytes, as well as infiltrating leukocytes within the lamina propria and submucosa (panel B). Of note, only minimal COX-2 immuno-staining (i.e., primarily in surface colonic epithelial cells) was present within the colon of a BC30/*C. difficile*/Vanco treated mouse (panel C).

Discussion

Other investigators have described the recurrence of CDAD following Vanco withdrawal in mice [10,19]. Chen et al. reported severe recurrent CDAD in mice following the removal of Vanco. CDAD was associated with severe diarrhea, prominent body weight loss, marked histological pathology, and a 58% mortality rate [10]. In contrast, Sun and colleagues found only mild diarrhea, transient body weight loss, and no evidence of mortality following Vanco withdrawal in mice. It should be mentioned that different strains of C. difficile (VPI10463 or UK 101) were used in the two studies, as well as somewhat different Vanco treatment regimens [10,11]. Despite the fact that we used the same strain of *C. difficile* (VPI10463) as Chen and colleagues, our mortality and stool consistency results (Figure 3) are more similar to those reported by Sun et al. [11]. Differences in these study results may also be related to alterations in endogenous bacterial flora populations within the colonies of mice. Certain types of bacteria that predominate in the colon (e.g., numbers of Firmicutes and Proteobacteria) have recently been shown by other investigators to critically influence the severity of *C. difficile* induced colitis in mice [20].

Interestingly, our results suggested that treatment of mice with BC30 slightly lowered the overall C. *difficile* infection rate (Figure 2A), as well as the measured levels of associated toxins in the stool (Figure 2B). However, statistically significant differences were not found compared to the corresponding cohort of vehicle treated animals. These results suggest the possibility that BC30 probiotic treatment may have lowered the actual numbers of *C. difficile* in the colonic lumen and/or mucosa. However, more detailed follow-up studies would be needed to critically test this possibility.

Previously, we found that pre-treatment of mice with B30 improved the stool consistency during the primary phase of *C. difficile* infection [18]. In a similar fashion, our results show that BC30 treatment significantly improved both the stool consistency scores and percentage of mice with normal stools (Figure 3) during the recurrence phase (days 11–17) following Vanco withdrawal in mice. Of note, mice treated with BC30 tended to have longer and firmer stools (increased stool size) than Vehicle/*C. difficile* treated mice. These results re-affirm the positive effects of this probiotic on stool consistency (Figure 3).

Other laboratories have found that toxin A secreted by *C. difficile* can activate the NF-κB and AP-1 signal transduction system in monocytes and colonic epithelial cells [6,8,21]. This process leads to secretion of a key pro-inflammatory chemokine (IL-8) and subsequent neutrophil influx into the colonic tissue [6,8,21]. Interestingly, BC-30 can significantly inhibit IL-8 directed migration

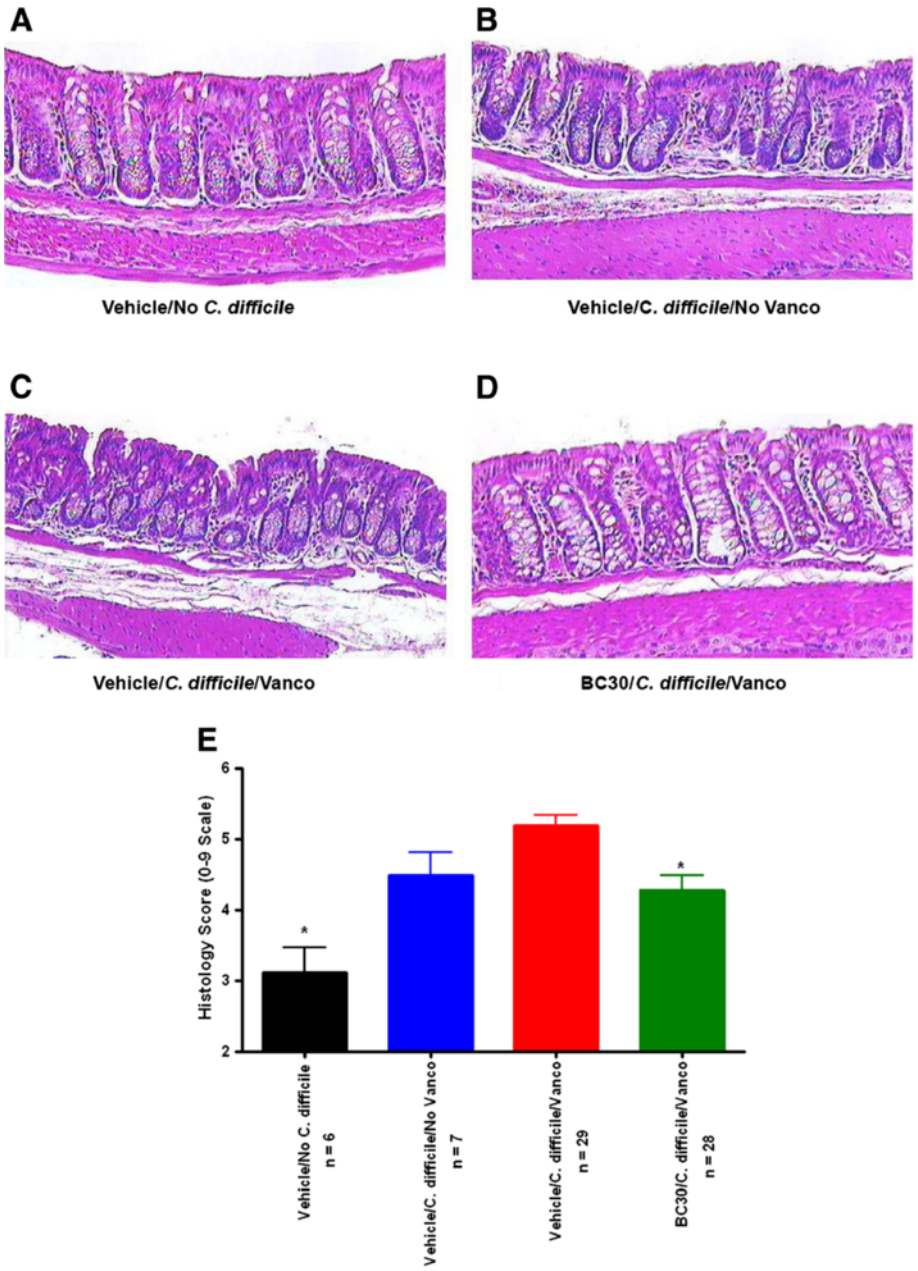

Figure 5 Colonic histology. Representative histology pictures from hematoxylin and eosin (H&E) stained colonic specimens are shown at a magnification of 200-fold. **A**) A relatively normal histological appearance is evident in the colon from a mouse not infected with *C. difficile*. **B**) Evidence of crypt damage, submucosal edema and the influx of inflammatory cells in the lamina propria and sub-mucosa is present in the colon of an animal infected with C. difficile but not treated with Vanco. **C**) In the colon of a mouse given *C. difficile* plus Vanco, there is evidence of crypt disruption, leukocyte influx and prominent sub-mucosal edema. **D**) Mild pathology is observed in the colon of a BC30 treated mouse that was also given *C. difficile* plus Vanco. Modest leukocyte influx is present in the lamina propria, as well as limited sub-mucosal edema, when compared to the vehicle control (compare panels **C** and **D**). **E**) This panel shows a summary of the colonic histology score data. * $p < 0.05$ vs. Vehicle/*C. difficile*/Vanco treatment group (compare red and green bars in the graph).

of human neutrophils *in vitro* [17]. Based on these results, we measured the effects of BC30 on colonic MPO, as well the murine chemokine (KC) content in the colons of *C. difficile* infected mice. Probiotic treatment resulted in a significant reduction in colonic MPO (Figure 4), as well as a diminution in the KC content. However, statistical significance was not achieved for reducing this chemokine, as compared to values in vehicle treated mice. Nevertheless, these positive effects of BC30 on parameters associated with neutrophil influx

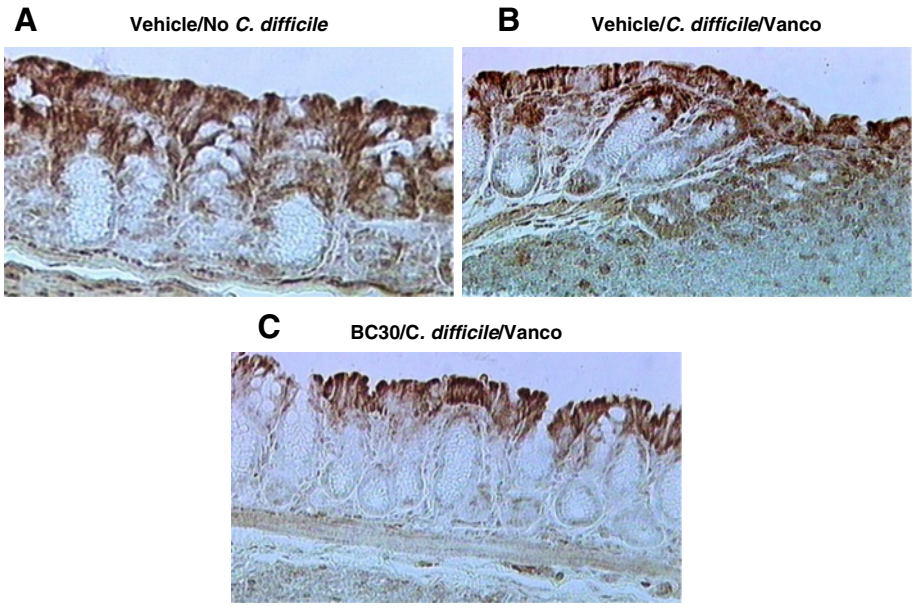

Figure 6 Colonic COX-2 immunohistochemistry. COX-2 immunohistochemistry was performed on representative histology slides from colonic samples of three treatment groups (panels **A**, **B** and **C**). As shown in **panel A,** Immuno-staining for COX-2 was evident primarily in the colonic epithelial cells from a mouse that was not infected with *C. difficile*. In the colon of a Vehicle/*C.difficile*/Vanco treated animal there was prominent brown COX-2 staining in colonocytes, as well as infiltrating leukocytes within the lamina propria and submucosa (panel **B**). Only minimal COX-2 immuno-staining (i.e., primarily in surface colonic epithelial cells) was present within the colon of a BC30/*C. difficile*/Vanco treated mouse (panel **C**).

into the colon may also contribute to the observed improvement in stool consistency observed in the probiotic-treated mice.

Murine CDAD is associated with a specific colonic histopathology that includes crypt damage, submucosal edema and influx of inflammatory cells [10]. These pathological changes were also evident during the recurrence phase in our Vehicle/*C. difficile*/Vanco treated mice (panel C, Figure 5). Interestingly, histological pathology also persisted to some degree in the Vehicle/C. difficile/ No Vanco cohort of mice (panel B, Figure 5), even at 8 to 11 days after the initial infection with *C. difficile*. In contrast, mice treated with BC30 showed evidence of improved colonic histopathology, including decreased leukocyte influx into the colon and diminished sub-mucosal edema (panel D, Figure 5). Importantly, the comparisons of mean colonic histology scores showed a statistically significant reduction in B30 treated mice compared to the corresponding vehicle cohort of animals (Figure 5E).

Other investigators have found evidence of *in vitro* and *in vivo* COX-2 induction in colonocytes or macrophages following exposure to *C. difficile* derived toxin A [9,22]. Moreover, inducible COX-2 may contribute through prostaglandin formation to the alteration in stool consistency that is a prominent feature of CDAD [10,18]. Therefore, it is interesting that colonic COX-2 immunostaining was dramatically diminished in the colon of BC30 treated mice (Figure 6). It is possible that this probiotic may affect the CREB-COX-2-PGE2 pathway, which promotes fluid secretion and contributes to CDAD in mice [9,10,18]. Future studies could focus on more critically evaluating the effects of BC30, as well as other *Bacillus coagulans* probiotic strains, on this important pathway of CDAD.

Conclusions

BC30 limited the recurrence of *CD*-induced colitis following vancomycin withdrawal in mice. Specifically, this probiotic significantly improved stool consistency of mice in this recurrence model of CDAD. BC30 also significantly attenuated histological and biochemical indices (MPO) of infectious colitis.

Methods

Bacillus coagulans GBI-30, 6086 (GanedenBC^{30})

BC30 and maltodextrin were obtained from Ganeden Biotech Inc. (Mayfield Heights, OH).

Clostridium difficile (VPI 10463)

VPI 10463 was obtained from Dr. Efi Kokkotu, (Beth Israel Deaconess Medical Center, Boston, MA) and ATCC (Manassas, VA).

Mice

Male C57 Bl/6 mice (≈ 9 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were

acclimated in our research facility for approximately 3 to 4 weeks, before use in experimental studies.

Murine *Clostridium difficile*-Induced colitis

The protocol for *Clostridium difficile* recurrence developed by Chen et al. was followed with slight modifications [10]. Briefly, an antibiotic cocktail (kanamycin (0.4 mg/mL), gentamicin (0.035 mg/mL),colistin (850 U/ml), metronidazole (0.215 mg/mL), and vancomycin (0.045 mg/mL).was given in the drinking water to mice on study days 0 to 3. Subsequently, clindamycin (10 mg/kg) was administered to mice by a single i.p. injection. On study day 6, mice were randomized to receive VPI 10463 ($\approx$ 5 x 10^4 CFU) by oro-gastric gavage. A negative disease control group of animals was administered vehicle (0.9% saline). Subsequently, on day 6, mice received either vancomycin (50 mg/kg) or 0.9% saline (vehicle) by oro-gastric gavage, until day 10. On study day 10, animals were randomized to receive either BC30 (2 x 10^9 CFU per day), or vehicle (50% maltodextrin in 0.9% saline), which were dosed by oro-gastric gavage until study day 16. Both body weight and stool consistency data were collected daily on study days 10 through 17. Stool samples from all mice were scored based on the consistency of the fecal sample, as shown here: 0 = normal, 1 = loose stool, 2 = loose/some diarrhea, 3 = diarrhea and 4 = severe watery diarrhea [18].

Based on preliminary time course studies, mice were euthanized on days 14, 16, or 17 (i.e., cohorts 1, 2 or 3) in Figure 1. On these study days, we confirmed the presence of *Clostridium difficile* and associated toxins (A and B) in stools with a Wampole^{TM} CD quick check complete kit from TECHLAB (Blacksburg, VA). Furthermore, the amount of toxins A and B in available stool samples was determined in a semi-quantitative fashion by use of a *C. DIFFICILE TOX A/B II*^{TM} ELISA KIT from TECHLAB (Blacksburg, VA). Also, in some mice, stool size (length in mm) was determined with electronic callipers from available specimens.

On these same study days (days 14, 16 or 17), mice were euthanized; and the distal colon was collected for evaluating morphometric (colon weight), histological and biochemical parameters. An overview of the study design is shown in Figure 1. This study was repeated twice and results were combined in the final data analyses. Since no significant differences in measured parameters of CDAD were found on study days 14, 16, and 17, these data were combined for data analyses. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the Penn State College of Medicine.

Colonic histology evaluation

Using coded slides from the distal colon, four areas from each slide were scored on a three-point severity scale: 0 = Normal, 1 = Mild, 2 = Moderate, 3 = Severe, for three different parameters. These three parameters were epithelial damage, mucosal/submucosal edema and leukocyte infiltration. Therefore, the total score for each slide (i.e., mouse) was between 0 and 9 [18]. Histology photographs (H&E staining) were captured at 200x magnification using an Olympus IMT-2 microscope (Olympus Corporation, Lake Success, NY) and EPIX-XCAP® image capture software (Buffalo Grove, IL).

Colonic MPO

Colonic myeloperoxidase (MPO) was utilized as an indicator of neutrophil influx into the mouse colon, as described previously by our laboratory [18]. Results were expressed as Units/2 cm colon.

Colonic KC (CXCL1) chemokine content

KC (keratinocyte derived chemokine) is a functionally relevant murine chemokine [7]. The colonic KC content was measured with an ELISA kit from R&D systems (Minneapolis, MN). Results are expressed as pg/2 cm colon.

COX-2 Immunohistochemistry: Mouse colon

Generally, we followed the procedures for immunohistochemistry with colonic tissue samples, which have been described previously by our laboratory [23]. For the cyclooxygenase-2 (COX-2) primary antibody, we used a 200-fold dilution, as suggested by the manufacturer (Cell Signaling, Danvers, MA). Representative, COX-2 immunohistochemistry photographs from mouse colons were captured at a 300x magnification, using the aforementioned Olympus IMT-2 microscope and EPIX-XCAP® image capture software program.

Statistical analyses

All statistical analyses were performed with a GraphPad Prism® (San Diego, CA). Differences in the percentages of mice with normal stools, as well as percentages of mice with *C. difficile* infection were determined with the Fisher's exact test. Stool consistency scores were evaluated by the Mann Whitney test. Biochemical and histological data were evaluated using unpaired t test analyses. A p value of < 0.05 was considered to be statistically significant for all parameters.

Ethical statement

This study, which utilized mice, was approved by the IACUC at the Penn State College of Medicine. The corresponding author was involved in the intellectual aspects of the study. GanedenBC^{30} is a patented strain of Ganeden Biotech Inc. All requests to use GanedenBC^{30} for further research should be made directly to the company and are evaluated on an individual basis.

Abbreviations
BC30: *Bacillus coagulans* GBI-30, 6086; KC: Keratinocyte derived chemokine; CDAD: *Clostridium difficile*-associated disease; CDI: Clostridium *difficile* infection.

Competing interests
None of the authors have any conflict of interest disclosures to make regarding this manuscript, with the exception of Sean Farmer and Dr. David Keller. Sean Farmer and Dr. Keller are paid employee of Ganeden Biotech Inc.

Authors' contributions
LRF contributed to the technical and intellectual aspects of the manuscript. WHG, KDK, SF and DK contributed to the intellectual aspects of the paper. JSS contributed to the technical aspect of the manuscript. All the authors read and approved the manuscript.

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IMPACT OF GANEDENBC^{30} (*BACILLUS COAGULANS* GBI-30, 6086) ON POPULATION DYNAMICS OF THE HUMAN GUT MICROBIOTA IN A CONTINUOUS CULTURE FERMENTATION SYSTEM

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ABSTRACT: *Interest in Bacillus probiotics, for use in humans and other animals, is increasing. GanedenBC^{30} (Bacillus coagulans GBI-30, 6086) is a commercially available probiotic that is considered safe for human use. Few data are available in relation to the impact of Bacillus coagulans supplementation on the gut microbiota of animals, with none available for humans. In vitro single-stage continuous culture fermentation experiments were performed to examine the effect of GanedenBC^{30} supplementation on the human gut microbiota and to determine persistence of the probiotic strain upon cessation of supplementation. Fluorescence in situ hybridization and denaturing gradient gel electrophoresis were employed to monitor bacterial populations within the fermentation systems, while cultivation work was performed to examine the aerobic spore-formers in samples. GanedenBC^{30} supplementation had no major effect on the microbiota in vitro, although post-treatment clustering was seen for DGGE profiles. Both cultivation and fluorescence in situ hybridization analysis (with probe Bcoa191) demonstrated persistence of GanedenBC^{30} in vitro post-treatment and indicated germination of spores in vitro. GanedenBC^{30} supplementation did not cause dysbiosis of the human gut microbiota in vitro, indicating GanedenBC^{30} is microbiologically safe for probiotic application in humans. Future work should examine the potential probiotic function(s) of GanedenBC^{30}, including its immunomodulatory and antimicrobial effects.*

KEY WORDS: *Bacillus coagulans*, Persistence, Probiotic, Spore-forming bacteria

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Abbreviations Used: DGGE, denaturing gradient gel electrophoresis; FISH, fluorescence *in situ* hybridization; SCFA, short-chain fatty acid.

INTRODUCTION

Probiotic bacilli, unlike the more traditional probiotics bifidobacteria and lactobacilli, can be produced aerobically as freeze-dried mono- or multiculture preparations that can be stored at room temperature with a long shelf life. They are administered orally in spore form. Although not as widely used as *Bifidobacterium* and *Lactobacillus* probiotics, different strains of probiotic *Bacillus* have been shown to be effective in the treatment of antibiotic-associated diarrhoea (Doron *et al.*, 2008), to have lipid-lowering effects (Paik *et al.*, 2005), to have immunomodulatory effects in allergic rhinitis patients (Ciprandi *et al.*, 2005a, b) and to reduce the duration of respiratory infections in children (Marseglia *et al.*, 2007). The ingestion of *Bacillus* strains rich in carotenoids has been suggested as a useful source of dietary carotenoids (Duc *et al.*, 2006). In addition, *in vitro* data have suggested probiotic bacilli could be useful in reducing the risk originating from genotoxic agents to gastrointestinal health (Cenci *et al.*, 2008).

To date, the known mechanisms by which *Bacillus* strains exert their probiotic actions are modulation of the immune system (Ciprandi *et al.*, 2005b; Huang *et al.*, 2008b) and competitive exclusion (La Ragione *et al.*, 2001; La Ragione & Woodward, 2003). Several studies have shown that bacilli can persist in the gastrointestinal tracts of animals (Donskey *et al.*, 2001; Hoa *et al.*, 2001; Tam *et al.*, 2006). Tam *et al.* (2006) demonstrated that biofilm-forming *Bacillus subtilus* strains isolated from human faeces persisted in the mouse gut longer than a laboratory *Bacillus* strain. They also showed that the bacilli were able to sporulate, germinate and re-sporulate in the mouse gut. Therefore, this bimodal lifecycle may be one mechanism by which bacilli could potentially enhance their probiotic effects. In addition, it has been suggested that the potential probiotic effects of *Bacillus* strains may be enhanced if strains of interest have an intimate interaction with host cells or the indigenous microbiota (Duc *et al.*,

2006). However, to date no studies have examined the effect of administration of probiotic bacilli on the indigenous gastrointestinal microbiota of humans.

Bacillus coagulans is a facultative, lactic acid-producing, Gram-positive bacterium that has received great interest as a probiotic in recent years. GanedenBC^{30} (*Bacillus coagulans* GBI-30, 6086) is a proprietary probiotic preparation that does not demonstrate mutagenic, clastogenic, or genotoxic effects, and is considered safe for human consumption (Endres *et al.*, 2009). It has recently been suggested that GanedenBC^{30} may be safely used as a therapeutic agent for enhancing T-cell responses to adenovirus and influenza A infections (Baron, 2009), and may be a safe and effective option for the relief of abdominal pain and bloating for patients with irritable bowel syndrome (Hun, 2009). Strains of *Bacillus coagulans* have been detected in the faeces of mice four days after cessation of feeding (Donskey *et al.*, 2001). The effect of oral administration of a lyophilized preparation of *Bacillus coagulans* CNCMI-1061 on the faecal microbiota of piglets has been examined (Adami & Cavazzoni, 1999). The strain was found to reduce the number of coliforms and enterococci in probiotic-treated compared with control animals, but statistically significant reductions in the numbers of these bacteria were seen only after >1 week of therapy. However, the ability of GanedenBC^{30} to persist in the human gastrointestinal tract and its impact on the human gastrointestinal microbiota have not been examined.

In vitro fermentation systems offer relative freedom from animal experimentation, are relatively cheap to run and the duration and environmental conditions of an experimental run can be carefully controlled (McBurney & Thompson, 1987). In addition, they have been successfully used to examine the impact of probiotics on the human faecal microbiota and its activities (Pereira *et al.*, 2003). Consequently, it was decided to use an *in vitro* continuous culture system mimicking conditions in the ascending colon to examine the effect of GanedenBC^{30} on the human gut microbiota, and whether this strain persisted in the system upon cessation of probiotic feeding.

MATERIALS AND METHODS

Probiotic

The probiotic used in this study was GanedenBC^{30} (*Bacillus coagulans* GBI-30, 6086), patented by Ganeden Biotechnology, Incorporated (Mayfield Heights, OH). The strain was provided freeze-dried and stored at 4 °C prior to use.

Donors and preparation of faecal homogenates

Healthy adult donors who had not taken prebiotics, probiotics or antibiotics for 3 months prior to the start of the study were recruited (n=4; 1 female, 3 males; age range 25–39 years). Faecal homogenates (20 % faeces: 80 % sterile anaerobic distilled water, w/w) were prepared in filter bags from fresh faecal samples by using a Seward stomacher (high speed, 2 min). The faecal homogenates were used immediately to inoculate the continuous culture systems as described below.

Continuous culture systems

Each system comprised a 100 ml fermentation vessel, to which 50 ml of sterile, anaerobic gut model medium were added. Feed vessels containing sterile, anaerobic gut model medium were connected to the fermentation vessels. The medium in the fermentation and feed vessels was sparged with O_2-free N_2 to maintain anaerobic conditions, and stirred continuously. Aliquots (50 ml) of faecal homogenate were added to each vessel. To mimic environmental conditions in the ascending colon, the pH of the vessels was maintained at 5.5 by the automatic addition (Electrolab U1000 pH controller) of 2 M NaOH or 2 M HCl. The temperature of the vessels was maintained at 37 °C by use of a circulating waterbath. Gut model medium (Macfarlane *et al.*, 1998) comprised (g per litre unless stated otherwise): starch from potato, 5.0; pectin from citrus fruit, 2.0; guar, 1.0; porcine gastric mucin (type III), 4.0; xylan from oatspelts, 2.0; arabinogalactan from larchwood, 2.0; inulin (Orafti), 1.0; casein from bovine salts, 3.0; peptone water (Oxoid), 5.0; tryptone (Oxoid), 5.0; bile salts no. 3 (Oxoid), 0.4; yeast extract (Oxoid), 4.5; $FeSO_4.7H_2O$ (AnalaR), 0.005; NaCl (Fisher Scientific), 4.5; KCl, 4.5; KH_2PO_4 (AnalaR), 0.5; $MgSO_4.7H_2O$ (AnalaR), 1.25; $CaCl_2.6H_2O$, 0.15; $NaHCO_3$, 1.5; L-cysteine hydrochloride, 0.8; Tween 80, 1.0; and hemin, 0.05 g dissolved in 1 ml of 1 M NaOH. All reagents were purchased from Sigma Aldrich unless stated otherwise.

Two continuous culture systems were run in parallel for each donor. After inoculation, samples were run as batch cultures

TABLE 1. Sampling and treatment schedules for the continuous culture systems used in this study. *t, time in hours. Vessel A, probiotic-treated; vessel B, control.

Samples taken/treatment*	Samples processed for:
t_0 Vessels A+B inoculated, then 5 ml samples taken from vessels A+B	DGGE, FISH, SCFA, cultivation
t_{12} – medium flow turned on	–
t_{84} – 2 ml samples taken from vessels A+B	DGGE, SCFA
t_{96} – 2 ml samples taken from vessels A+B	DGGE, SCFA
t_{108} – 5 ml samples taken from vessels A+B, then 132 mg GanedenBC^{30} added to vessel A	DGGE, FISH, SCFA, cultivation
t_{132} – 132 mg GanedenBC^{30} added to vessel A	–
t_{156} – 132 mg GanedenBC^{30} added to vessel A	–
t_{168} – 2 ml samples taken from vessels A+B	DGGE, SCFA
t_{180} – 2 ml samples taken from vessels A+B, then 132 mg GanedenBC^{30} added to vessel A	DGGE, SCFA
t_{192} – 5 ml samples taken from vessels A+B	DGGE, FISH, SCFA, cultivation
t_{204} – 5 ml samples taken from vessels A+B	DGGE, FISH, SCFA, cultivation
t_{228} – 5 ml samples taken from vessels A+B	DGGE, FISH, SCFA, cultivation
t_{252} – 5 ml samples taken from vessels A+B	DGGE, FISH, SCFA, cultivation

for 12 h, then the medium feed was started: medium was fed into the systems at a rate of 10 ml h^{-1}, to give a system retention time of 10 h. Table 1 details the way in which the control and probiotic systems were treated and sampled. Samples were processed as described below.

Processing of samples

Cells from samples (2x 1 ml) were pelleted by centrifugation at 13,000 rpm (MSE Microcentaur; Sanyo) for 5 min at room temperature. Supernatants were removed and centrifuged for a further 20 min; they were then passed through a 13 mm 0.2 µm filter (Millipore) and stored at -20 °C until required for HPLC analysis of short-chain fatty acids (SCFAs). The cell pellets were resuspended in 1 ml of sterile PBS/glycerol (1:1, v/v) and stored at -20 °C until DNA was extracted from the samples. Aliquots (2x 375 µl) of samples were processed for fluorescence *in situ* hybridization (FISH) analysis as described by Martín-Peláez *et al.* (2008).

Cultivation work was carried out from ethanol-treated samples as described by Barbosa *et al.* (2005). Briefly, in the presence of oxygen, 1 ml aliquots of samples were diluted with 1 volume of ethanol and shaken at 50 rpm for 1 h at room temperature. Dilution series (10^{-1} to 10^{-6}) were prepared from the treated samples and 100 µl aliquots were spread in triplicate onto tryptone soya agar (Oxoid) plates for each dilution. The plates were then incubated for 3 days at 37 °C in an anaerobic cabinet (MARKIII with satellite; Don Whitley), and the number of viable cells was recorded in terms of cfu (ml sample)$^{-1}$.

FISH analysis

Details of the probes used in this study and their target bacteria/groups can be found in Table 2. Cyanine 3-labelled probes were obtained from Sigma Genosys (Sigma). Hybridizations and enzyme treatments were carried out as described by Martín-Peláez *et al.* (2008).

TABLE 2. Details for the oligonucleotide probes used for FISH analysis in this study. *These probes were used together in equimolar concentrations (50 ng μl^{-1}). †Enzyme pre-treatment with lysozyme according to Martin-Pelaez *et al.* (2008).

Probe(s)	Sequence (5' to 3')	Formamide concn (%)	Hybridization temp. (°C)	Washing temp. (°C)	Target group /molecule	Ref.
EUB 338 mix*	GCTGCCTCCCGTAGGAGT GCAGCCACCCGTAGGTGT GCTGCCACCCGTAGGTGT	35	46	48	Most *Bacteria*/16S rRNA	Daims *et al.* (1999)
Ato291	GGTCGGTCTCTCAACCC	0	50	50	*Cryptobacterium curtum, Gordonibacter pamelaeae, Paraeggerthella hongkongensis,* all *Eggerthella, Collinsella, Olsenella* and *Atopobium* species/16S rRNA	Harmsen *et al.* (2000)
Bif164	CATCCGGCATTACCACCC	0	50	50	Most *Bifidobacterium* species and *Parascardovia denticolens*/16S rRNA	Langendijk *et al.* (1995)
Bac303	CCAATGTGGGGGACCTT	0	46	48	Most members of the genus *Bacteroides,* some *Parabacteroides* and *Prevotella* species, *Paraprevotella, Xylanibacter, Barnesiella* species and *Odoribacter splanchnicus*/16S rRNA	Manz *et al.* (1996)
Bcoa191†	GCCGCCTTTCCTTTTTCCTCC	20	46	48	*Bacillus coagulans*/16S rRNA	Sakari & Ezaki (2006)
Chis150	TTATGCGGTATTAATCTYCCTTT	0	50	50	Most members of *Clostridium* clusters I and II/16S rRNA	Franks *et al.* (1998)
Erec482	GCTTCTTAGTCARGTACCG	0	50	50	*Clostridium* cluster XIVa/ 16S rRNA	Franks *et al.* (1998)
Lab158†	GGTATTAGCAYCTGTTTCCA	0	50	50	All *Oenococcus, Vagococcus, Melissococcus, Tetragenococcus, Enterococcus, Catellicoccus, Paralactobacillus, Pediococcus* and *Lactococcus* species, most *Lactobacillus, Weissella* and *Leuconostoc* species/16S rRNA	Harmsen *et al.* (1999)
Prop853	ATTGCGTTAACTCCGGCAC	0	50	50	*Clostridium* cluster IX/16S rRNA	Walker *et al.* (2005)
Rbro730/ Rfla729*†	TAAAGCCCAGYAGGCCGC AAAGCCCAGTAAGCCGCC	20	50	50	*Anaerotruncus colihominis, Ruminococcus bromii, Ruminococcus flavefaciens, Ruminococcus albus, Desulfotomaculum alcoholivorax*/16S rRNA	Harmsen *et al.* (2002)

DNA extraction, PCR and denaturing gradient gel electrophoresis (DGGE)

Cells stored in PBS/glycerol were removed from the freezer and placed on ice. The samples were vortexed, and 250 µl aliquots were transferred to sterile 1.5 ml microcentrifuge tubes and

centrifuged at 13,000 rpm for 5 min. To remove the glycerol from the samples, the cell pellets were washed twice in 1 ml PBS. After the second wash, the cell pellets were resuspended in 500 µl TES buffer and processed for DNA extraction as described by Hoyles (2009). Quantification of DNA, and PCR and DGGE with the universal primers of Muyzer *et al.* (1993) were carried out as described by Hoyles (2009). Silver staining of gels was carried out as described by Roger (2008).

Analysis of SCFAs

Filtered supernatants were removed from the freezer and placed on ice. Aliquots (400 µl) of sample were transferred to 0.6 ml vials (Chromacol Ltd) and 50 µl of 2-ethylbutryric acid (20 mM; internal standard) was added to each vial. The samples were run using a Rezex ROA-organic acid H^+ column (300 x 7.80 mm, 8 micron; Phenomenex) at 84 °C on a HP1050 series (Hewlett Packard) HPLC system equipped with UV detector and autosampler. The eluent was 0.0025 M H_2SO_4 in HPLC-grade water, and the flow rate was 0.5 ml min^{-1}. Detection was performed at 210 nm, and data were acquired using ChemStation for LC3D software (Agilent Technologies).

Statistical analysis

Paired, two-tailed Student's *t*-test was used to determine statistically significant differences within treatments (control or GanedenBC^{30}-supplemented) over time and between treatments at each time point. For the purposes of statistical analysis of FISH counts, the detection limit [232065.9 cells (ml sample)$^{-1}$] was used when levels were below the detection limit. Statistical significance was accepted at $P<0.05$.

RESULTS

Enumeration of aerobic spore-formers via cultivation

Results are presented for three donors, as growth of a biofilm on the glass spreader used for distributing samples around plates affected results obtained for Donor 2. It can be seen from Fig. 1 that aerobic spore-formers could be cultivated from control vessels at all time points. None of the strains isolated from the control vessels was characterized; therefore, we can not comment on the relevance of this finding. The results of cultivation work from the probiotic-fed vessels demonstrated that ethanol treatment of samples followed by plating on tryptone soy agar is a suitable method for following persistence of the probiotic GanedenBC^{30} in the continuous culture system. Incubation under anaerobic conditions aided in recovery of the strain due to it being a facultative anaerobe, whereas most currently recognized species of *Bacillus* are obligate/facultative aerobes. Partial 16S rRNA gene sequencing confirmed that the predominant colony morphotype of the GanedenBC^{30}-supplemented systems after probiotic treatment was most closely related to *Bacillus coagulans* (GenBank accession no. AB271752). Furthermore the increase in colony counts after GanedenBC^{30} supplementation suggests that the probiotic strain was able to germinate within the *in vitro* fermentation system.

FIGURE 1. Enumeration of aerobic spore-formers in control and GanedenBC^{30}-supplemented single-stage continuous culture fermentation systems. Data are presented as mean±standard deviation (n=3; data from run 2 were excluded due to biofilm formation on the glass spreader used). Black squares, GanedenBC^{30}-supplemented systems; grey diamonds, control systems. GanedenBC^{30} was added to GanedenBC^{30}-supplemented systems daily between SS1 and SS2.

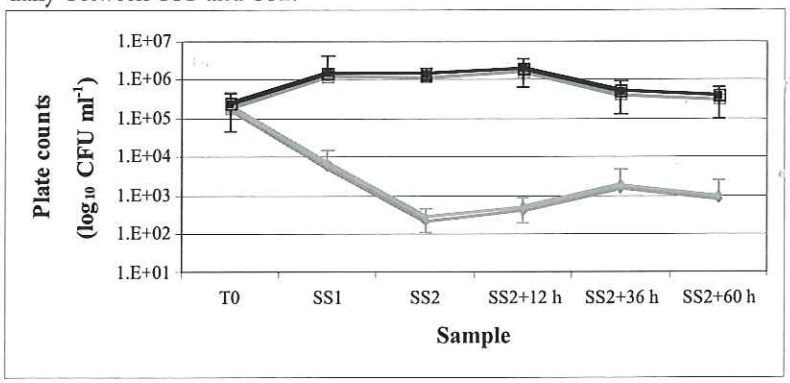
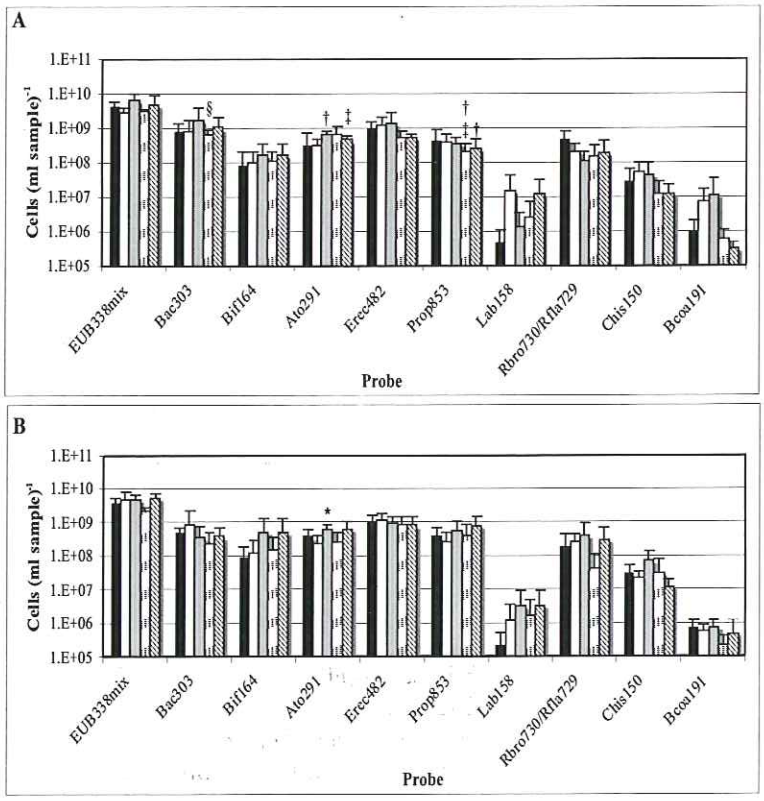


FIGURE 2. Effect of GanedenBC^{30} supplementation on FISH counts obtained from single-stage continuous culture fermentation systems. Data are presented as mean+standard deviation (n=4). A, GanedenBC^{30}-supplemented systems; B, control systems. Black bars, SS1; white bars, SS2; grey bars SS2+12 h; dotted bars, SS2+36 h; hatched bars, SS2+60h. GanedenBC^{30} was added to GanedenBC^{30}-supplemented systems daily between SS1 and SS2. *, significantly different from SS1; †, significantly different from SS2; ‡, significantly different from SS2+12 h; §, significantly different from probe count for control system at same sample time.



DGGE analysis

Analysis of the DGGE profiles generated from the systems inoculated with samples from the four different donors demonstrated that GanedenBC^{30} supplementation elicited a notable effect on the microbiota, beyond that of microbiological drift (seen in control systems) (Fig. 3). This was consistent for all four runs, even though distinctive profiles were evident for each homogenate and subsequently each run. Overall, SS1 samples clustered together (both systems), subsequent samples then clustered according to treatment (system), with marginally greater similarity seen between the control cluster and SS1 cluster than for the GanedenBC^{30} cluster and other clusters. Furthermore, post-treatment profiles for the probiotic systems showed greater profile variation from that of SS2 (92.7 % similarity; Fig. 3) compared to control systems (96 % similarity; Fig. 3).

SCFA production

Lactate was either not detected or present in only trace amounts throughout the four runs (both systems). While statistically significant differences were seen in the amounts of acetate, butyrate and propionate detected (Table 3), the only significant difference seen between treatments was propionate levels at t_{84} (i.e. SS1-24 h), reflecting the relatively low levels seen in control systems at t_{84}. Furthermore, neither treatment elicited marked changes in SCFA levels; namely, SS2 (192 h) compared to SS1 (108 h). Overall, inter-run variation was observed in SCFA levels; however, GanedenBC^{30} supplementation did not significantly alter the SCFA profiles compared to those of control systems.

DISCUSSION

We employed *in vitro* single-stage continuous culture fermentation systems whose conditions mimicked those of the ascending colon to examine the effect of GanedenBC^{30} supplementation on the human gut microbiota. As far as we are aware, this is the first study investigating the effect of a *Bacillus* probiotic on the human gastrointestinal microbiota. In general, there were no major shifts in the microbiota after GanedenBC^{30} supplementation, compared to control systems. However, notable clustering of post-treatment DGGE profiles was seen within runs according to treatment. The ability to cultivate aerobic spore-formers from all systems at all time points [~10^5 cfu (ml sample)$^{-1}$

FIGURE 3. Effect of GanedenBC^{30} supplementation on universal DGGE profiles during single-stage continuous culture fermentations (donor 1 systems). The dendrogram was generated using Dice/UPGMA. Clustering was confirmed by using Pearson/UPMGA (data not shown). A, GanedenBC^{30}-supplemented system; B, control system. GanedenBC^{30} was added to probiotic-fed system daily between SS1 (A4) and SS2 (A7). Sample numbering (e.g. 1–10) corresponds to 0, 84, 96, 108 (SS1), 168, 180, 192 (SS2), 204, 228 and 252 h, respectively.

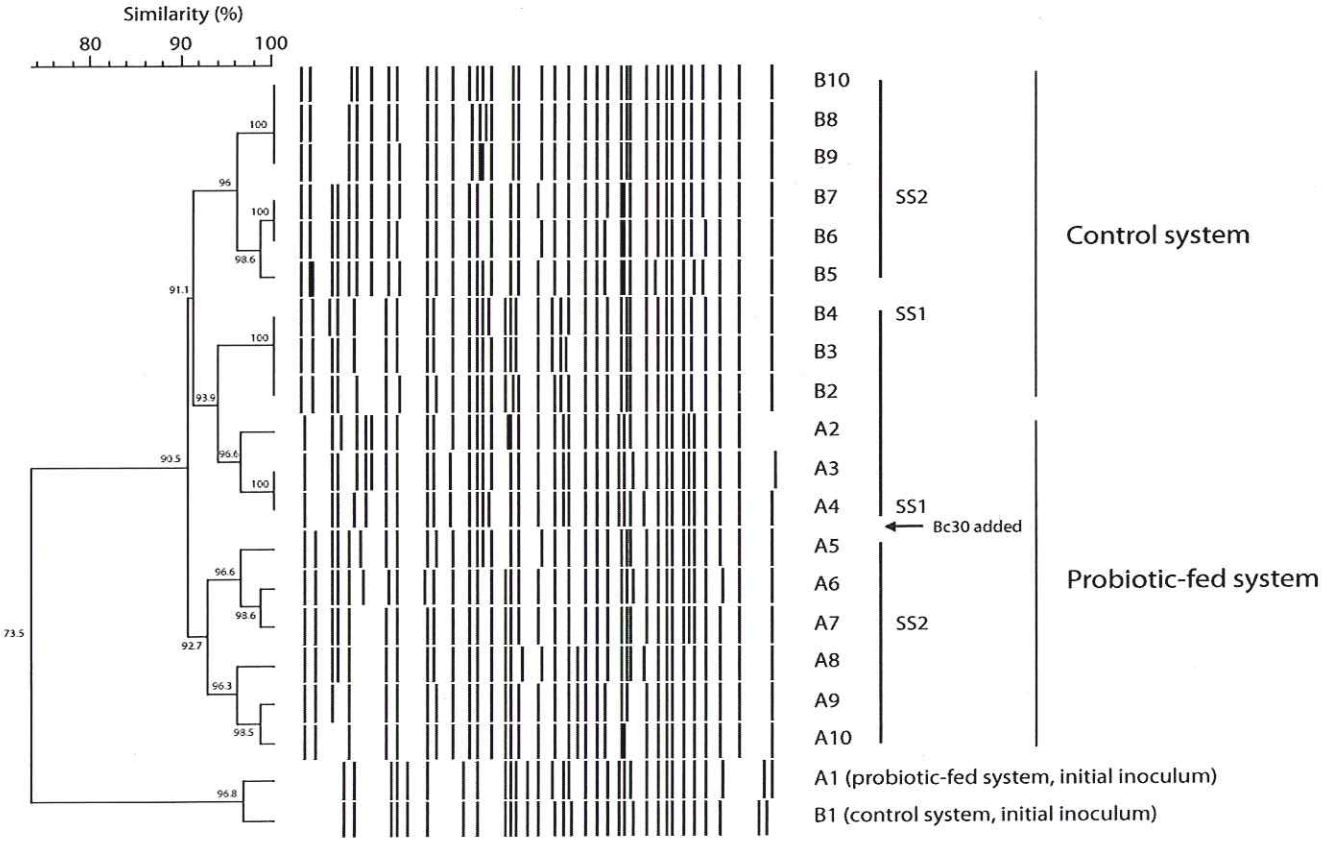


TABLE 3. Investigation of the effects of GanedenBC^{30} supplementation on SCFA levels during single-stage continuous culture fermentations. Data are presented as mean±standard deviations (n=4). Lactate is not included in the table as it was either not detected or present in only trace amounts in samples; *Significantly different from 0 h sample; †Significantly different from 84 h sample; ‡Significantly different from SS1 sample; §Significantly different from control systems at same time.

Time (h)	Control system			GanedenBC^{30}-supplemented system		
	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)
0	5.57±2.63	2.44±2.59	0.73±1.26	4.02±4.09	3.57±4.47	0.72±1.25
84	41.80±21.29	8.24±5.62	38.73±32.94	49.10±7.01*	27.43±5.70*§	32.03±30.31
96	40.30±17.52	19.60±17.09	32.98±18.79	45.70±13.87*	28.90±13.26*	36.24±18.86
108 (SS1)	36.03±15.79*	26.65±12.22*	29.05±20.84	42.88±6.79*	29.57±6.68*	34.85±14.55
168	50.63±13.36*	32.92±20.88	37.45±16.97	59.04±22.32	38.26±7.51*†	36.81±13.21*
180	39.81±5.45*	24.85±14.20	29.14±7.05*	62.50±30.98	39.13±13.01	37.93±16.31
192 (SS2)	43.82±4.89*	28.15±16.94	32.65±8.52*	52.37±23.87	33.90±8.86*†	33.42±14.29
204	42.66±7.05*	28.05±16.75	33.83±8.92*	48.00±18.75	31.72±6.41*†	32.29±12.04
228	40.37±4.02*	24.83±15.21	33.86±13.86	55.61±23.75	35.74±10.82*†	37.27±18.66
252	47.33±12.13	27.95±27.52	33.66±6.30‡	42.77±3.61*†	31.11±8.35	28.30±0.81*†

at t_0, both systems] in this study does, however, support the growing body of evidence suggesting that *Bacillus* species are part of the resident human gut microbiota (Hong *et al.*, 2005; Fakhry *et al.*, 2008; Hong *et al.*, 2009). The marked decrease in aerobic spore-formers in control systems during *in vitro* fermentation (Fig. 1), compared to the relative increase in counts seen in GanedenBC^{30}-supplemented systems, probably reflects the facultative anaerobic nature of GanedenBC^{30} – i.e. it is able to germinate under the anaerobic *in vitro* conditions employed.

We have provided clear evidence that the administered GanedenBC^{30} spores germinated and persisted *in vitro*, with increased levels post-probiotic supplementation [as confirmed by cultivation work and FISH analysis (probe Bcoa191)]. Furthermore, the continuous culture systems described herein were set up to resemble the conditions of the human ascending colon. As such, it is possible that GanedenBC^{30} persistence within the human gastronintestinal tract may be further enhanced by delivery of vegetative cells to the lower regions of the colon, which may offer more favourable conditions than those of the ascending colon. The lack of any major modulation of the human gut microbiota does not preclude GanedenBC^{30} from eliciting health effects on the host. Indeed, the germination of *Bacillus* probiotics has been proposed as a mediator of probiotic effect (Sanders *et al.*, 2003). In addition, the germination of GanedenBC^{30} spores in the ascending colon may have a cascade effect on the transverse and/or descending colonic microbiota.

To date, the proposed mechanisms for probiosis by bacterial spore-formers include immune stimulation, production of antimicrobial compounds, competitive exclusion, conversion of genotoxic compounds to unreactive products and metabolic activities (Sanders *et al.*, 2003; Hong *et al.*, 2005). Published studies from work with humans and animal models have demonstrated immunomodulation by spores and/or vegetative cells of a number of *Bacillus* species. This immunomodulation has been associated (in combination with *Bacteroides fragilis*) with the development of the gut-associated lymphoid tissue and preimmune antibody repertoire in rabbits, and the ability of spores to stimulate cell proliferation in Peyer's patches (Rhee *et al.*, 2004; Hong *et al.*, 2005; Huang *et al.*, 2008a, b). Induction of cytokines, including proinflammatory cytokines (i.e. TNF-α and IL-6), in mesenteric lymph nodes and spleen has also been demonstrated for certain *Bacillus* strains. More recently, Baron (2009) demonstrated that 30-day GanedenBC^{30} supplementation in nine healthy humans significantly enhanced T-cell response to adenovirus and influenza A Texas 1/77 (H3N2) exposure.

In summary, the current study has shown that GanedenBC^{30} supplementation does not detrimentally alter the human gut microbiota *in vitro* even though the probiotic is able to germinate and persist beyond administration in the *in vitro* continuous culture fermentation system used. This work gives further support to the case for a 'bimodal life cycle' of certain bacilli within the human gastrointestinal tract.

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Clinical microbiology

Effect of prebiotics on the fecal microbiota of elderly volunteers after dietary supplementation of *Bacillus coagulans* GBI-30, 6086

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ABSTRACT

In advancing age, gut populations of beneficial microbes, notably *Bifidobacterium* spp., show a marked decline. This contributes to an environment less capable of maintaining homoeostasis. This *in vitro* investigation studied the possible synergistic effects of probiotic supplementation in modulating the gut microbiota enabling prebiotic therapy to in elderly persons. Single stage batch culture anaerobic fermenters were used and inoculated with fecal microbiota obtained from volunteers after taking a 28 day treatment of *Bacillus coagulans* GBI-30, 6086 (GanedenBC^{30} (BC30)) or a placebo. The response to prebiotic supplements fructooligosaccharides (FOS) and galactooligosaccharides (GOS) in the fermenters was assessed. Bacterial enumeration was carried out using fluorescent in situ hybridisation and organic acids measured by gas chromatography. Baseline populations of *Faecalibacterium prausnitzii*, *Clostridium lituseburense* and *Bacillus* spp. were significantly higher in those having consumed BC30 compared to the placebo. Both prebiotics increased populations of several purportedly beneficial bacterial groups in both sets of volunteers. Samples from volunteers having ingested the BC30 also increased populations of *C. lituseburense, Eubacterium rectale* and *F. prausnitzii* more so than in persons who had consumed the placebo, this also resulted in significantly higher concentrations of butyrate, acetate and propionate. This shows that consumption of BC30 and subsequent use of prebiotics resulted in elevated populations of beneficial genres of bacteria as well as organic acid production.

1. Introduction

The complex ecosystem that exists in the human gut plays host to around $10^{11} - 10^{12}$ bacterial cells per gram of stool [1,2], as the entire microbiome has been suggested to posses in the region of 100 times more genes than is present in the human genome this reveals reflects on its capability to host many metabolically active events and thus play an important role in host health and disease [3].

Benefits resulting from metabolic activities within the large gut are often attributed to its ability to hydrolyse complex carbohydrates which escape digestion in the upper gastrointestinal tract. This produces short chain fatty acids (SCFA) which are not only the primary source of energy for the luminal epithelium, but are also linked with other advantageous health outcomes, including anti-inflammatory affects [4] and modulation of diet induced obesity via regulation of gut hormones [5]. Microbial populations in the gut are also responsible for the production of other bioactive compounds including vitamins and minerals [6] and a reduction of pathogen colonisation via competitive exclusion.

The maturity of the gut microbiota occurs around the age of two years old, due in part to an increase in diversity following weaning and consumption of solid foods [7–10]. Prior to this, changes seen are very much dependent of mode of birth, immediate environment and diet. During adult life the microbiota has been regarded as remaining fairly stable, unless disrupted via the presence of an antagonist, like antibiotics, or the onset of ageing [11].

Dysbiosis occurs when the natural balance of the microbiota is disturbed, leading to other pathophysiological changes which can affect health. This occurs in advancing age leading to incidences of increased gut permeability, increase in baseline inflammatory status [11], reduced biodiversity in microbial populations [12] and increase in colonisation of pathogens [13]. Immunosenescence describes a general decline in immune function due to natural

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ageing. This is often characterised by a reduction in the capacity to respond adequately to infections, leading to reduced functionality of cell mediated immunity [14]. A connection between reduced microbial diversity, an increase in colonisation of pathogens and an increase in baseline inflammatory status maybe interconnected [11,15]. The presence, or increased opportunity, for several types of antigens to interact with immunoactive cells increases the capacity for the host immune system to respond by elevating inflammatory status.

Changes that occur within the microbiota in healthy ageing have been extensively reviewed by Biagi et al. [16] and Cheng et al. [17], both reaching a consensus that the difficulty in analysis of studies is with the definition of older persons used, the current health status of volunteers, and the dominant motive for the study, this may result in other changes being overlooked whilst in search of specific outcomes.

The dominant phyla in healthy adults (age 18 – 50) are Firmicutes, Bacteroidetes and Euyarchaeota. As ageing begins post 65 years, changes noted to occur include a reduction in Firmicutes, specifically the bifidobacteria genus and an increase in Proteobacteria, which contain several opportunistic pathogens [10].

One observation in the variance of the data concerned differing nationalities [18], which may also encompass differences in diet and lifestyle. Mueller et al. [18] conducted a cross sectional study of the fecal microbiota of different European populations in order to investigate age, gender and differences related to nationality, they showed that higher levels of bifidobacteria were seen in the 20 – 50 year olds in Italy and Sweden in comparison to their over 60 counterparts. However, this was comparable between age groups in France and Germany but with those in Italy possessing an average 1 – 2 $\log^{10}$ higher populations than other countries. *Bacteroides* spp., were higher in the under 50's in Italy and the over 60's in Germany, whereas *E. rectal*e group were higher in the under 50's in Italy but higher in the over 60's in Germany. This shows that variation exist in what would be considered age related changes in the microbiota that relate to location and probably dietary preferences.

Modulation of the human gut microbiota is one way to help counteract the affect of dysbiosis and there are several methods of achieving this which have been investigated. One is direct transplantation of a healthy microbiota into a host who suffers from a gut related disorder such as Inflammatory Bowel Disease. [19] or *Clostridium difficile*-associated diseases [20,21]. Faecal transplant is the method of using a liquid suspension of a fecal sample from a healthy (disease free) donor and transplanting it into an individual through a nasogastric, nasoduodeanal or rectal catheter.

The consumption of probiotics and prebiotics is a more frequently used way of modulating the gut microbiota. Prebiotics are essentially substrates for the beneficial commensal bacteria which already exist within the gut microbiota, they surpass dissimilation in the upper GI tract in order to be fermented by specific bacteria in the large gut [22]. Probiotics are live microbial cultures of species known to elicit beneficial effects such as the affect on intestinal cell proliferation [23], production of vitamins and minerals [6] and modulation of immune function by production of anti-inflammatory cytokines [24]. As mentioned, recent studies on changes in the gut microbiota with age can vary by nationality [18], those which are more detrimental to gut health are the reduction of bifidobacteria and increase in pathogenic species. Probiotic use is often limited to *Lactobacillus* spp. and *Bifidobacterium* spp. which have shown positive effects when orally administered in studies with older volunteer [25–27].

Synbiosis is the combined use of both prebiotics and probiotics. Prebiotic fructooligosaccharide (FOS) consist of fructose units linked with β (2-1) glycosidic bonds with a terminal D-glucose unit and galactooligosaccharides (GOS) are comprised of galactose units with a terminal D-glucose unit. Several studies have shown beneficial effects from prebiotic supplementation in both *in vitro* and *in vivo* situations using samples from a younger (18 – 50 years) cohort [28,29].

Bacillus coagulans GBI-30, 6086 (GanedenBC^{30} (BC30)) is a spore forming lactic-acid producing bacterium. Being spore formers they have the capacity to resist adverse conditions of stomach acid and bile in the gastrointestinal tract [30,31]. *In vitro* studies using continuous culture have investigated the ability of *B. coagulans* to affect pathogen survival in the human gut microbiota. This research found that through maintenance of populations in a mixed community, which included pathogens, the strain could competitively exclude transient pathogens [32]. Such bacteria are also known to excrete anti-microbial peptides [33,34]. Moreover, use of *B. coagulans* both *in vitro* and *in vivo* demonstrated an ability to reduce distension in adults with post prandial intestinal gas related symptoms [35] and the probiotic has also shown immunomodulatory effects [36]. However no direct study has been completed to evaluate its ability to significantly increase markers identified in known probiotic strains of *Lactobacillus* spp and *Bifidobacterium* spp.

This study aimed to investigate the possibility that dietary manipulation of the human gut microbiota by a potential probiotic BC30 could concomitantly influence prebiotic fermentation.

2. Methodology

2.1. Human study design

The study from which the samples were taken was a double blind placebo controlled trial which consisted of 28 day treatments of either BC30 or a placebo (microcrystalline cellulose). Faecal samples were taken at the beginning for baseline measurements and the end of each treatment.

2.2. Human study subjects

Volunteers on the study were of ages 65 – 80 years, with a BMI: 18 – 31 kg/m^2 and in general good health and free from any chronic diseases including gastrointestinal (GI) illnesses at the time of recruitment. They were required to refrain from consuming any pharmaceuticals, including immunosuppressive drugs and anti-inflammatory agents, for 6 weeks preceding the study and for the trial duration. Volunteers were also asked to refrain from consumption of any prebiotics, probiotics and antibiotics for the duration of the study and for at least 3 months prior to the study and to discontinue the use of any laxatives or any foods with a laxative affect. Diseases and disorders within the exclusion criteria included any inflammatory disorder, asthma, arthritis or dermatitis. Furthermore, any volunteers who had a colonic irrigation in the previous 3 months, were suffering from severe food allergies, following a calorie restricted diet, suffering from any mental illness, or had a history of drug and alcohol abuse were also excluded. The six volunteers who provided fecal samples for this work had a BMI of between 25 and 31.

Written consent was gained for all volunteers and all were screened to ensure they were in good health. The research ethics committee at the University of Reading approved the study (Project Ref: UREC 12/24). The probiotic BC30 or the placebo were consumed once a day as capsules for 28d, with capsule of BC30 containing 1×10^7 CFU. The study design was of a blinded random crossover nature with a 21 day washout period. At the end of each treatment arm, fecal samples from 6 volunteers (three on BC30 and 3 on the placebo) were used to inoculate anaerobic batch culture fermenters.

2.3. Probiotic and prebiotic substrates

The BC30 (*B. coagulans* GBI-30, 6068) (10^7 CFU/capsule) was supplied by Ganeden Biotechnology, Mayfield, USA, as was the placebo. They were packaged in a manner that was anonymous to the study investigators and volunteers. Galactooligosaccharides (GOS) (Clasado BiMuno product) were obtained from a local supermarket and fructooligosaccharides (FOS) (Orafti P95) were from Beneo Orafti, Wimbledon, London.

GOS comprised of 48% galactooligosaccharides with an average degree of polymerisation around 2 or 3; FOS had an inulin/oligofructose content of 95% and an average degree of polymerisation around 4.

2.4. Faecal sample collection and preparation

Feces were obtained from six healthy volunteers in a double blind placebo controlled dietary intervention study consisting of treatments of either probiotic or placebo. Faecal samples were obtained on day 28 of the study periods.

Freshly voided feces were collected in plastic pots and placed in an anaerobic cabinet (H_2:CO_2:N_2, 10:10:80 by volume at 37 °C) (Don Whitley Scientific, West Yorkshire, UK) and processed within 2 h. Samples were diluted 1 into 10 weight by weight using 0.1 M, pH7.4, phosphate buffered saline (PBS) (Oxoid, Hampshire, UK). This slurry was homogenised in a stomacher (Seward, stomacher 80, Biomaster) for 120 s at normal speed, sieved and fermenter vessels then inoculated with the resultant 10% (w/v) slurry.

2.5. Batch culture basal nutrient medium and substrates

Basal nutrient medium was prepared with chemicals obtained from Sigma, Poole, UK unless otherwise stated. In one litre: 2 g peptone water, 2 g yeast extract (Oxoid, Hampshire, UK), 0.1 g NaCl, 0.04 g K_2HPO_4 (BDH, Poole, UK), 0.04 g KH_2PO_4 (BDH), 0.01 g $MgSO_3.7H_2O$ (Fischer scientific, Loughborough, UK), 0.01 g $CaCl_2.6H_2O$ (Fluka, Poole, UK), 2 g $NaHCO_3$ (Fischer), 0.5 g L–cysteine HCl, 2 mL tween 80, 10 μL vitamin K1, 0.05 g haemin, 0.05 g bile salts (Oxoid), 4 mL resazarin (pH7).

2.6. pH controlled, stirred batch culture fermentation

Vessels with an operating volume of 300 mL were used. 135 mL of basal nutrient medium was autoclaved (121 °C for 15 min) and aseptically poured into sterile vessels. This system was left overnight with oxygen-free nitrogen pumping through the medium at a rate of 15 mL min^{-1} pH metres (Electrolab pH controller, Tewksbury, UK) were connected to each vessel to regulate pH at 6.8 by automatic addition of 0.5 M HCL or NaOH as appropriate. Each vessel was also temperature controlled at 37 °C and stirred using a magnetic stirrer. 1% (w/v) of pure prebiotic was added to a separate vessel prior to inoculation with 1% (w/w) of inocula. Samples were removed from the fermenters after 0, 12 and 24 h incubation. Control vessels containing basal medium only were also ran.

2.7. Bacterial enumeration by fluorescent in situ hybridisation (FISH)

A 375 μl sample of batch culture fluid was fixed in 1125 μl filtered cold (4 °C) 4% (v/v) paraformaldehyde (PFA) and samples stored at 4 °C for 4 h. These were then washed thoroughly with phosphate buffered saline (PBS) to remove PFA and re-suspended in a 300 μL mixture of 1:1 v/v PBS/ethanol. Samples were then stored at −20 °C until required. FISH was carried out as described by Franks et al. [16]. DNA oligonucleotide probes used were Bif164 (targeting *Bifidobacterium* spp.), LAB158 (targeting *Lactobacillus* spp. and *Enterococcus* spp.), EREC482 (which targets *Clostridium coccoides*, *Eubacterium rectale*) group (*Clostridium* cluster XIVa and XIVb), CLIT135 (targeting *Clostridium lituseburense* group (*Clostridium* cluster XI)), BAC303 (targeting *Bacteroidaceae* spp., *Prevotellaceae* spp. and some Porphyromonadaceae spp.), Fprau645 (targeting *Faecalibacterium* spp.), and relatives, and Bcoa191 (targeting *Bacillus* spp.) 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI 50 ng μL^{-1}) was used to detect total bacteria [37–39]. All microbial counts are presented as $\log_{10}$ cells per mL^{-1} sample.

2.8. Organic acid analysis

Batch culture samples were first centrifuged at 13,226 × g for 10 min and the supernatant filtered through a 0.22 μm filter (Milipore, UK). 1 mL sample had 50 μL of 0.1 M 2-ethylbutyric acid (internal standard) added and was acidified using 0.5 mL of concentrated hydrochloric acid (6 N) with 2 mL diethyl ether being added and vortexed for 1 min to separate the fatty acids. Samples were centrifuged at 931× *g* for 10 min and the diethyl ether layer collected. Analysis was carried out using a Hewlett Packard GC (Agilent Technologies Ltd, UK) 5890 series II GC system with a volume of 1 μL injected at 220 °C (with a split of 100:1) onto a Rtx-1 (10 m × 0.18 mm with a 0.20 μm coating). Injection and detector temperatures were set at 275 °C with initial column temperature being held at 63 °C for three minutes and then increased by 10 °C/min to 190 °C, carrier gas helium was delivered at a flow rate of 0.7 mL/min.

External standards of short chain and branched chain fatty acids formic acid, acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, n-valeric acid, lactic acid and succinic acid were prepared at concentrations of 10 mM, 30 mM, 20 mM, 5 mM, 20 mM, 5 mM, 5 mM, 10 mM and 20 mM respectively. Organic acids were then dissolved in 2-propanol and analysed using the same method as for samples. Concentrations were calculated and presented in mM.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism for windows, version 5. Analysis of variance (ANOVA) was carried out on values of bacterial populations in CFU mL^{-1} and organic acid concentrations in mM, with a confidence level of 95%. Bonferoni's post-tests were carried out to determine significance of the BC30 feeding effect, time and prebiotic substrate on bacterial groups and fatty acid concentrations where $n = 3$ per treatment.

3. Results

Faecal samples were taken from three volunteers having consumed BC30 for 28 days and three volunteers having been on the placebo for 28 days on a double blind placebo controlled cross over study. These faecal samples were then used to inoculate anaerobic single stage fermenters in order to investigate the effect of the study treatment on the response of the faecal microbiota to two different types of prebiotic.

3.1. In vitro effects of prebiotic fermentations on bacterial populations measured by FISH

Changes in bacterial counts are shown in Table 1 and Figs. 1–3, during 24 h fermentations. Both GOS and FOS had a positive overall effect on populations of *Lactobacillus* spp., ($p < 0.001$, $p < 0.0001$), *Bifidobacterium* spp., ($p < 0.0001$ for both), *E. rectale* ($p = 0.009$,

Table 1
Mean changes in bacterial numbers (Log10 cells/mL^{-1}) determined by fluorescent in situ hybridization from 24 h in vitro batch culture fermentations of different prebiotics.

Probe	Time (h)	Cell numbers (Log10 cells mL^{-1} ± SD) by FISH						Sig#
		Placebo ($n = 3$)			BC30 ($n = 3$)			
		Control	GOS	FOS	Control	GOS	FOS	
DAPI	0	8.83 ± 0.14	8.61 ± 0.04	8.58 ± 0.12	8.71 ± 0.07	8.74 ± 0.07	8.61 ± 0.15	b
	12	8.72 ± 0.02	8.89 ± 0.15	9.17 ± 0.36*	8.66 ± 0.22	9.22 ± 0.2*	**9.15 ± 0.06****	
	24	8.64 ± 0.13	8.77 ± 0.5	9.17 ± 0.08*	8.61 ± 0.09	8.9 ± 0.14	**9.09 ± 0.14****	
Bif164	0	7.72 ± 0.17	**7.89 ± 0.16** **	**7.77 ± 0.15** ***	7.5 ± 0.21	**7.69 ± 0.25*****	**7.58 ± 0.14****	b
	12	7.77 ± 0.11	**8.87 ± 0.19****	**8.86 ± 0.2*****	7.81 ± 0.09	**8.92 ± 0.06*****	**8.79 ± 0.27****	
	24	7.55 ± 0.3	8.84 ± 0.28	8.84 ± 0.07	7.44 ± 0.29	8.96 ± 0.06	8.78 ± 0.47	
Lab158	0	7.62 ± 0.26	7.57 ± 0.40	7.52 ± 0.36	7.67 ± 0.18	7.96 ± 0.3	7.87 ± 0.3	b
	12	7.56 ± 0.07	8.25 ± 0.16	8.49 ± 0.06*	7.73 ± 0.11	8.16 ± 0.22	8.4 ± 0.43	
	24	7.66 ± 0.16	8.25 ± 0.25	8.21 ± 0.28*	7.8 ± 0.18	8.21 ± 0.11	8.32 ± 0.33	
Bac303	0	7.9 ± 0.32	8.01 ± 0.26	7.82 ± 0.43*	7.98 ± 0.30	8.2 ± 0.37	8.06 ± 0.33	b
	12	8.28 ± 0.35	8.46 ± 0.54	8.66 ± 0.32*	8.31 ± 0.15	8.55 ± 0.07	8.08 ± 0.33	
	24	8.24 ± 0.005	8.81 ± 0.07	8.73 ± 0.2	8.14 ± 0.20	8.5 ± 0.23	8.35 ± 0.02	
Erec482	0	8.05 ± 0.06	8.1 ± 0.16	7.8 ± 0.23	8.04 ± 0.3	8.22 ± 0.1	7.98 ± 0.24	a, b
	12	8.04 ± 0.2	**8.34 ± 0.3****	8.71 ± 0.14	8.05 ± 0.18	8.5 ± 0.3	8.7 ± 0.45	
	24	7.92 ± 0.19	8.36 ± 0.31 *	8.41 ± 0.23	8.2 ± 0.4	8.67 ± 0.4	9.03 ± 0.04	
Clit135	0	6.12 ± 0.2	6.14 ± 0.36	6.2 ± 0.57	6.95 ± 0.3	6.81 ± 0.22	6.79 ± 0.37	a, b
	12	6.39 ± 0.27	6.83 ± 0.45	6.56 ± 0.29	7.1 ± 0.43	7.75 ± 0.32*	7.42 ± 0.47	
	24	6.25 ± 0.3	6.43 ± 0.44	6.52 ± 0.35	6.37 ± 0.59	7.19 ± 0.4	6.89 ± 0.13	
Fprau 645	0	7.51 ± 0.32	7.54 ± 0.5	7.47 ± 0.36	7.85 ± 0.11	8.03 ± 0.03	7.85 ± 0.02	a, b
	12	7.66 ± 0.11	8.07 ± 0.16	8.28 ± 0.13*	7.72 ± 0.19	8.18 ± 0.44	8.19 ± 0.39	
	24	7.17 ± 0.13	8.23 ± 0.13	7.86 ± 0.39	7.74 ± 0.21	7.99 ± 0.27	8.09 ± 0.25	
Bcoa191	0	6.41 ± 0.67	6.4 ± 0.6	6.36 ± 0.58	6.83 ± 0.23	6.94 ± 0.23	6.81 ± 0.26	a
	12	6.07 ± 0.12	6.35 ± 0.22	6.5 ± 0.58	6.7 ± 0.2	7.13 ± 0.29	6.95 ± 0.32	
	24	6.49 ± 0.44	6.49 ± 0.56	6.35 ± 0.54	6.54 ± 0.33	6.9 ± 0.58	6.69 ± 0.33	

Table shows cell numbers in Log10 cells/mL during a 24 h batch culture fermentation of FOS and GOS using fecal innocula from volunteers after consumption of either the placebo or BC30, in comparison to the control fermenter which had no innocula and no added carbohydrate. A significant difference in microbial populations compared to 0 h within each treatment and substrate $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. #Significant difference between treatments and substrates, $n = 3$ in each group.
a – Significant effect of treatments (probiotic, placebo); b – significant effect of substrates (FOS, GOS).
GOS = galactooligosaccharides) and FOS = fructooligosaccharides.

$p = 0.002$), *Faecalibacterium prausnitzii* ($p = 0.0002$, $p = 0.001$) (Fig 2) and total bacteria ($p = 0.038$, $p = 0.001$) respectively. Many of these have been associated with increased production of organic acids and their positive attributes [22].Increased *Bifidobacterium* spp. is important as this particular genus is known to decline during advancing age.

GOS specifically increased populations of *Bacteroides* spp., ($p = 0.012$) and *C. lituseburense* spp., ($p = 0.012$), both groups may elicit a more detrimental effect on colon health due to the association with metabolites which exert unfavourable affects, [22]. However, this is more so in clostridia strains more closely related to *Clostridium histolyticum* where negative effects are associated with fermentation of proteins and amino acids [40]. As both groups contain saccharolytic and proteolytic species, it is important to note that members of several species in clostridia clusters XIVa and b, IV, IX and XVI produce large concentrations of beneficial butyrate, acetate and propionate amongst other SCFA from hexose sugars [40]. Therefore, an increase in the population of *Clostridium* spp. and *Bacteroides* spp. is not necessarily detrimental to host health, as it is partially dependent on the precursor macromolecule to which they are exposed. In this case, the growth substrate was a carbohydrate (prebiotic), leading to benign fermentation end products.

Although some groups of bacteria significantly responded to only one of the prebiotics, no major differences were found between the two test materials. This suggests that GOS and FOS may not result in significantly different modulations of the microbiota. The groups which exhibited significant differences between treatments (comparing with and without probiotic) were *C. lituseburense*, *F. prausnitzii* and *E. rectale* (Figs. 1–3).

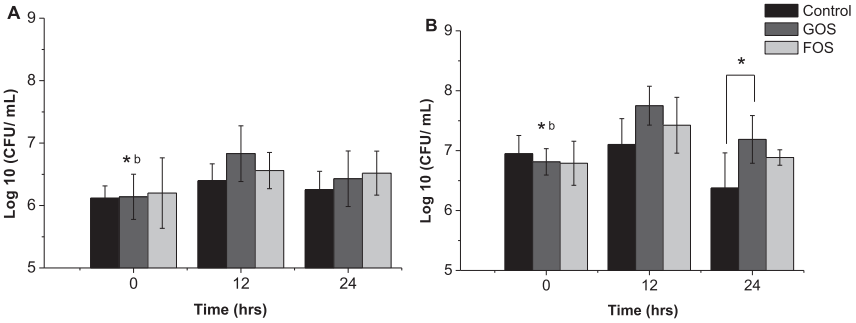

Fig. 1. Shows that the faecal microbiota from volunteers consuming probiotic increased *Clostridium lituseburense* significantly after 24 h fermentation. Values are mean values at three time points from six faecal donors per treatment ± standard deviation. A ($n = 3$) – Volunteers ingesting placebo, B ($n = 3$) – Volunteers ingesting BC30; * ($p \leq 0.05$), ** ($p \leq 0.001$), *** ($p \leq 0.0001$) as compared to the control with no substrate added; *b significantly different to the same sample as compared to the different treatment.

Although no significant overall effect was detected in *Bacillus* spp., in response to the prebiotics, a clear difference in populations showed that those on BC30 had significantly higher populations than on the placebo, irrespective of prebiotic effects (Fig 3).

3.2. In vitro effects on short chain fatty acid production

Changes in organic acid production measured by GC are shown in Table 2. Carbohydrate supplementation into the fermenters resulted in significantly different concentrations of organic acids with a probiotic treatment effect also being observed with acetic acid for both GOS and FOS ($p = 0.001$, $p < 0.0001$) (Table 2). Such effects were only seen with GOS supplementation for propionic acid ($p = 0.0006$), and valeric ($p = 0.0017$), and FOS with formic acid ($p = 0.04$). Other organic acids increased with time but no differences were seen between treatments. Overall concentrations of the three major fatty acids (acetate, propionate, butyrate) increased by a larger magnitude with BC30 compared to the placebo.

4. Discussion

Compositional changes in the microbiota can affect host health, the use of probiotic and prebiotic intervention is often suggested after destabilisation of the microbiota due to illness or antibiotic therapy [40,41]. Although the structure of prebiotics remains unchanged until arrival in the lower gut, survival rates of live bacteria (probiotics) may be affected by adverse conditions found in gastric juices and the small gut, leading to a potential fall in viability [42,43]. Several methods have been used to overcome this, including encapsulation [42,44].

Much is known about the beneficial effect of probiotics and prebiotics on the human gut microbiota and the favourable affects gained by an increase in specific gut genera [25,26,45]. However, the use of spore forming probiotics increases potential benefits to the host, particularly when combined with the positive benefits of a prebiotic. This is because spores offer improved survivability over vegetative cells. When looking at initial populations of the groups analysed, a paired *t*-test shows some significant differences between starting populations of specific groups of relevance (Table 3). Table 3 shows that after 28 day supplementation with either BC30 or the placebo, modulation of the faecal microbiota was such that populations of *Bacillus* spp. were significantly higher in those having consumed the placebo, as were *C. litsueburense* and *F. prausnitzii*. Table 1 shows that three of the groups seen to be significantly different at baseline did in fact respond better to the addition of the prebiotics increasing their populations significantly in during the fermentation. This suggests that consumption of BC30 was able to significantly increase populations of microbial groups known to elicit some beneficial effects [46]. *Bacillus* spp., differed significantly between treatments, and this may have a positive effect as they are linked to production of metabolites beneficial to gut health via creation of vitamins, minerals [36] and organic acids – which can alter the environment creating conditions adverse to pathogen colonisation.

Beneficial effects of saccharolysis (carbohydrate fermentation) are partly based on the resulting increased production of SCFA [47–49] (Table 1). Acetate and butyrate both significantly increased as a response to the addition for both prebiotics to the fecal microbiota of volunteers having consumed BC30, however propionate only responded to the addition of GOS, this appears to be in part due to the larger variance in the response to the FOS

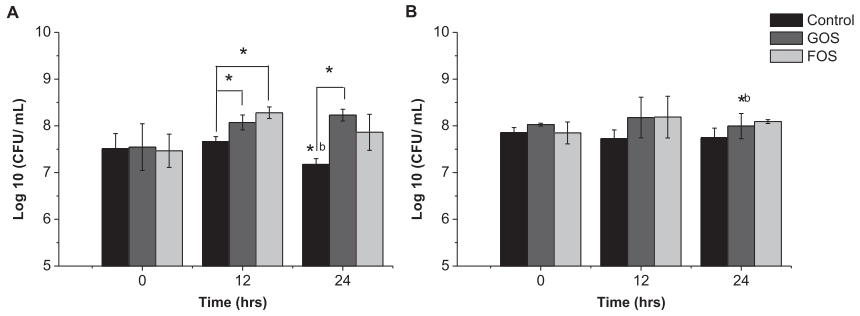

Fig. 2. Shows that 12 and 24 h supplementation of GOS and FOS significantly increased *Faecalibacterium prausnitzii*. Values are mean values at three time points from six faecal donors per treatment ± standard deviation. A ($n = 3$) – Volunteers ingesting placebo, B ($n = 3$) – Volunteers ingesting BC30; * ($p \leq 0.05$), ** ($p \leq 0.001$), *** ($p \leq 0.0001$) as compared to the control with no substrate added; *b significantly different to the same sample as compared to the different treatment.

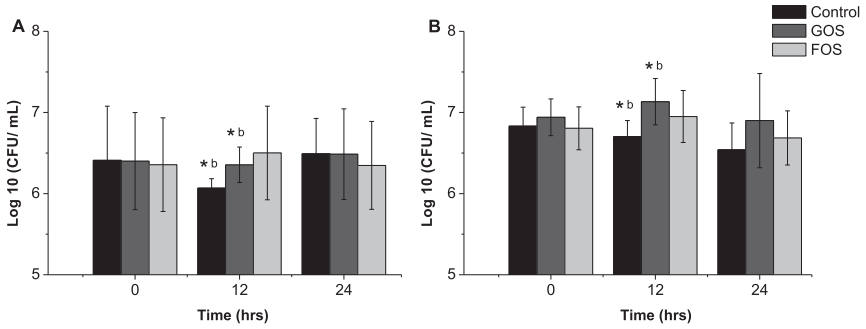

Fig. 3. Bacterial changes in *Bacillus* spp., as detected by Bcoa191 probe, over time as $\log^{10}$ CFU mL^{-1} in a single stage batch culture fermentation with 1% (w/v) galactooligosaccharides (GOS) and fructooligosaccharides (FOS) added with 1% (w/v) faecal inocula as analysed by fluorescent in situ hybridisation (FISH). Values are mean values at three time points from six faecal donors per treatment ± standard deviation. A ($n = 3$) – Volunteers ingesting placebo, B ($n = 3$) – Volunteers ingesting BC30; * ($p \leq 0.05$), ** ($p \leq 0.001$), *** ($p \leq 0.0001$) as compared to the control with no substrate added; *b significantly different to the same sample as compared to the different treatment.

Table 2
Short chain fatty acid production in the presence of different prebiotic substrates during pH-controlled batch culture fermentations with 1% fecal slurry inoculums from participants after 28 days on the placebo or GBC30.

	Time (h)	Concentrations of fatty acids in mM/mL						Sig#
		Placebo ($n = 3$)			BC30 ($n = 3$)			
		Control	GOS	FOS	Control	GOS	FOS	
Formic acid	0	1.24 ± 0.14	ND	1.25 ± 0.02	0.96 ± 0.1	0.52 ± 0.15	0.98 ± 0.2	a, b
	12	0.96 ± 0.07	ND	ND	0.43 ± 0.15	20 ± 18	39 ± 32	
	24	5.71 ± 0.7	1.7 ± 0.9	14.2 ± 0.48	2.86 ± 4	5.37 ± 8	14 ± 24	
Propionic acid	0	0.24 ± 0.07	0.25 ± 0.05	0.2 ± 0.04	1.26 ± 0.38	0.82 ± 0.12	1.4 ± 0.89	a, b
	12	2.25 ± 0.23	6.4 ± 5.2	6.5 ± 4.7	9.6 ± 1	**29 ± 8****	24 ± 14*	
	24	2.85 ± 0.12	12.4 ± 4.6	12 ± 9	12.1 ± 2.3	**29 ± 8****	28 ± 15*	
Acetic acid	0	1.49 ± 0.42	1.79 ± 0.71	11.4 ± 17.4	4.2 ± 0.77	2.3 ± 0.02	4.62 ± 2	a, b
	12	17.9 ± 1.7	59 ± 22	49.6 ± 16	434 ± 10	**165 ± 38*****	**175 ± 45** ***	
	24	23 ± 2.5	65 ± 15*	58.9 ± 3	60 ± 13	**137 ± 45*****	**149 ± 22*****	
Isobutyric acid	0	0.18 ± 0.22	0.044 ± 0.01	0.049 ± 0.004	0.1 ± 0.05	0.078 ± 0.06	0.11 ± 0.03	NS
	12	0.44 ± 0.54	0.33 ± 0.34	0.18 ± 0.08	0.54 ± 0.46	0.62 ± 0.5	0.55 ± 0.6	
	24	0.69 ± 0.64	0.66 ± 0.24	1.36 ± 0.03	1.29 ± 0.84	1.4 ± 1*	1.4 ± 1*	
Butyric acid	0	0.13 ± 0.06	0.16 ± 0.07	0.097 ± 0.01	0.82 ± 0.11	0.49 ± 0.16	0.93 ± 0.44	a, b
	12	1.87 ± 0.46	2.7 ± 1.9	8.3 ± 4.9	11.5 ± 4	20 ± 18*	28 ± 25*	
	24	2.97 ± 0.72	9.5 ± 2	13 ± 6.1	13.5 ± 1	**48 ± 16** ***	**63 ± 8** ***	
Isovaleric acid	0	0.04 ± 0.02	0.052 ± 0.02	0.36 ± 0.54	0.03 ± 0.013	0.03 ± 0.002	0.026 ± 0.005	b
	12	0.26 ± 0.19	0.1 ± 0.04	0.12 ± 0.03	0.19 ± 0.19	0.15 ± 0.2	0.081 ± 0.1	
	24	0.43 ± 0.23	0.27 ± 0.04*	0.18 ± 0.9	0.4 ± 0.28	0.2 ± 0.2*	0.18 ± 0.12	
Valeric acid	0	0.22 ± 0.05	0.22 ± 0.04	0.21 ± 0.07	0.12 ± 0.025	0.08 ± 0.06	0.12 ± 0.01	a
	12	5.78 ± 2.5	4.2 ± 2.4*	4.23 ± 1.8	3.5 ± 4	2.5 ± 2	2.2 ± 2	
	24	10.8 ± 1.9	**7.4 ± 1.4*****	3.3 ± 2.4*	5.2 ± 1.6	3.3 ± 2	3.3 ± 2	
Lactic acid	0	0.53 ± 0.02	0.65 ± 0.16	0.45 ± 0.06	1.9 ± 0.26	1.4 ± 0.71	1.8 ± 0.35	NS
	12	0.33 ± 0.37	11.9 ± 1.1	10.9 ± 7.8	0.47 ± 0.04	31 ± 35	41 ± 39*	
	24	0.06 ± 0.002	ND	0.41 ± 0.03	0.34 ± 0.07	0.31 ± 0.1	0.4 ± 0.01	
Succinic acid	0	21 ± 2.9	19.7 ± 2.2	21.2 ± 2.1	16.3 ± 2	23 ± 4	16 ± 5	NS
	12	18 ± 1.4	21.5 ± 0.9	22.5 ± 1.4	33 ± 29	18 ± 3*	21 ± 4	
	24	19 ± 1.03	20.2 ± 0.67	21 ± 3	23 ± 9	16.3 ± 2	21 ± 2	

GraphPad Prisim 5 2-way ANOVA with Bonferoni's post hoc tests multivariate comparison test were used to determine a significant difference in microbial populations compared to 0 h within each treatment and substrate $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. # Significant difference between treatments and substrates. $N = 3$ in each group. Values in bold show where a significant treatment effect was seen.
a – Significant effect of treatments (probiotic, placebo); b – significant effect of substrates (FOS, GOS).
GOS = galactooligosaccharides) and FOS = fructooligosaccharides.

supplementation nevertheless the average values were not too dissimilar and therefore this study could benefit from an increased number of volunteers to demonstrate this. *F. prausnitzii* and *E. rectale* showed both a substrate and treatment effect, with increased populations after consumption of BC30. Both groups are known to produce SCFA from dissimilation of hexoses sugars [50]. Clostridia are often associated with detrimental effects based on the known negative effect of many metabolites produced [22].However, these metabolites are from the dissimilation of dietary protein in the large gut (not carbohydrate) and they are large contributors to SCFA production in the gut.

In this study, the three main SCFA (acetate, propionate and butyrate) all increased over time in response to prebiotic fermentation, with higher concentrations produced from the faecal microbiota of volunteers with initial elevated *Bacillus* spp. Concentrations of SCFA were also higher than previously detected during fermentation of non-digestible carbohydrate using similar *in vitro* methods [51,52]. Branched chain fatty acids are primarily associated with the dissimilation of proteins and have been shown to increase during fermentation of several meat types [53]. Isovaleric and isobutyric acid did not increase in a response to addition of prebiotics, however treatment did appear to have an effect on valeric acid concentrations with samples from those on the placebo producing higher concentrations in response to GOS.

Table 3
Baseline comparisons in Log10 cells/mL^{-1} of different microbial groups.

Probe	*P*-value	Treatments
Bif164	0.004	a > b
Bac303	0.5	ns
Fpra645	0.01	a < b
Erec482	0.94	ns
CLit135	0.0005	a < b
Lab152	0.12	ns
Bcoa191	0.03	a < b

Paired *t*-test showing significant differences between fecal groups in the faecal microbiota of volunteers after a 28 supplementation of either a – placebo or b – BC30.

In summary, increased numbers of positive bacterial groups were observed in response to prebiotic supplementation. The fecal microbiota from persons ingesting BC30 showed increased numbers of *E. rectale, F. prausnitzii* and *C. lituseburense*, groups which are known to significantly affect the concentrations of organic end products. Increased concentrations of acetate, propionate and butyrate were also observed after the addition of prebiotic substrates to the fecal microbiota from volunteers after consuming a 28 day course of the probiotic BC30. Showing that the increase in probiotic populations and subsequent prebiotic supplementation may pose another route for improving gut health of older persons and those in a state of diminishing favourable activities of the gut microbiota.

5. Conclusions

Using *in vitro* fecal batch culture fermenters as a model for the microbiota we have demonstrated that samples which possessed an increased population of *Bacillus* spp. at baseline had a markedly elevated response to prebiotic supplementation than those which did not. Host consumption of BC30 and subsequent exposure of their fecal microbiota to prebiotics resulted in elevated populations

of other beneficial genera of bacteria which significantly increased production of many beneficial organic acids.

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Pathogenesis and Toxins

Use of a continuous culture fermentation system to investigate the effect of GanedenBC^{30} (*Bacillus coagulans* GBI-30, 6086) supplementation on pathogen survival in the human gut microbiota

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ABSTRACT

Single-stage continuous fermentation systems were employed to examine the effects of GanedenBC^{30} supplementation on the human gastrointestinal microbiota in relation to pathogen challenge *in vitro*. Denaturing gradient gel electrophoresis analysis demonstrated that GanedenBC^{30} supplementation modified the microbial profiles in the fermentation systems compared with controls, with profiles clustering according to treatment. Overall, GanedenBC^{30} supplementation did not elicit major changes in bacterial population counts *in vitro*, although notably higher Bcoa191 counts were seen following probiotic supplementation (compared to the controls). Pathogen challenge did not elicit significant modification of the microbial counts *in vitro*, although notably higher Clit135 counts were seen in the control system post-*Clostridium difficile* challenge than in the corresponding GanedenBC^{30}-supplemented systems. Sporulation appears to be associated with the anti-microbial activity of GanedenBC^{30}, suggesting that a bi-modal lifecycle of GanedenBC^{30} *in vivo* may lead to anti-microbial activity in distal regions of the gastrointestinal tract.

1. Introduction

Probiotics, including *Bacillus* species, are widely used for humans and other animals to confer health benefits on the host [1,2]. *Bacillus* species are aerobic or facultatively anaerobic Gram-positive spore-formers that have generally been considered allochthonous members of the gut microbiota. However, a number of recent studies have isolated several *Bacillus* species from the intestinal tracts of humans and other animals, suggesting they may be rare members of the commensal microbiota [3–6]. A number of studies have also demonstrated persistence of bacilli in the gastrointestinal tracts of animals and provided evidence for a bi-modal lifecycle of *Bacillus* strains in the mouse gut [7–9]. While a range of probiotic effects (including immunomodulatory, anti-microbial, and lipid-lowering effects) have been shown for different *Bacillus* strains, the best proven mechanisms of action to date are competitive exclusion [10,11] and modulation of the immune system [12–16]. Information in relation to the anti-microbial activity of *Bacillus* species is limited, but it is an area of interest.

Bacillus coagulans is currently available in commercial probiotic products, including GanedenBC^{30} (*B. coagulans* GBI-30, 6086), a proprietary probiotic preparation that is considered safe for human consumption and has been associated with easing of gastrointestinal symptoms (such as bloating and abdominal pain) in irritable bowel syndrome [17,18]. The anti-microbial potential of a number of *B. coagulans* strains has been reported. Hyronimus *et al.* [3] showed that *B. coagulans* I4 (originally isolated from cattle faeces) had anti-microbial activity via production of a bacteriocin-like inhibitory substance named coagulin. No data have been published in relation to the anti-microbial activity of GanedenBC^{30}, although the strain has been shown to reduce vancomycin-resistant enterococci (VRE) in mice via an unknown mechanism, and persist in the faeces of VRE-colonised mice post-probiotic supplementation [7]. The aim of the current study was to determine whether GanedenBC^{30} supplementation would reduce pathogen levels in continuous culture fermentation systems inoculated with human gastrointestinal microbiota and challenged with *Escherichia coli*, *Salmonella* Typhimurium, *Clostridium difficile* or *Clostridium perfringens*. Using the continuous culture system, we have previously demonstrated germination of GanedenBC^{30} spores and demonstrated persistence of the strain *in vitro* [19]. Similar

Abbreviations: DGGE, denaturing gradient gel electrophoresis; FISH, fluorescence *in situ* hybridization; GC, gas chromatography; SCFA, short-chain fatty acid; SS, steady state.

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systems have also been used for studying the effects of probiotics and synbiotics on intestinal pathogens [20,21].

2. Materials and methods

2.1. Bacterial strains

The probiotic used in this study was *B. coagulans* GBI-30, 6086 strain (GanedenBC^{30}), patented by Ganeden Biotechnology, Incorporated (Mayfield Heights, OH). The strain was provided freeze-dried and stored at 4 °C prior to use. Indicator pathogen strains, *C. difficile* CCUG 19126 (isolated from canine wound, toxigenic), *C. perfringens* (canine isolate), *E. coli* NCFB 1989^{T} and *Salmonella enterica* serovar Typhimurium LT2, were kindly supplied by Dr. Bernard M. Mackey and Dr. Eva Oguebon (Department of Food & Nutritional Sciences, University of Reading). Pathogens were incubated in nutrient broth at appropriate conditions (aerobically at 37 °C for *E. coli* and *Salmonella* Typhimurium, anaerobically (MARKIII with satellite; Don Whitley Scientific, UK; 80% N_2; 10% CO_2; 10% H_2) at 37 °C for *Clostridium* species) until late-exponential phase (10^7 to 10^8 cells/ml). Cells were harvested from 1 ml of late-exponential phase culture by centrifugation (11,714 *g*, 10 min) and resuspended in 1 ml phosphate-buffered saline (PBS; Oxoid, UK) prior to use.

2.2. Donors and preparation of faecal homogenates

Faecal samples were obtained from three healthy adults (2 females and 1 males; between 24 and 39 years old) who had not taken antibiotics nor consumed probiotics or prebiotics in the 3 months prior to the start of the study. Faecal homogenates (20%, w/w) were prepared from fresh faecal samples as described previously [19].

2.3. Continuous culture systems

Continuous culture fermentation systems were set up and run as described by Honda *et al.* [19]. Two continuous culture systems were run in parallel for each run: one was the control system and the other the probiotic-supplemented system. Different donors were used for each run, with duplicates being performed for each pathogen with the exception of *C. difficile* (triplicate runs were conducted). Following inoculation, the systems were run as batch cultures for 12 h, prior to continuous feeding with fresh medium at a rate of 10 ml/h for the duration of the experiment. Systems were operated to steady state (SS1; 108 h); thereafter GanedenBC^{30} (132 mg) was added daily to the probiotic-supplemented system. Systems were again run to steady state (SS2, 192 h) prior to pathogen challenge and then run for a further 96 h (with or without probiotic supplementation). Samples were taken from both systems (control and probiotic-supplemented) throughout the fermentation experiments (Table 1) and processed for denaturing gradient gel electrophoresis (DGGE), fluorescence *in situ* hybridization (FISH) as described previously [19]. Samples were also processed for cultivation work and short-chain fatty acid (SCFA) analysis.

2.4. Processing of samples for gas chromatography (GC) analysis of SCFAs

Aliquots (1 ml) of each sample were centrifuged at 11,714 *g* for 5 min at room temperature. Supernatants were transferred into new sterile 1.5 ml microcentrifuge tubes and stored at −20 °C for GC analysis of SCFAs. Samples were extracted and derivatized according to Richardson *et al.* [22]. Briefly, aliquots (500 μl) of samples were transferred into glass vials along with 50 μl of 2-ethylbutyric acid (10 mM; internal standard). Concentrated HCl (250 μl) and 1 ml diethyl ether were added and samples vortexed for 1 min. Samples were centrifuged at 2000 *g* for 10 min, and the diethyl ether layers transferred to new vials. A second extraction was conducted using a further 500 μl of diethyl ether. The diethyl ether layers were pooled and 400 μl of this pooled extract was mixed with 50 μl *N*-methyl-*N*-*t*-butyldimethylsilyltrifluoroacetamide (MTBSTFA; Cheshire Sciences) and incubated at 80 °C for 20 min. The samples were left at room temperature for 48 h in order to complete the derivatization of lactic acid. Derivatized samples (1 μl) were run on a 5890 SERIES II Gas Chromatograph (Hewlett Packard) with flame ionization detector, 30 m × 0.25 mm fused silica capillary column coated with 0.25 μm DBI-1 (Agilent Technologies). Injector and detector temperature was 275 °C, the column temperature was programmed from 60 °C for 3 min to 275 °C at 10 °C/min. Helium was used as a carrier gas (flow rate 1 ml/min; head pressure 8.4 psi). External standards (mM): sodium formate, 10; acetic acid, 30; propionic acid, 20; iso-butyric acid, 5; n-butyric acid, 20; iso-valeric acid, 5; n-valeric acid, 5; sodium lactate, 10; sodium succinate, 20. Chemstation B.03.01 (Agilent Technologies) was used for recording peak areas as well as calibration.

Table 1
Sampling and treatment schedules for the continuous culture systems used in this study.

Samples taken/treatment[a]	Samples processed for
Vessels A + B inoculated	
5 ml samples taken from vessels A + B	DGGE, FISH, SCFA, cultivation
t_{12} − medium flow turned on	
t_{84} − 2 ml samples taken from vessels A + B	DGGE, SCFA
t_{96} − 2 ml samples taken from vessels A + B	DGGE, SCFA
t_{108} − 5 ml samples taken from vessels A + B, then 132 mg GanedenBC^{30} added to vessel A	DGGE, FISH, SCFA, cultivation
t_{132} − 132 mg GanedenBC^{30} added to vessel A	
t_{156} − 132 mg GanedenBC^{30} added to vessel A	
t_{168} − 2 ml samples taken from vessels A + B, then 132 mg GanedenBC^{30} added to vessel A	DGGE, SCFA
t_{180} − 2 ml samples taken from vessels A + B	DGGE, SCFA
t_{192} − 5 ml samples taken from vessels A + B, then 132 mg GanedenBC^{30} added to vessel A, then pathogen added to vessels A + B and 2 ml samples taken from vessels A + B for pathogen cultivation	DGGE, FISH, SCFA, cultivation
t_{204} − 5 ml samples taken from vessels A + B, then 132 mg GanedenBC^{30} added to vessel A	DGGE, FISH, SCFA, cultivation
t_{216} − 5 ml samples taken from vessels A + B, then 132 mg GanedenBC^{30} added to vessel A	DGGE, FISH, SCFA, cultivation
t_{240} − 5 ml samples taken from vessels A + B, then 132 mg GanedenBC^{30} added to vessel A	DGGE, FISH, SCFA, cultivation
t_{264} − 5 ml samples taken from vessels A + B, then 132 mg GanedenBC^{30} added to vessel A	DGGE, FISH, SCFA, cultivation
t_{288} − 5 ml samples taken from vessels A+B	DGGE, FISH, SCFA, cultivation

[a] *t*, time in hours. Vessel A, probiotic-supplemented; vessel B, control.

2.5. Processing of samples for cultivation work

Cultivation work to enumerate spore-formers was done from ethanol-treated samples as described previously [19]. Enumeration of pathogens was performed by spread-plating 100 μl of serially (ten-fold) diluted samples onto appropriate selective agars (in triplicate). MacConkey agar No. 3 (Oxoid) was used for *E. coli* and *Salmonella* Typhimurium, and Tryptose Sulphite Cycloserine agar (Oxoid) was used for *C. perfringens*. Cultivation work was not

conducted for *C. difficile* since no selective agar was available for this bacterium. Following appropriate incubation, selective colony counts were performed, with results expressed as cfu/ml sample.

2.6. FISH analysis

FISH was conducted as described by Martín-Peláez *et al.* [23], using a broad range of probes (Table 2). Cyanine 3-labelled probes were supplied by Sigma Genosys (Sigma).

2.7. DNA extraction from samples

Samples stored at −20 °C in PBS/glycerol were vortexed and placed on ice. DNA was extracted as described by Hoyles [6]. Briefly, aliquots (250 μl) were washed twice in 1 ml PBS, then resuspended in 0.5 ml TES buffer. Samples were incubated at 37 °C in the presence of 8 μl lysozyme (10 mg/ml) and 2 μl mutanolysin (4500 U/ml) for 30 min. Proteinase K (10 μl; 20 mg/ml) and RNase (10 μl; 10 mg/ml) were added to the samples, which were mixed by vortexing and incubated at 65 °C for 1 h; 100 μl of 10% sodium dodecyl sulfate was added to the samples, and they were incubated for a further 15 min at 65 °C. Phenol/chloroform extractions were done on the samples. Samples were then centrifuged at 6500 rpm for 10 min. DNA was precipitated from the upper aqueous layer with ice-cold ethanol, then the samples were centrifuged at 13,000 rpm for 10 min. Supernatants were removed carefully, and the DNA dried before being eluted in 50 μl sterile H_2O. The quality of DNA in each sample was assessed by running 5-μl aliquots of the samples on 1.5% ultraPURE agarose gels in 1 × TAE buffer containing ethidium bromide (0.4 mg/ml in sterile H_2O; Sigma), and visualizing the bands using UV light. The amount (ng/μl) of DNA in each sample was assayed by using a ND-1000 NanoDrop spectrophotometer, and samples diluted to 5 ng/μl for use in PCR.

2.8. PCR-DGGE and silver staining of gels

The universal primers of Muyzer *et al.* [24] were used in PCRs, with mixtures comprising 5 μl of 10 × $MgCl_2$-free buffer (Promega), 5 μl dNTPs (12.5 mM each; Promega), 1 μl each primer (20 pmol), 6 μl $MgCl_2$ (25 mM; Promega), 30 μl H_2O, 1 μl *Taq* (1.25 U DNA polymerase; Promega) and 1 μl template DNA. Amplification products were examined by using agarose gel electrophoresis; products were stored at −20 °C until required. Electrophoresis was run at a constant voltage of 100 V using the V20-HCDC DGGE system (BDH); PCR samples (5 μl of each) were applied directly onto polyacrylamide gels. Silver staining of gels was carried out as described by Roger [25]. Gels images were scanned and TIFF files saved at 600 dpi were analysed using GelCompar II (Applied Mathematics, Belgium). Bands within a profile with peak areas of less than 1% of the total peak area for the profile were excluded from all analyses.

2.9. Statistical analysis

Paired, two-tailed Student's *t*-test was used to determine statistically significant differences within treatments (control or GandenBC^{30}-supplemented) over time and between treatments at each time point. For the purposes of statistical analysis of FISH counts, the detection limit [232,065.9 cells/ml sample] was used when levels were below the detection limit. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Enumeration of bacteria via cultivation

Overall, aerobic spore-formers were observed in all samples that were analysed, regardless of system (control or probiotic-

Table 2
Details for the oligonucleotide probes used for FISH analysis in this study.

Probe(s)	Sequence (5′ to 3′)	Enzyme pre-treatment	Formamide concn (%)	Hybridization temp. (°C)	Washing temp. (°C)	Target group/molecule	Reference(s)
EUB338/ EUB338II/ EUB338III[a]	GCTGCCTCCCGTAGGAGT GCAGCCACCCGTAGGTGT GCTGCCACCCGTAGGTGT	No	35	46	48	Most bacteria/16S rRNA	[29]
Ato291	GGTCGGTCTCTCAACCC	Lysozyme	0	50	50	*Cryptobacterium curtum*, *Gordonibacter pamelaeae*, *Paraeggerthella hongkongensis*, all *Eggerthella*, *Collinsella*, *Olsenella* and *Atopobium* species/16S rRNA	[30]
Bac303	CCAATGTGGGGGACCTT	No	0	46	48	Most members of the genus *Bacteroides*, some *Parabacteroides* and *Prevotella* species, *Paraprevotella*, *Xylanibacter*, *Barnesiella* species and *Odoribacter splanchnicus*/16S rRNA	[31]
Bcoa191	GCCGCCTTTCCTTTTTCCTCC	Lysozyme	20	46	48	*Bacillus coagulans*/16S rRNA	[32]
Bif164	CATCCGGCATTACCACCC	Lysozyme	0	50	50	Most *Bifidobacterium* species and *Parascardovia denticolens*/16S rRNA	[33]
Chis150	TTATGCGGTATTAATCTYCCTTT	No	0	50	50	Most members of *Clostridium* clusters I and II (including *C. perfringens*)/16S rRNA	[34]
Clit135	GTTATCCGTGTGTACAGGG	No	0	50	50	Part of *Clostridium* cluster XI (including *C. difficile*)/16S rRNA	[34]
EC1531	CACCGTAGTGCCTCGTCATCA	No	35	37	37	*Escherichia coli*/23S rRNA	[35]
Erec482	GCTTCTTAGTCARGTACCG	No	0	50	50	*Clostridium* cluster XIVa/16S rRNA	[34]
Lab158	GGTATTAGCAYCTGTTTCCA	Lysozyme	0	50	50	All *Oenococcus*, *Vagococcus*, *Melissococcus*, *Tetragenococcus*, *Enterococcus*, *Catellicoccus*, *Paralactobacillus*, *Pediococcus* and *Lactococcus* species, most *Lactobacillus*, *Weissella* and *Leuconostoc* species/16S rRNA	[36]
Prop853	ATTGCGTTAACTCCGGCAC	No	0	50	50	*Clostridium* cluster IX/16S rRNA	[37]
Rbro730/ Rfla729[a]	TAAAGCCCAGYAGGCCGC AAAGCCCAGTAAGCCGCC	Lysozyme	20	50	50	*Anaerotruncus colihominis*, *Ruminococcus bromii*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Desulfotomaculum alcoholivorax*/16S rRNA	[38]
Sal3	AATCACTTCACCTACGTG	No	0	45	45	*Salmonella* subspecies I, IIIb and VI/23S rRNA	[39]

[a] These probes were used together in equimolar concentrations (50 ng μl^{-1}).

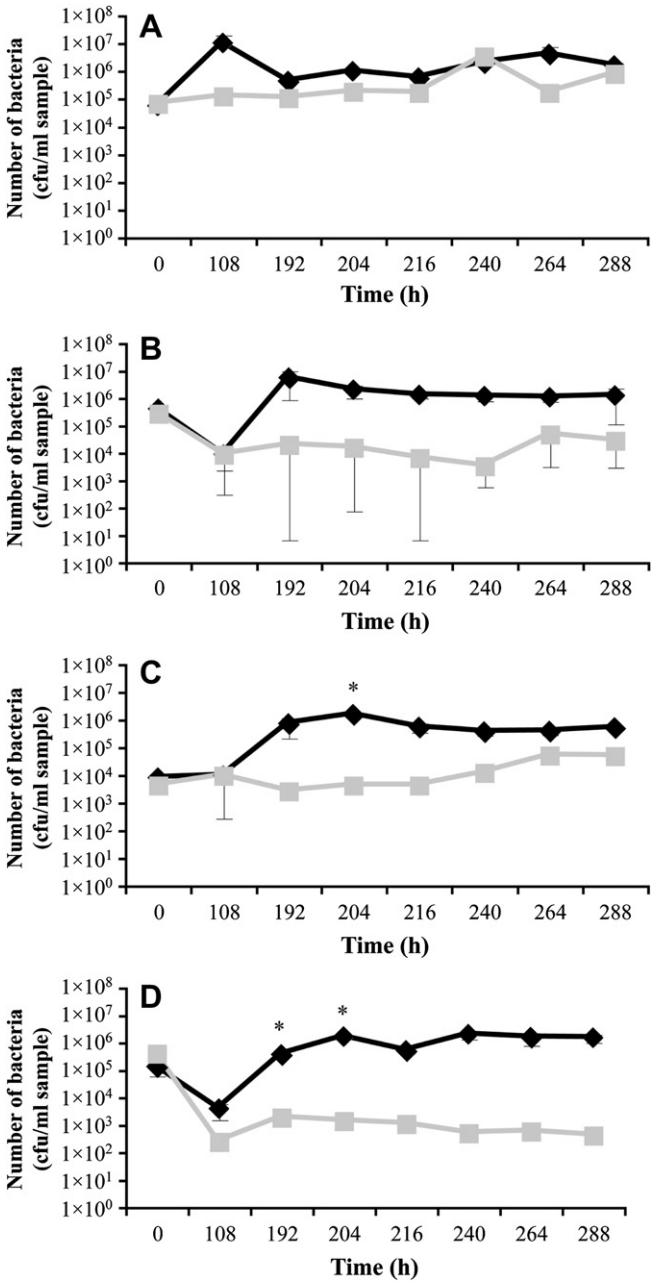

Fig. 1. Enumeration of aerobic spore-formers in control and GanedenBC^{30}-supplemented single-stage continuous culture fermentation systems before and after pathogen challenge. A, *C. difficile* challenge; B, *C. perfringens* challenge; C, *E. coli* challenge; D, *Salmonella* Typhimurium challenge. Data are presented as mean ± standard error. Black diamonds, GanedenBC^{30}-supplemented systems; grey squares, control systems. GanedenBC^{30} was added daily to GanedenBC^{30}-supplemented systems after t_{108} (SS1) and pathogens were added to both systems immediately after t_{192} (SS2) sample was taken. *Significantly different ($P < 0.05$) from control systems at the same time point.

supplemented) and pathogen (Fig. 1). With the exception of the systems that were subsequently challenged with *C. difficile*, aerobic spore-former counts of around 10^4 cfu/ml sample were generally seen at SS1 (108 h fermentation; Fig. 1B–D). These levels were essentially maintained in the control systems thereafter, although some fluctuation was seen 72 h and 96 h post-pathogen challenge for *C. perfringens* and *E. coli* (Fig. 1B and C). Probiotic supplementation generally corresponded with increased counts of aerobic spore-formers (SS2; 192 h fermentation), and was not affected by pathogen challenge. As noted above, the aerobic spore-former counts for the *C. difficile* systems gave somewhat anomalous results, with higher SS1 counts than the other systems and no distinction between the probiotic-supplemented and control systems (Fig. 1A). This correlated with additional colony morphotypes other than that of GanedenBC^{30}, not seen during the other runs.

Selective cultivation of *C. difficile* was not achieved in this study, due to background contamination from the faecal microbiota (data not included). Overall, GanedenBC^{30} supplementation did not alter the mixed microbiota effect on the pathogen colony counts for *E. coli*, *C. perfringens* or *Salmonella* Typhimurium (data not included). Counts for *C. perfringens* and *E. coli* were essentially maintained at inoculation levels in both control and probiotic-supplemented systems post-challenge, while *Salmonella* Typhimurium counts diminished in both systems during the first 12 h post-challenge and then remained around 5 $\log_{10}$(cfu/ml sample) thereafter. During one run of *E. coli* challenge systems high counts were obtained in both systems 72 h post-challenge (264 h), but decreased to similar levels as seen in all other post-challenge samples by 96 h post-challenge (288 h) (data not included).

3.2. Enumeration of specific bacteria/bacterial populations via FISH

Overall, FISH analysis did not identify significant modification of the microbiota *in vitro* following GanedenBC^{30} supplementation, although notably higher Bcoa191 counts were seen in the probiotic-supplemented systems compared to the controls after SS1. Pathogen levels (i.e. *E. coli*, *Salmonella* Typhimurium and *C. perfringens*) were not significantly affected by GanedenBC^{30} supplementation (data not shown). However, *C. difficile* appeared to be inhibited in the probiotic-supplemented vessels, with notably higher Clit135 counts seen in the control system post-*C. difficile* challenge than in the corresponding GanedenBC^{30}-supplemented systems (Fig. 2; Clit135). Inter-run variations were evident which reduced the significance of this observation, although we still conclude that some inhibitory activity was apparent. Some fluctuations in probe counts were also observed over the course of the fermentation experiment (i.e. within systems over time), although these were generally minor (i.e. <1 $\log_{10}$ count changes) and statistical significance was attained in some instances. It is possible that the observed inter-run variations were due to the inherent subjectivity associated with counting bacteria via FISH: use of a negative control with hybridized samples could be incorporated into future studies to overcome this. Importantly, pathogen challenge did not elicit significant modification of the microbiota *in vitro* (both control and probiotic-supplemented systems). The proportion of the total microbiota detected for each sample was generally >60%, although 30–50% coverage was seen for some of the samples (including SS1 and/or SS2) from the *E. coli* and *Salmonella* Typhimurium challenge systems inoculated with faceal homogenate from donor 1.

3.3. Investigation of microbial diversity using DGGE analysis

DGGE analysis of individual runs was performed (i.e. per gel) using GelCompar II. Consecutive samples taken leading up to calculated steady states (84 h, 96 h and 108 h [SS1]; 168 h, 180 h and 196 [SS2]) clustered together, indicating obtention of steady state (data not included). Subsequent DGGE analysis was performed using 0 h, SS1, SS2 and post-pathogen challenge samples only, to examine the effects of probiotic supplementation and pathogen challenge on the microbiota.

In general, initial samples (0 h, taken immediately after inoculation of the systems) clustered together with high similarity (>90%) (Fig. 3). The one exception to this was the second run for *Salmonella* Typhimurium challenge, where a number of bands were not evident in the 0 h profile of the system subsequently

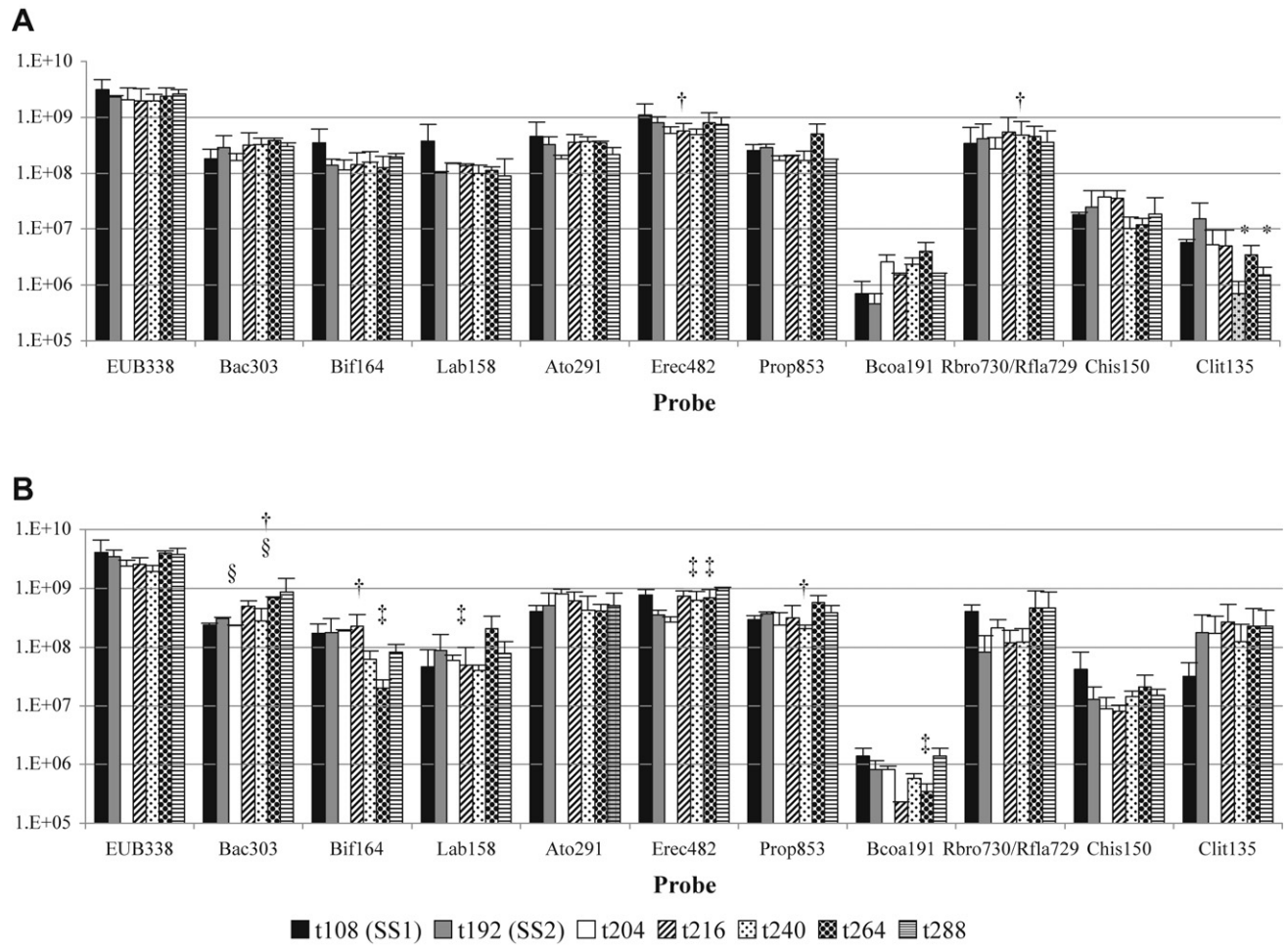

Fig. 2. *In vitro* investigation of the effects of GanedenBC^{30} supplementation on the faecal microbiota and its response to *C. difficile* challenge, using FISH. Data are presented as mean + standard deviation. A, GanedenBC^{30}-supplemented system; B, control system. GanedenBC^{30} was added daily to GanedenBC^{30}-supplemented systems after t_{108} and *C. difficile* was added to both systems immediately after t_{192} samples were taken. *Significantly different from t_{108}; †Significantly different from t_{192}; ‡Significantly different from the previous sample time; §Significantly different from probe count for control system at same sample time. Statistical significance was taken as $P < 0.05$.

supplemented with GanedenBC^{30} (data not shown). In most runs, clustering was also seen according to treatment, control versus probiotic supplementation (i.e. clustering of profiles taken from the same system at and after SS2). However, one run displayed very similar profiles across both systems throughout, with 97.4% similarity between the control and GanedenBC^{30}-supplemented systems' SS2 profiles (data not included), and similar subtle changes to profiles of each system seen after *C. perfringens* challenge.

Overall, DGGE analysis demonstrated that GanedenBC^{30} supplementation modified the microbial profile distinctly different from the drift seen in control systems (between SS1 and SS2) and that minor shifts in profiles were observed in both control and GanedenBC^{30}-supplemented systems post-pathogen challenge (Fig. 3).

3.4. SCFA analysis

Total SCFAs were quite low (7–14 mM) in initial samples (0 h; immediately after inoculation) from all systems (data not included). Thereafter, total SCFAs were between 55 and 142 mM with butyric acid identified as the major SCFA (on average), followed by acetic acid and propionic acid in both control and GanedenBC^{30}-supplemented systems. Overall, no statistically significant differences were observed between SCFA levels in probiotic and control systems at the same time point (data not included). Pathogen challenge did not elicit any notable shifts in SCFA profiles of any systems.

4. Discussion

The current study extended our previous research investigating the effect of GanedenBC^{30} supplementation on the human gastrointestinal microbiota *in vitro* [19], examining the effect of such supplementation on reducing pathogen levels *in vitro*. In accordance with the previous study, GanedenBC^{30} supplementation did not elicit major changes in the microbiota *in vitro* as determined by FISH, although the microbial diversity of control and GanedenBC^{30}-supplemented systems was somewhat divergent (DGGE profiles clustered separately). This is typical in probiotic studies *in vitro* and *in vivo* (i.e. inclusion of one or more microbial species does not markedly affect composition at the genus level). Overall, pathogen challenge of the *in vitro* systems showed little effect microbiologically, whether in control or GanedenBC^{30}-supplemented systems. Our view is that this reflects a 'healthy gut microbiota' in both systems, neither of which was perturbed by pathogen challenge. This is further supported by the fact that *in vitro* pure culture work done in our laboratory has shown that GanedenBC^{30} has antimicrobial activity against *C. difficile*, *C. perfringens* and *Listeria monocytogenes* (to varying degrees) (Kuforiji and McCartney, unpublished data). Anti-microbial activity was assessed using a colony overlay assay modified from Barbosa *et al.* [4]. Seven *Bacillus licheniformis* strains (previously isolated from healthy humans' faecal samples and identified as potential 'probiotics'; [6])

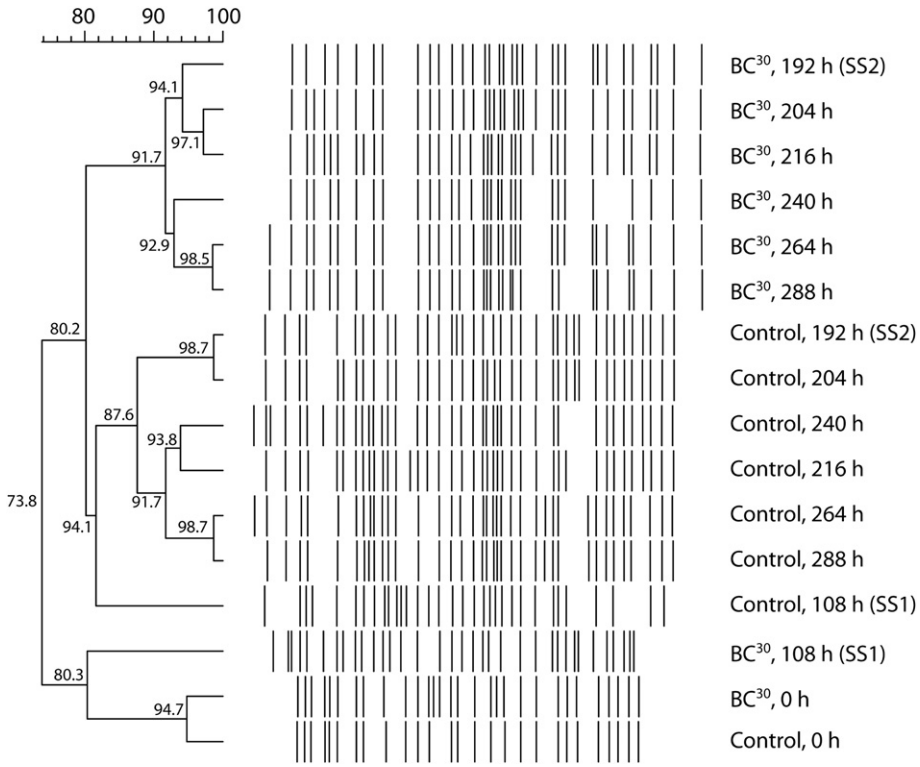

Fig. 3. Investigation of the effects of GanedenBC^{30} supplementation on universal PCR-DGGE profiles during single-stage continuous culture fermentations (*C. perfringens* challenge, donor 1). The dendrogram was generated using Dice/UPGMA. BC^{30}, GanedenBC^{30}-supplemented system. Scale bar, similarity (%).

and GanedenBC^{30} were grown on different substrates and screened for anti-microbial activity against *C. difficile*, *C. perfringens*, *L. monocytogenes* and *Staphylococcus aureus*. GanedenBC^{30} did not grow well on some substrates, but clear zones of inhibition (0.5–4.9 mm) were observed for *C. difficile* and *C. perfringens* when GanedenBC^{30} was grown on resistant starch.

A major influence of anti-microbial activity of GanedenBC^{30} during the *in vitro* fermentation runs may be due to the state of the probiotic cells, namely that sporulation had generally not occurred within the systems and thus the concentration of anti-microbial compounds was low. This is in accord with previous work by Ducluzeau *et al.* [26,27] which demonstrated anti-microbial activity of *B. licheniformis* in monoxenic/dixenic mice. Ducluzeau *et al.* suggested sporulation and anti-microbial production were associated, and they speculated that sporulation only occurred in the upper gastrointestinal tract of the gnotobiotic mice – although anti-microbial activity was rarely evidenced in the upper gastrointestinal tract samples, yet constantly present in the cecum and colon samples [27]. The data from Ducluzeau *et al.* [27] does, however, clearly demonstrate the bi-modal lifecycle of *B. licheniformis* in the mouse gastrointestinal tract, although this may be affected by the presence of other bacteria in the gastrointestinal tract. Indeed, the order of establishment was identified as an important facet in anti-microbial production of *B. licheniformis* [26]. Although it should be noted that the protocol for initial experiments did not guarantee delivery of vegetative *Bacillus* cells to *C. perfringens* monoxenic mice who were housed with *B. licheniformis* monoxenic mice. However, taken together with the results from their second study [27], the data suggest that establishment and sporulation of *B. licheniformis* in the upper gastrointestinal tract of gnotobiotic mice is diminished in the presence of *C. perfringens* and *Lactobacillus* 313.

The culture medium used here is rich and not designed to induce sporulation. In patients where antibiotic intake is occurring the situation is likely to differ and therefore our previously observed anti-clostridial activity is feasible. A prospective human study would clarify this. This is supported by our previous work, which clearly demonstrated persistence of GanedenBC^{30} post-supplementation *in vitro*, indicating administered spores (or some thereof) had entered vegetative state [19] (even in the presence of a mixed microbiota) and by *in vitro* pure culture work by Osmanova [28] which demonstrated anti-microbial activity of *B. licheniformis* 8-2AIA correlated with sporulation. We propose that both the level of growth (i.e. number of vegetative cells) and phase of growth (i.e. exponential phase, late-exponential phase, or sporulation) are important factors in the expression of anti-microbial compounds from *Bacillus* probiotics. Taken further, this may suggest that a bi-modal lifecycle of GanedenBC^{30} *in vivo* may lead to anti-microbial activity in distal regions of the gastrointestinal tract. This is an important facet given the prevalence of gut disorders in the left side of the large intestine and warrants further investigation.

In conclusion, GanedenBC^{30} supplementation did not elicit major changes in bacterial population counts *in vitro*, although notably higher Bcoa191 counts were seen following probiotic supplementation (compared to the controls) and DGGE analysis demonstrated clustering according to treatment. The fact that the GanedenBC^{30} was able to maintain its populations in the mixed community (including in the presence of pathogen challenge) shows that it is a robust strain.

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Bacillus coagulans as a probiotic

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Abstract

Probiotics are live microbial feed additions that improve human or animal health. Their activities are towards improving the composition of the gastrointestinal microbiota in a manner that reduces the risk of disorder. In some cases, probiotics are also used therapeutically. Most probiotics use lactobacilli or bifidobacteria as the main constituents. These produce lactic acid as well as other anti-pathogenic attributes. Traditionally, probiotics are incorporated in dairy products (yoghurts or fermented drinks) or in lyophilised form. Because of stability and viability factors, heated products are not usually a target for probiotic use. This is because they are temperature sensitive. However, a spore-forming genus would have the ability to overcome this limitation. Here, we discuss evidence for the spore-forming Gram-positive bacterium *Bacillus coagulans* as a probiotic.

Keywords: probiotics, *Bacillus*, spore formers

1. Introduction

The gut microflora acts as an effective barrier against opportunistic and pathogenic microorganisms, and this 'colonisation resistance' is one of its most important functions (Gibson and Rastall 2006). However, the gut flora itself can be subdivided into benign, beneficial and potentially harmful species, although certain genera contain species belonging to both groups, e.g. bacteroides may be saccharolytic (beneficial) or proteolytic (potentially harmful). Bacterial metabolism can result in a number of advantageous effects, including the production of vitamins, modulation of the immune system, enhanced digestion and absorption, inhibition of harmful species and removal of carcinogens and other toxins (Conway 1995). Furthermore, bacterial metabolism results in the production of short-chain fatty acids (SCFA) upon which the colonic mucosa is dependent. Negative effects include the production of toxins and carcinogens, constipation or diarrhoea, liver damage, predisposition towards gut disorder and intestinal putrefaction. More specifically, the resident microflora is known to contain pathogens such as yeasts and clostridia which, if allowed to overgrow, can disrupt normal gut function (Cummings and Macfarlane 1991).

In addition, the gut can be 'contaminated' by pathogens that cause acute effects. Clinical features of acute gastroenteritis vary greatly and can range from mild to life threatening. Symptoms may include vomiting, nausea, abdominal cramps and/or diarrhoea depending on the causative agents (Mitsuoka 1992; Tancrède 1992; Guarner 2006). Whilst viruses probably cause the most cases of gastroenteritis in children and adults, it is bacteria that are most problematic in terms of spread and ubiquity. They are able to upset gut homeostasis by invasive means as well as toxin generation (Macfarlane and McBain 1999).

Health-promoting effects of the microflora may include immunostimulation, improved digestion and absorption, vitamin synthesis, inhibition of the growth of potential pathogens, cholesterol reduction and lowering of gas distension. Harmful effects are carcinogen production, intestinal putrefaction, toxin production, diarrhoea/constipation, liver damage and intestinal infections (Fooks *et al*. 1999; Guarner and Malagelada 2003). Bifidobacteria and lactobacilli are considered to be examples of health-promoting constituents of the microflora. Lactobacilli may aid digestion of lactose in lactose-intolerant individuals, reduce constipation and infantile diarrhoea, help to resist infections such as salmonellae, prevent traveller's diarrhoea

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and help in irritable bowel syndrome (IBS). Bifidobacteria are thought to stimulate the immune system, produce B vitamins, inhibit pathogen growth, reduce blood ammonia and blood cholesterol levels and help to restore the normal flora after antibiotic therapy. Both probiotics and prebiotics are functional foods that fortify the lactate flora of the human or animal gut (Fuller 1989, 1992, 1997; Gibson and Roberfoid 1995).

2. Background to probiotics

The most frequently used dietary method of influencing the gut flora composition is that of probiotics, whereby live microbial additions are made to appropriate food vehicles (Bengmark 1998). The first recorded intake of deliberate bacterial consumption goes back more than 2000 years ago. However, the situation was put onto a scientific note by the work of Metchnikoff at the Pasteur Institute (Metchnikoff 1907). He hypothesised that longevity in Bulgarians was associated with their elevated intake of "soured milks", i.e. dairy-based drinks containing live bacteria, what would now be recognised as probiotics.

Important findings that helped the development of probiotics included the observation that faeces contained 'protective' factors that could help to promote resistance to infections such as salmonellae and *Clostridium difficile*. The protection could not be replicated in germ-free animals. One of the most popular definitions of probiotics is "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller 1992). A recent formal definition of probiotics was agreed by a working part of European scientists and is given as "a live microbial feed supplement that is beneficial to health" (www.isapp.net). This emphasised the importance of definitive improvements in health as well as the possibility that probiotics could have effects systemic to the gut, e.g. vagina, skin, and mouth.

Over the years, many species of microorganisms have been used. They consist mainly of lactic acid bacteria (lactobacilli, streptococci, enterococci, lactococci, bifidobacteria) but also *Bacillus* spp. and fungi such as *Saccharomyces* spp. and *Aspergillus* spp. The commonest probiotics belong to the genera *Lactobacillus* (e.g. *L. casei*, *L. acidophilus*, *L. rhamnosus*, *L. plantarum*, *L. johnsonii* and *L. reuteri*) and *Bifidobacterium* (e.g. *B. bifidum*, *B. lactis*, *B. longum* and *B. breve*). Probiotics are commercially available for consumption, mainly either as specific freeze-dried preparations such as tablets, sprays, capsules or powders, or as foods that are not subject to heat treatment, e.g. dairy products and fruit drinks (Tannock 1999, 2001).

For probiotics, there is likely to be no upper dose. However, given the vast numbers of bacteria present in the gastrointestinal tract, it is not likely that a product will exert any significant effect at an intake dose less than 10^7. Probiotics are marketed as health, or functional, foods whereby they are ingested for their purported positive advantages in the digestive tract and/or systemic areas like the liver, vagina or bloodstream. Unlike new drugs or pharmaceuticals, which are intensively screened for safety and effectiveness, probiotics are not so rigorously assessed. Nevertheless, consumers should be provided with an accurate assessment of physiological, microbial and safety aspects. Several criteria for the appropriate use of probiotics exist and may be summarised as follows (Goldin 1998; Gibson *et al*. 2000; Dunne *et al*. 2001):

- They should exert a proven beneficial effect on the consumer, preferably with a mechanistic explanation of how this occurred.
- They should be non-pathogenic, non-toxic and free of adverse side effects. This should be assessed by strain and produce a standard that is comparable with the USDA's GRAS (generally recognised as safe) criteria. It is not acceptable to base safety assessments on historical data, which may even include related but not identical strains.
- They should maintain stability in the product. This should be the case at the end of shelf life, not just the start.
- They should contain a large number of viable cells. It is unlikely that a daily dose of below 10^7 per millilitre product is likely to be effective.
- They should survive well in the gastrointestinal tract.
- They should have good sensory and mouth-feel properties.
- They should have accurate product labelling and content.

Much effort has concentrated on identifying probiotic bacteria and characterising their beneficial credentials. It is generally considered that probiotic bacteria must possess certain properties. The probiotic must survive passage through the upper regions of the gastrointestinal tract and persist in the lower gut. There must be no adverse host response to the bacterium, its components or metabolic end products. Advances in the genetics of probiotic strains (usually lactic acid bacteria or yeasts) have enabled the determination of mechanisms involved in probiotic function, such as production of antimicrobials, mucosal adhesins and organic acids. However, this also offers the possibility of modifying existing strains to increase survival and efficacy in the human gastrointestinal tract (Gilliland 1985; Fooks *et al*. 1999).

Many of these traits also apply to prebiotics although being non-viable dietary components, product survival characteristics are not applicable. The active ingredient should be defined, any product impurities should be quan-

tified and operable dosage should be suggested (Gibson and Roberfroid 1995, 1999).

One concerning aspect of the probiotic market is that labelling is not always accurate in terms of microbial content and numbers; this should be addressed by the use of molecular diagnostic procedures. It is imperative that labelling and actual contents should be identical. When growing microorganisms in bulk culture, such as for use as probiotics, it is reasonable to assume that some genetic drift in their integrity occurs. Moreover, it will be the case that certain products become contaminated during processing and/or storage. Regular quality control (preferably using molecular-based methods of identification) should be carried out. Testing for every lot should include enumeration, full microbial testing to ensure no contamination and sequencing of the product to ensure no genetic drift.

3. Benefits of probiotics

Major advances have been made in the application of pharmaceutical-based interventions for improving human health. Nevertheless, the incidence of acute and chronic disorder continues to rise, with many new diseases being untreatable. Whilst this is partly related to better detection and diagnosis of disorder, the fact remains that drug-based therapies are often inefficient, expensive and may have unwarranted side effects. Moreover, the indiscriminate use of antibiotics has led to a reduction in their potency and efficiency. As the population continues to age, there is increasing susceptibility among consumers to morbidity and mortality. In later years, there is more emphasis upon long-term care rather than treatment. This necessitates a more prophylactic approach towards improved health not only for the elderly but also for the younger generations who are increasingly exposed to risk. However, for the approach to succeed, a multi-disciplinary science programme that has long-term vision but short to medium term benefits is required. Crucially, the research should be underpinned by investigating mechanisms that underlie diet–disease relationships across the entire food chain, as well as the social interactions required. Attention has therefore markedly turned towards dietary ingredients that may offer improved health bonuses. Public and industrial perceptions of the importance of gut microbiology in human health and nutrition have led to a major increase in prebiotic and prebiotic-based products. However, for the full value to be realised, it is imperative that such developments are based upon sound scientific principles and research that provide irrefutable information on efficacy, and this is now happening.

Several different avenues are being explored for pre/probiotics. These are largely mediated by effecting an increase in beneficial bacteria within the gut flora. The different avenues are as follows (e.g. Salminen *et al*. 1998; Gibson *et al*. 2000; Marteau *et al*. 2001):

- **Improved tolerance to lactose**: it is thought that probiotics may help, in this regard, through their lactase activity, which is an enzyme that degrades lactose. Probiotics are generally saccharolytic in nature.
- **Protection from gastroenteritis**: beneficial gut flora components are inhibitory towards bacterial and viral pathogens. This may occur through the excretion of antimicrobial substances (acids and antibiotics), competition for colonisation sites and nutrients. This also has relevance for more chronic diseases caused by pathogens (e.g. ulcerative colitis).
- **Reduction of toxins**: stimulating a more beneficial community will reduce toxin levels, perhaps including carcinogens.
- **Cholesterol reduction**: it has been suggested that some probiotics can degrade cholesterol in the gut as well as produce metabolites that interfere with its synthesis in the liver.
- **Vitamin synthesis**: some probiotics can synthesise various vitamins, largely of the B group.
- **Irritable bowel syndrome**: IBS is a major consumer of general practitioner's time, and recent evidence has implicated a 'dysfunctional' gut flora. This may be addressed through probiotics.
- **Improved digestion and gut function**: an active gut flora helps to adequately digest the 60–80 g of food that enters the adult colon each day. Probiotics have a major role in carbohydrate degradation.
- **Food allergy**: it has been suggested that gut flora modulation may down-regulate gut inflammation and hypersensitivity that would otherwise lead to atopic eczema.
- **Immune regulation**: a stimulation of the non-specific immune response through non-pathogenic means may help to improve resistance to infection.
- **Mineral bioavailability**: a reduced pH in the bowel because of lactic fermentation enables better sequestration of calcium and magnesium.

A recent (July 2010) search of available research articles on PubMed shows around 6000 reported studies of probiotics, with almost 700 human studies.

The mechanism by which protection is offered by these probiotics has not yet been fully established. However, one or more of the following are possible (Fuller 1992; Fooks and Gibson 2002; Rolfe 2002):

- Competition for nutrients
- Secretion of antimicrobial substances
- Reduction of gut pH through SCFA formation
- Blocking of adhesion sites
- Blocking of toxin receptor sites

- Immune stimulation
- Suppression of toxin production.

4. *Bacillus coagulans*

Bacillus coagulans is a lactic acid, spore-forming bacterial species of the genus *Bacillus*. It has been called a 'spore-forming *Lactobacillus*', as it is phylogenetically close to the lactobacilli. *B. coagulans* is a Gram-positive rod (0.9 μm by 3.0 μm to 5.0 μm in size), catalase positive, spore-forming, motile and a facultative anaerobe. Its optimum temperature for growth is 50°C; the range of temperatures tolerated are 30–55°C.

This ability to resist high temperatures and develop spores is attractive from the viewpoint of withstanding baking temperatures and opening up more food delivery systems for probiotic use. Casula and Cutting (2002) addressed the issue over how *Bacillus* spores could enhance the normal microbial flora of the gastrointestinal tract. They used RT-PCR to develop a molecular-based method for the detection of spore germination in the mouse gastrointestinal tract. They suggested that the small intestine, in a murine model, was briefly colonised by spore-forming bacilli. However, the authors also suggested that the spore itself exerted an immunostimulatory effect adding to the antimicrobial effect of the strain. Given this, the other potential probiotic traits of a spore-forming *B. coagulans* is worthy of attention.

Here, we overview the traits of a proprietary strain of *B. coagulans*, known as GanedenBC^{30} (*Bacillus coagulans* GBI-30, 6086 (BC30); Figure 1), for its probiotic attributes.

4.1 Safety

A series of toxicology testing assays was conducted on BC30. These are summarised in Table 1, and these assays show that there were no safety issues, even when the product was consumed in high quantities. Seven tests were conducted including mutation assays, chromosomal aberrations, oral toxicity in rats, surface irritations in rabbits and a subchronic toxicity test (Endres *et al.* 2009).

4.2 Digestive function

4.2.1 Human

BC30 has been shown to support digestive function. In a randomised, double-blind, placebo-controlled trial of 44 subjects with IBS, it was found that BC30 was an effective option for relieving abdominal pain and bloating (Hun 2009). This was followed by a second randomised, double-blind, placebo-controlled trial of 52 subjects with IBS-D (diarrhoeal form), where a significant reduction in the number of bowel movements was seen daily (Dolin 2009). The probiotic was suggested to exert a positive effect upon transit time.

A randomised, double-blind, placebo-controlled trial of 61 adults with post-prandial intestinal gas found a statistically significant reduction in abdominal pain and a strong trend for improvement of abdominal distention (Kalman *et al.* 2009).

A randomised, double-blind, placebo-controlled trial of 35 subjects with Crohn's disease was also carried out. Of those, 11 subjects were excluded from the study, with none related to the study product. Of patients who completed the study, there was a strong trend in decreasing the Crohn's disease activity index and total number of daily stools.

4.2.2 Laboratory

BC30 produces various enzymes that have been shown to be able to break down a wide variety of carbohydrates (Table 2). In a validated simulated gut model TIM-1 (TNO, Zeist, The Netherlands), the survival and activity of BC30 during passage through a simulated upper gastrointestinal tract were assessed (Maathuis *et al.* 2010). BC30 was tested in a dynamic, validated, *in vitro* model of the stomach and small intestine (TIM-1) on survival and its potential to aid the digestion of milk protein, lactose and fructose. The survival of BC30 was high (70%), although germination of its spore was minimal (<10%) under the conditions tested. Survival of the strain in the presence of lactose and fructose was lower (56–59%) than in the absence of the sugars. The amount of digested milk protein available for absorption was higher when BC30 was added to the milk. When BC30 was tested with lactose or fructose added to the meal, extra lactate was produced when compared with the BC30 alone. These results show the potential of BC30 to aid in protein digestion and in the digestion of lactose and fructose.

Co-culture work showed that BC30 could exert antimicrobial activity against *C. difficile*, *C. perfringens* and *Listeria monocytogenes* (to varying degrees). These initial experiments were further investigated using complex model systems and with *C. difficile* as the target. In a culture system designed to favour *C. difficile* growth, the pathogen was repressed by the probiotic (Honda *et al.* in press). The rich culture medium used would not induce sporulation by BC30. In the human situation where persons at risk of *C. difficile*-associated diarrhoea are likely to be taking antibiotics, BC30 sporulation is likely. It is suggested that both the level of growth (i.e. number of vegetative cells) and phase of growth (i.e. log phase, late log phase or sporulation) are important factors in the expression of anti-microbial compounds from *Bacillus* probiotics. We also suggest that the (unique among probiotics) bimodal lifecycle of BC30 may lead to anti-microbial activity in distal regions of the gastrointestinal tract.

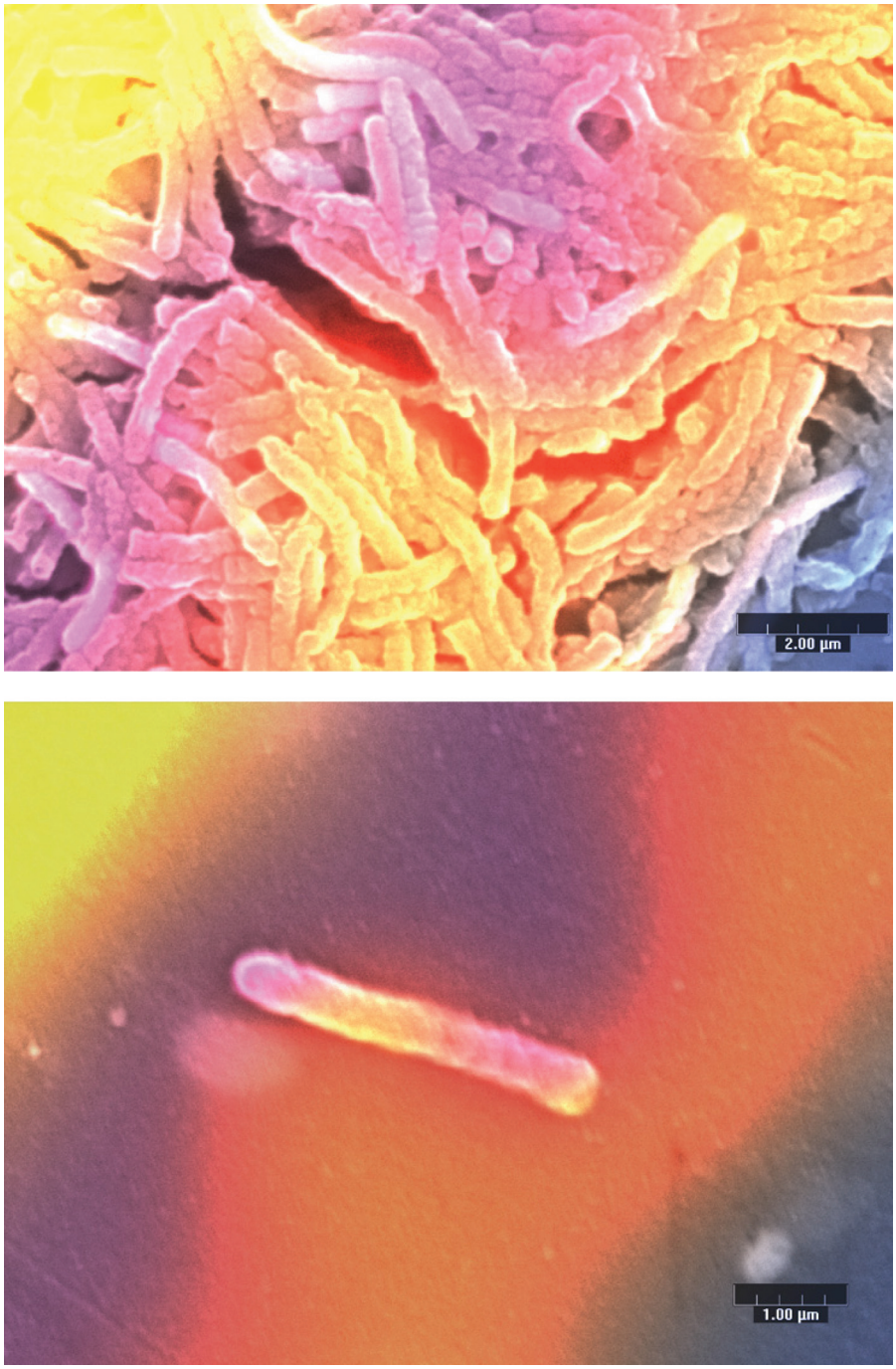

Figure 1. Scanning electron micrographs of *Bacillus coagulans* BC30.

This is an important facet given the prevalence of gut disorders in the left side of the large intestine.

4.3 Immunoregulatory properties

4.3.1 Human

Multiple human clinical trials have been run to demonstrate that BC30 has the ability to affect the immune system. Ten subjects had blood drawn at baseline, were given two billion cfu of BC30 per day for 30 days and had blood drawn again at conclusion. The blood was then exposed to various antigens, and the cytokine reactivity was measured. It demonstrated that there was an increase in various cytokines to various antigens (Baron 2009). This study was repeated again at a dosage of 500 million cfu/day, and again found increases of various cytokines to

Table 1. Summary of safety assessments for *Bacillus coagulans* BC30 (Endres *et al.* 2009)

Test	Outcome
Bacterial reverse mutation (AMES) study	No mutagenic potential
Micronucleus assay in mice	No signs of toxicity
In vitro chromosomal aberration study	Negative response for induction of structural aberrations both with and without metabolic activation system
Acute oral toxicity in rats	No evidence of any toxicity in the study
Acute eye irritation study in rabbits	According to the EC Directive 2004/73/EC criteria and OECD Guideline TG405, BC30 is not an eye irritant
Acute skin irritation study in rabbits	According to the EC Directive 2001/59/EC criteria and OECD Guideline TG405, BC30 is not an skin irritant
Subchronic 13 week oral toxicity study in rats	Resulted in an expert panel concluding that the NOAEL is greater than 1000 mg/kg/day. This equates to a safety factor ranging from 3173 to 95 200 times the normal daily dosage for humans

Table 2. Carbohydrate preferences of *Bacillus coagulans* BC30

L-Arabinose	*N*-Acetyl-D-glucosamine	Succinic acid
D-Trehalose	D-Mannose	D-Sorbitol
Glycerol	D,L-α-Glycerol phosphate	D-Xylose
L-Lactic acid	D-Mannitol	L-Glutamic acid
D,L-Malic acid	D-Ribose	D-Fructose
α-D-Glucose	Maltose	Thymidine
L-Asparagine	α-D-Lactose	Lactulose
Sucrose	β-Methyl-D-glucoside	Maltotriose
2-Deoxy adenosine	*m*-Inositol	Fumaric acid
D-Cellobiose	Inosine	Methyl pyruvate
L-Malic acid	D-Psicose	L-Lyxose
Pyruvic acid	D-Galacturonic acid	

Study carried out by Ganeden (internal company data). Phenotypic assays were conducted on *B. coagulans* (strain BC30) to ascertain the level and scope of carbohydrate metabolism of the organism when incubated for 24 h at body temperature. The results show that the organism has a very broad carbohydrate substrate range (35 individual carbohydrates) and that the time to peak (the time necessary for the organism to exhaust the substrate) was well beyond the 24 h incubation period. The lag phase for many of the carbohydrates indicated that, in some cases, a considerable amount of time elapsed before the organism adapted to the conditions necessary for spore germination and carbohydrate utilisation leading to growth. This observation in addition to the time to peak observance suggests that the organism requires at least 24 h to provide maximum digestive benefits related to carbohydrate metabolism.

various antigens. In addition, flow cytometry was conducted in the second study that showed an increase in the %CD3-CD69 cells (Kimmel *et al*. 2010).

4.3.2 Animal

In a mouse model, animals were given lethal strains of influenza A. This study demonstrated that BC30 was able to significantly protect the animals against these two strains (internal data, not yet published).

4.3.3 Laboratory

An extensive laboratory workup of BC30 demonstrated the ability to affect multiple immune and inflammatory markers, including PMN cells, NK cells, IL-4, IL-6, IL-10, TNF-α and IFN-γ (Jensen *et al*. 2010).

4.4 Joint health

A study was conducted with BC30 in patients with arthritis. In a randomised, double-blind, placebo-controlled trial, 45 subjects with rheumatoid arthritis were monitored for 60 days. Subjects on BC30 had significant decreases in pain, along with greater improvement in mobility. In addition, a greater decrease in c-reactive protein (CRP) was seen (Mandel *et al*. 2010).

4.5 Survival in the gut

Aside from the *in vivo* studies mentioned earlier, *in vitro* fermenters (continuous culture) were used to assess the persistence of BC30 in the background of a complex mixed microbiota. Survival in such an ecosystem is one of the main drawbacks to probiotic success, as they can often be easily outcompeted. Modern molecular-based technologies were used to assess microbial changes in the fermenters. Our studies (Honda *et al*. in press) showed that BC30 was effective at persisting in the reactors for the duration of the experimental period. Culture-based methods suggest that this was due to the spore-forming nature of the strain and subsequent germination. The results were reproducible using faecal flora from three different persons. No negative effects of supplementation on the normal flora were determined. In conclusion, the probiotic was able to persist in a complex mixture of gut bacteria; this was due to its spore-forming capacity.

5. Conclusions

Probiotics are a very successful sector of the functional foods industry. They offer a relatively harmless route of

improving consumer health, largely from the viewpoint of reducing the risk of gastrointestinal-mediated disorders. Several attributes are needed to produce good probiotics. Individual strains may be better suited to individual conditions and it is unlikely that any one probiotic will 'serve all' requirements. Recently, the use of *B. coagulans* has increased. Its ability to produce spores could give it several advantages that may not occur with vegetative forms.

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Ganeden GRAS Approval Summary

Ganeden is in the process of having an expert panel review all the data on its' patented GanedenBC-30 bacteria. This summary will outlines the steps involved in this process.

In October 2006, Ganeden entered into an agreement with AIBMR Life Sciences (Puyallup, Washington) to head this process. AIBMR is the oldest dedicated full-service nutraceutical products consulting company in the world. All staff of AIBMR have at least 10 years experience in their respective fields. The firm specializes in nutraceutical research, foreign and domestic regulatory compliance, preclinical and clinical trials management, safety testing (toxicological studies), product pharmacokinetics, and product development. The Senior Director of AIBMR Life Sciences, Alexander G. Schauss, Ph.D, FACN, has been a member of two National Institutes for Health (NIH) advisory committees, reviewed monographs for the U.S. Pharmacopoeia (USP), represented the United States as a voting member of the World Health Organization (WHO) Study Group on Health Promotion, and for over 14 years chairs the safety subcommittee of the Compliance and Label Integrity Committee (ComPLI) for the Natural Products Association. AIBMR also maintains an extensive database and reference library on natural products research to assist its clients and staff, headed by a full-time professional librarian and her support staff. The library contains over 12,000 books and journals, 400 periodical subscriptions and over 120,000 reference papers.

The process proceeded as follows:

1. Ganeden supplied AIBMR with all background material on our company and bacteria. This included
    a. History of company and how the bacteria was isolated
    b. History of Use in Ganeden OTC products
    c. Extensive manufacturing and testing records for the bacteria
    d. Clinical Trials conducted with the bacteria
    e. Current food products available in the US and abroad using B. Coagulans
    f. Continue to supply all testing data from animal and human studies when available
2. AIBMR conducted the following research and complied the data :
    a. Historical search for uses of B. Coagulans
    b. Full literature search and analysis
    c. Full regulatory search in US
3. AIBMR developed a plan on testing that would be adequate to show an expert panel the safety of the strain.
    a. Acute Toxicology Test
        i. Animals were given 5mg/kg of the BC-30 in a single dose and monitored for 21 days for any adverse or toxicity. A necropsy was conducted on day 21 to check for any gross organ changes. This dose was equivalent to 1,620 times the highest dose in any

Ganeden product (Arthritis Advantage). It ranged from 1,620 to over 12,000 times Ganedens' normal dosage.

ii. Results: No adverse events were noted in any of the animals and no gross changes were noted on necropsy.

b. AMES Test

i. This test is to demonstrate if other bacteria can share genes with BC-30 and mutate in its' presence. Four strains of Salmonella and one of E. Coli were tested. This test is run in duplicate to ensure validity of the results.

ii. Results: No mutations of any of the strains were seen in either of the test.

c. Subacute Toxicology Test

i. This is a 90 day test where animals are separated into three groups and administered a low, medium and high dose for 90 days. Daily blood and urine analysis along with behavior observation is done. Necropsy of the animals and detailed histopathology on all organs is conducted at the end of 90 days.

ii. Status: The animals started dosing on 5/22/07. Estimated final report date 10/15/07.

d. Draize Test

i. This is a combination of two tests. The first is to demonstrate that there is no dermal irritation to the bacteria. The second is that there is no ocular irritation to the bacteria.

ii. Results:

Dermal: According to EEC directive 2001159/EEC, the test item GANEDEN BC-30 has not been classified as irritating for the skin.
Ocular: According to the EC criteria for classification and labeling requirements for dangerous substances and preparations, the test item does not have to be classified and has no obligatory labeling requirement for eye irritation.

e. Sequencing

i. We will conduct an s16RNA analysis of BC-30 and a know ATTC strain of B. Coagulans Hamer. This will demonstrate that our bacteria is in the B. Coagulans family.

4. AIBMR develops binder on BC-30 for Panel

a. When all of the above has been concluded, AIBMR will create a binder including all of the above information that will demonstrate to the panel proof that the BC-30 should be GRAS.

b. Status: This binder is currently in the works and will be completed in mid October when the 90 Subactute Study has concluded.

5. AIBMR will assemble expert panel

a. AIBMR has already spoken with one of the former heads of Toxicology for the FDA who will chair this panel. He will pick others as he feels needed to complete the panel.

b. The panel with write an expert opinion that will say based on all the supplied data, Ganeden BC-30 is GRAS.

c. Status: We expect that the opinion will be completed October/November 2007.

Original Research: *Bacillus coagulans* Significantly Improved Abdominal Pain and Bloating in Patients with IBS

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Abstract

Background: Symptoms of irritable bowel syndrome (IBS) can have a profound impact on emotional health and quality of life, and current treatments are sometimes unsatisfactory for patients facing this lifelong disease. Probiotics, which can normalize gastrointestinal microflora, may alleviate symptoms of IBS. **Objective:** This preliminary controlled study was conducted to evaluate the effects of the probiotic *Bacillus coagulans* GBI-30, 6086 on IBS symptoms. **Methods:** This was a randomized, double-blind, parallel-group, placebo-controlled clinical trial involving 44 subjects who received either placebo or *B coagulans* GBI-30, 6086 once a day for 8 weeks. Self-assessments of the severity of IBS symptoms (abdominal pain and bloating) were recorded every day for 8 weeks. Because baseline values were significantly different between the 2 study groups, within-group analysis was conducted. **Results:** Improvements from baseline abdominal pain and bloating scores in the *B coagulans* GBI-30, 6086 group were statistically significant for all 7 weekly comparisons ($P < 0.01$). In the placebo group, only changes in abdominal pain scores at weeks 6 and 8 achieved statistical significance ($P < 0.05$). No treatment-related adverse events or serious adverse events were reported during the 8-week study period. **Conclusions:** Preliminary data suggest that the patented *B coagulans* GBI-30, 6086 probiotic may be a safe and effective option for the relief of abdominal pain and bloating for patients with IBS. Larger, extended trials are needed to verify these results.

Keywords: probiotics; irritable bowel syndrome; *Bacillus coagulans*; GanedenBC[30]; lactic acid-producing bacteria

Introduction

Irritable bowel syndrome (IBS) is a chronic gastrointestinal disorder involving abdominal pain or discomfort and alterations in bowel habit (frequency, form, and passage).[1,2] The prevalence of IBS in the United States varies widely because of the use of different diagnostic criteria and survey methods. The 2 most recent surveys reported a total prevalence of 7% to 14%, with a higher prevalence in women.[3,4] Although IBS is not life threatening, the symptoms of IBS can have a profound impact on the patient's emotional health and quality of life. Irritable bowel syndrome accounts for 12% of primary care visits and is the most common complaint of patients seen by gastrointestinal (GI) specialists;[5,6] however, the majority of patients with IBS symptoms either go undiagnosed or do not seek medical care.[2,4] For those patients who are diagnosed with IBS, there are few Food and Drug Administration (FDA)-approved therapeutic options. Clinical intervention typically includes patient education, reassurance, and

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dietary modification to alleviate symptoms,[1] all of which can be unsatisfactory to patients who are facing this lifelong disease.

While the precise pathophysiology of IBS remains unclear, symptoms can be triggered by changes in GI function caused by infection, altered diet, or stress.[2] Immune activation and inflammation have also been implicated in IBS.[1] Both retrospective and prospective studies have documented the onset of IBS following bacterial gastroenteritis,[7–9] and others have provided evidence of low-grade mucosal inflammation and immune activation in patients with IBS.[10,11]

Modifications in the normal gut flora may also be a cause or a consequence of IBS. Individuals with this disease have been shown to have altered gut flora that may represent or lead to disordered GI function. The fecal flora of IBS patients differs from that of normal patients.[12] Some individuals with IBS may harbor bacterial overgrowth and their symptoms may be ameliorated by its eradication.[13,14]

Probiotics are live or attenuated bacteria or bacterial products that have been shown to re-establish balance in the gut microflora as well as modulate the mucosal immune response.[15,16] Probiotics modulate bacterial or virus-related diarrhea and could modify the course of postinfective IBS.[16] Probiotics have been demonstrated to exert anti-inflammatory effects at the mucosal surface.[17,18] By reducing mucosal inflammation, probiotics may decrease immune-mediated activation of intestinal motor and sensory neurons and modify neural traffic between the gut and the central nervous system.[17] Probiotics may also alter the volume and/or composition of stool and gas[19] or increase intestinal mucus secretion,[20] effects that may influence intestinal handling of its contents and thus modulate symptoms of IBS such as constipation and diarrhea.[18]

Bacillus coagulans GBI-30, 6086 (Ganeden Biotech, Inc., Mayfield Heights, OH) is a patented strain of lactic acid-producing bacteria that can sustain the low pH of stomach acid and become active in the intestine.[21,22] The objective of this preliminary study was to evaluate the effects of 8 weeks of *B coagulans* GBI-30, 6086 therapy on specific IBS symptoms.

Materials and Methods

Study Design

This was a randomized, double-blind, parallel-group, placebo-controlled clinical study to evaluate the effects of a marketed probiotic preparation, *B coagulans* GBI-30, 6086, on the symptoms of IBS. Fifty males and females between 23 and 70 years old were randomized to receive a probiotic preparation of GanedenBC[30] (≈800 million CFU) or placebo once a day for 8 weeks. Patients gave informed consent at study inclusion. The study protocol, informed consent, and test product(s) information received institutional review board (IRB) approval before the beginning of the study.

Patient Population

All subjects met the Rome II Criteria for IBS with diarrhea (ie, must have had, during the 12 months prior to evaluation, and for a total of at least 12 weeks [not necessarily consecutively], abdominal discomfort or pain that had 2 of 3 features: [1] relieved with defecation, [2] onset associated with a change in frequency of stool, or [3] onset associated with a change in appearance of stool). Individuals with any organic gastrointestinal conditions or diseases, previous intestinal surgery, immunodeficiency, or lactose intolerance, or who were pregnant or lactating, were excluded from the study. Patients who had taken commercially available probiotic medications within 30 days of the study were also excluded.

Fifty subjects with IBS symptoms were randomized. Six subjects did not complete the study for reasons unrelated to the study and were not included in the analysis. The majority of the subjects in the study were white (90%) and female (82%), and the average age was 48 years (Table 1). All subjects had diarrhea-prominent IBS.

Treatment and Follow-up

Subjects were required to make a total of 3 visits to the study site during the 8-week treatment period. At the first visit, subjects were screened, randomized, and provided with a sufficient supply of the assigned study product to last for the duration of the study, along with written instructions for daily product use.

Table 1. Subject Demographics

Study Population (N = 50)	
Race, n (%)	
White	45 (90%)
Asian	1 (2%)
Black	3 (6%)
Other	1 (2%)
Gender, n (%)	
Female	41 (82%)
Male	9 (18%)
Age (years)	
Average	48.36
Range	23–70

Subjects were instructed to consume 1 caplet with water once daily, at approximately the same time each day, regardless of meals. Subjects received self-assessment and product use diaries to be completed daily at home. All responses were self-reported using the following scale: 0 = none, 1 = very mild, 2 = mild, 3 = moderate, 4 = severe, and 5 = very severe.

Self assessments of the severity of IBS symptoms (abdominal pain and bloating) were recorded in the diary each day for 8 weeks, starting at the end of the first visit (Day 0). Subjects began taking the study product the next day (Day 1). All subject responses used a 5-point scale to describe symptom severity (0 = none, 1 = very mild, 2 = mild, 3 = moderate, 4 = severe, 5 = very severe).

Subjects returned for a second visit approximately 28 days after randomization and then for a final visit approximately 56 days after randomization. Compliance with study product use was determined by caplet count and review of product use diaries.

All adverse events were reported regardless of whether they were related to the study drug. Event duration, severity, and causal relationship to the study drug were recorded.

Statistical Analysis

Changes in symptom severity compared with baseline in the placebo and *B coagulans* GBI-30, 6086 groups were determined by Student's t-test, with $P < 0.05$ considered statistically significant.

Results

Forty-four subjects, 22 subjects in the *B coagulans* GBI-30, 6086 group and 22 subjects in the placebo group, completed the study. Average daily self-reported abdominal pain severity (Figure 1) and bloating (Figure 2) scores during the 8-week treatment period significantly improved both IBS symptoms in subjects who received *B coagulans* GBI-30, 6086, but not in subjects who received placebo.

Because baseline (Week 1) abdominal pain and bloating scores were significantly different between the 2 study groups, within-group change from baseline was used to evaluate efficacy (Tables 2, 3). Subjects who received *B coagulans* GBI-30, 6086 achieved consistently better weekly scores in change from baseline in abdominal pain and bloating during the 8-week treatment period compared with subjects who received placebo. Changes from baseline abdominal pain and bloating scores in the *B coagulans* GBI-30, 6086 group were statistically significant for each weekly comparison throughout the study ($P < 0.01$). In the placebo group, changes in abdominal pain scores at weeks 6 and 8 achieved statistical significance ($P < 0.05$).

Figure 1. Mean daily patient-reported severity scores for abdominal pain during the 8-week treatment period in 22 subjects in the *Bacillus coagulans* GBI-30, 6086 group (circles) and 22 subjects in the placebo group (squares).

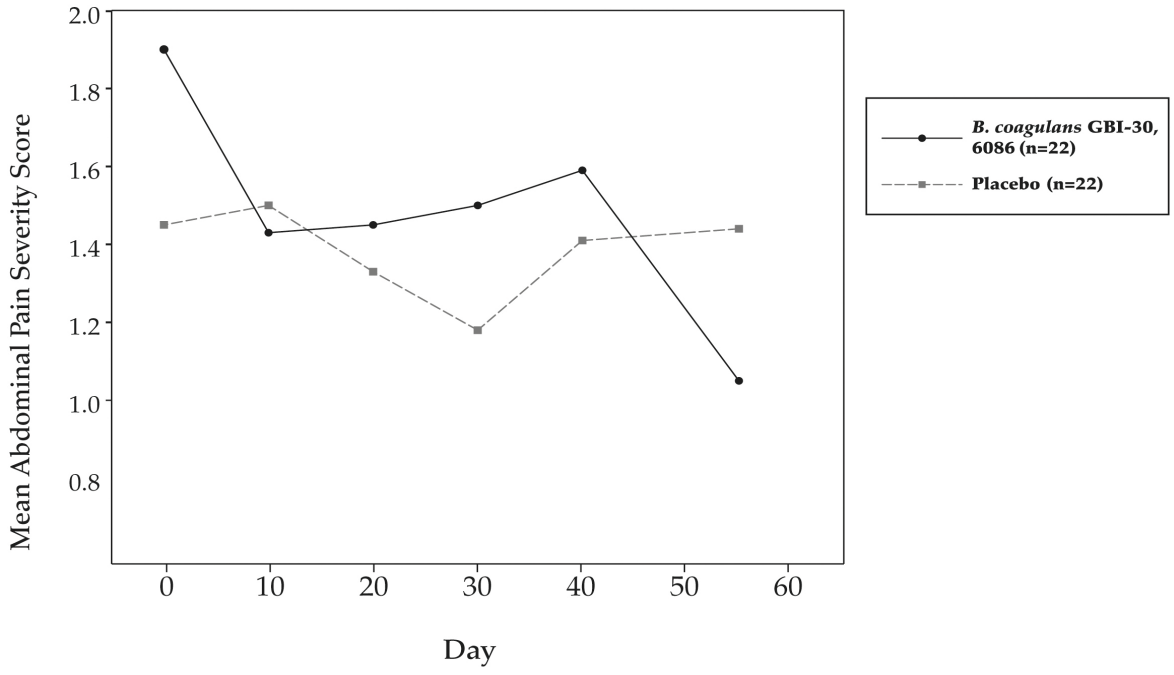
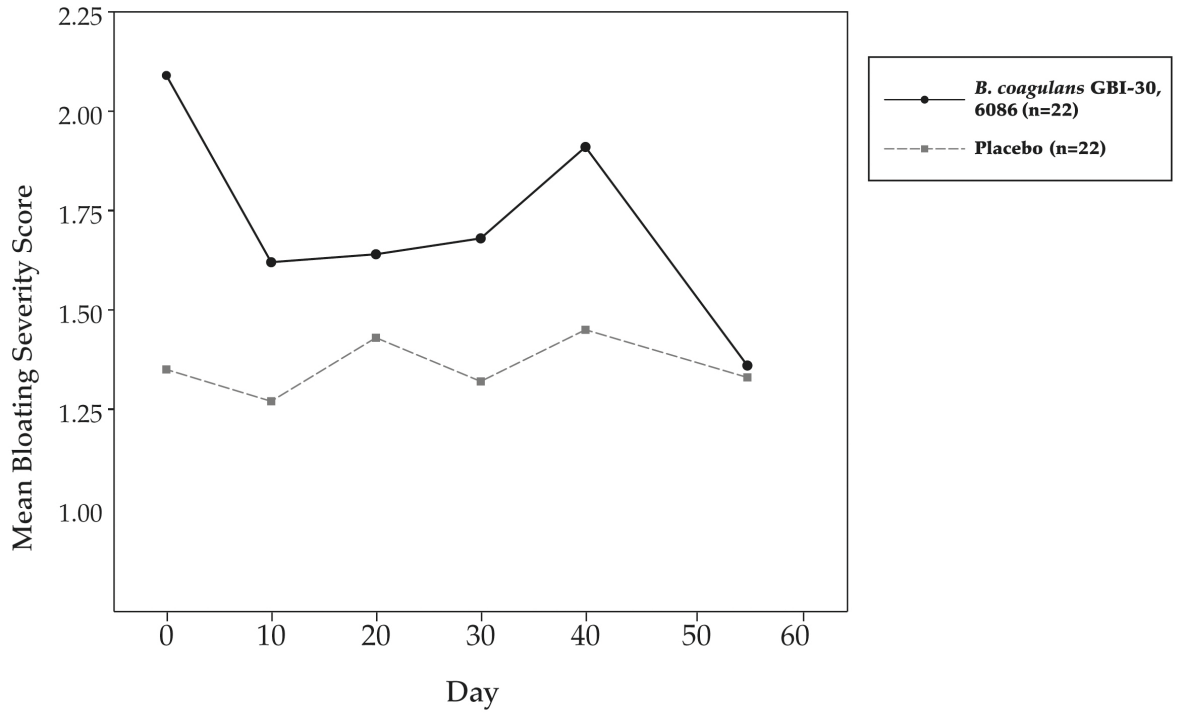


Figure 2. Mean daily patient-reported severity scores for bloating during the 8-week treatment period in 22 subjects in the *Bacillus coagulans* GBI-30, 6086 group (circles) and 22 subjects in the placebo group (squares).



There were 4 adverse events reported in the placebo group and 2 in the study group, all of which were unrelated to the treatments. No treatment-related adverse events or serious adverse events were reported during the 8-week study period.

Discussion

Studies of probiotics for the treatment of IBS have yielded contradictory results, and most studies have not offered convincing evidence that probiotics are effective for treating symptoms of IBS.[23]

A randomized study by O'Mahony et al[18] (N = 75) compared the effect of *Lactobacillus salivarius* or *Bifidobacterium infantis* on symptoms of IBS and cytokine ratios. The results showed *B infantis* was superior to *L salivarius* and placebo for relieving IBS symptoms, and the response was associated with normalization of the anti-inflammatory to proinflammatory cytokine ratio. In a large-scale, multicenter clinical trial[24] of women with IBS (N = 362), it was determined that encapsulated *B infantis* at a dose of 1×10^8 CFU was significantly superior to placebo and other doses of *B infantis* for relieving abdominal pain, bloating, bowel dysfunction,

Table 2. Average Weekly Change in Abdominal Pain Severity versus Baseline (Week 1)

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
***Bacillus coagulans* GBI-30, 6086 (n = 22)**								
Mean	1.79	1.41	1.32	1.42	1.30	1.30	1.27	1.39
Change from baseline	–	–0.37	–0.46	–0.37	–0.49	–0.49	–0.52	–0.40
P value	–	0.006[a]	0.0001[b]	0.004[a]	0.0002[b]	0.0001[b]	0.0001[b]	0.007[a]
Placebo (n = 22)								
Mean	1.43	1.44	1.40	1.32	1.20	1.25	1.27	1.16
Change from baseline	–	0.00	–0.03	–0.11	–0.23	–0.18	–0.16	–0.27
P value	–	0.92	0.77	0.28	0.06	0.04[c]	0.12	0.02[c]

[a]$P \leq 0.01$; [b]$P \leq 0.001$; [c]$P \leq 0.05$.

Table 3. Average Weekly Change in Bloating Severity versus Baseline (Week 1)

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
***Bacillus coagulans* GBI-30, 6086 (n = 22)**								
Mean	1.98	1.60	1.55	1.64	1.50	1.52	1.51	1.57
Change from baseline	–	–0.38	–0.43	–0.34	–0.48	–0.46	–0.46	–0.41
P value	–	0.003[a]	0.0003[b]	0.003[a]	0.0001[b]	0.0007[b]	0.0001[b]	0.002[a]
Placebo (n = 22)								
Mean	1.31	1.37	1.31	1.28	1.33	1.33	1.33	1.16
Change from baseline	–	0.06	0.00	–0.03	0.01	0.02	0.01	–0.15
P value	–	0.65	0.98	0.84	0.91	0.92	0.91	0.27

[a]$P \leq 0.01$; [b]$P \leq 0.001$.

incomplete evacuation, straining, and gas at the end of the 4-week study. The 2 other doses of *B infantis* (1×10^6 and 1×10^{10}) were not significantly different than placebo.

Finally, a 4-week, randomized study compared the composite probiotic VSL#3 containing *Bifidobacterium* (*B longum, B infantis*, and *B breve*), *Lactobacillus* (*L acidophilus, L casei, L delbrueckii* ssp. *bulgaricus*, and *L plantarum)*, and *Streptococcus salivarius* ssp. *thermophilus*) with placebo.[25] There was no statistical difference in bloating, pain, urgency, or number of stools. However, flatulence scores were reduced among patients treated with VSL#3 versus patients taking placebo.

In the present study, patients with IBS who were treated for 8 weeks with *B coagulans* GBI-30, 6086 demonstrated improvement of abdominal pain and bloating. The improvements were statistically significant at each weekly comparison. Subjects in the placebo group experienced statistically significant improvement in abdominal pain at weeks 6 and 8. According to a meta-analysis, this outcome is consistent with a number of other placebo-controlled IBS studies that have shown a high placebo effect among this patient population.[26]

Results from an earlier case-control study that investigated the efficacy of a probiotic preparation containing *L sporogenes* (*B coagulans*), *L acidophilus*, and *Streptococcus thermophilus* for alleviating the symptoms of IBS reported similar data.[27] Thirty-seven patients were given the probiotic preparation and followed for 6 months. Compared with baseline values, those who received the probiotic treatment reported significantly reduced abdominal pain ($P < 0.000001$), abdominal distention ($P = 0.003$), and constipation ($P = 0.03$), as well as reduced alternating constipation and diarrhea ($P = 0.01$).

Probiotics must survive gastric and bile acids[28] in order to reach the intestinal tract, colonize the host epithelium, and exhibit a beneficial effect.[29] Most conventional forms of lactobacilli-type probiotics are nonspore forming and, therefore, are inactivated by bile and low gastric pH.[30,31] Strains of *B coagulans* produce coagulin, which is a heat-stable, protease-sensitive, bacteriocin-like inhibitory substance with activity against gram-positive bacteria.[32] Spores of *Bacillus* are resistant to heat and hostile gastrointestinal conditions and, therefore, are able to reach the intestine where they can germinate and proliferate within the host.[21,22,30]

In addition, probiotics selected for commercial use must survive industrial manufacturing and storage to ensure long-term viability and activity.[21] Most cells of conventional lactobacilli die at 70°C, while spore-bearing lactic acid-forming bacteria do not show a decrease in viable cells even after heating in saline at 85°C for 30 minutes.[31] In addition to surviving heat and a hostile gastrointestinal environment, *B coagulans* GBI-30, 6086 maintains spore viability after 5 years of storage without the need for refrigeration (unpublished communication, Ganeden Biotech, Inc., Mayfield Heights, OH), making it particularly suitable for commercial use.

One limitation of this study was the need to use self-assessment diaries to measure outcomes, which contributed to wide variations in the average reported symptom changes during the study period. It is also likely that the small study size may have impacted the statistical outcomes. Nevertheless, the present study provides preliminary evidence that *B coagulans* GBI-30, 6086 probiotic has the ability to relieve abdominal pain and bloating in patients with IBS. No adverse events were reported with the use of this probiotic agent. Therefore, *B coagulans* GBI-30, 6086 may be a safe and effective alternative for patients with IBS who currently have limited therapeutic options. These results justify the design of larger scale, controlled clinical trials to verify our findings.

Conflict of Interest Statement

Larysa Hun, MD, FAAP discloses no conflicts of interest.

Disclosure

This study was performed at Research Testing Laboratories, an independent laboratory in Great Neck, NY.

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Draft Genome Sequence of *Bacillus coagulans* GBI-30, 6086, a Widely Used Spore-Forming Probiotic Strain

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***Bacillus coagulans* GBI-30, 6086 is a safe strain, already available on the market, and characterized by certified beneficial effects. The draft genome sequence presented here constitutes the first pillar toward the identification of the molecular mechanisms responsible for its positive features and safety.**

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Bacillus coagulans strain GBI-30, 6086 is a safe, spore-forming strain, as testified by the GRAS (Generally Recognized As Safe) status received in 2012 from the United States FDA; the strain is authorized for human consumption and already available in a wide selection of functional foods or as dietary supplement (1). This strain is characterized by certified beneficial effects in gastrointestinal disorders, such as irritable bowel syndrome, intestinal gas, and colitis (2, 3, 4, 5); in rheumatoid arthritis (6); and in common viral infections of the respiratory tract (7). As a spore-former, *B. coagulans* GBI-30, 6086 can be incorporated into foods, where it can survive the mild heat-treatments used for sterilization andwithstand the harsh conditions of the gastrointestinal tract, i.e., the low pH of the gastric barrier (8).

Here we report the draft genome sequence of *B. coagulans* GBI-30, 6086 in order to unveil the genetic basis of its safety and probiosis. To the best of our knowledge, this is the first published fully assembled genome of a commercial *B. coagulans* probiotic strain.

The whole-genome sequencing was performed using the Illumina GAIIx platform at CRA-Genomics Research Centre (Piacenza, Italy) with a paired-end library; the reads were *de novo* assembled using the CLC Genomic Workbench version 7.0. The genome sequence was annotated by the NCBI Prokaryotic Genomes Annotation Pipeline.

A total of 14,500,000 paired-end reads of 110-bp length on average (genome coverage of 840×) were assembled into 224 contigs (N_{50} length of 44,706 bp), with the largest assembled contig of 125,999-bp length. The draft genome consists of 3,458,655 bp with GC % content of 46.38.

A total of 3,373 genes were predicted, of which 3,197 are coding sequences (CDS), 18 are rRNAs, and 82 are tRNAs; 79 were identified to be pseudogenes, and 1 was identified as ncRNA. The genome also contains 3 CRISPR arrays, which could be involved as a defense mechanism toward foreign genetic elements (9).

The strain is predicted to encode for about 500 proteins involved in central carbohydrate metabolism, including glycolysis, pentose phosphate, and xylose utilization pathways (9, 10). As expected, about 80 genes regarding dormancy and sporulation were annotated, in particular, spore DNA protection, sporulation mechanisms, spore core dehydration, and spore germination. Furthermore, the genome contains determinants involved in the adhesion (i.e., fibronectin- and mucus-binding proteins) and active metabolism in the host (as a biotin biosynthesis pathway).

The complete genome of *B. coagulans* GBI-30, 6086 obtained in the present study will contribute to a wider and deeper insight into the safety features of this strain, and the comparative genomic analysis with other *Bacillus* strains genomes (11, 12, 13) might shed new light on the molecular mechanisms at the basis of its probiotic and beneficial properties.

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited in DDBJ/EMBL/GenBank under the accession number JPSK00000000. The version described in this paper is the first version, JPSK01000000.

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GanedenBC$^{30™}$ cell wall and metabolites: anti-inflammatory and immune modulating effects *in vitro*

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Abstract

Background: This study was performed to evaluate anti-inflammatory and immune modulating properties of the probiotic, spore-forming bacterial strain: *Bacillus coagulans*: GBI-30, (PTA-6086, GanedenBC30TM). In addition, cell wall and metabolite fractions were assayed separately to address whether biological effects were due to cell wall components only, or whether secreted compounds from live bacteria had additional biological properties. The spores were heat-activated, and bacterial cultures were grown. The culture supernatant was harvested as a source of metabolites (MTB), and the bacteria were used to isolate cell wall fragments (CW). Both of these fractions were compared in a series of *in vitro* assays.

Results: Both MTB and CW inhibited spontaneous and oxidative stress-induced ROS formation in human PMN cells and increased the phagocytic activity of PMN cells in response to bacteria-like carboxylated fluorospheres. Both fractions supported random PMN and f-MLP-directed PMN cell migration, indicating a support of immune surveillance and antibacterial defense mechanisms. In contrast, low doses of both fractions inhibited PMN cell migration towards the inflammatory mediators IL-8 and LTB4. The anti-inflammatory activity was strongest for CW, where the PMN migration towards IL-8 was inhibited down to dilutions of 10^{10}.
Both MTB and CW induced the expression of the CD69 activation marker on human $CD3^-$ $CD56^+$ NK cells, and enhanced the expression of CD107a when exposed to K562 tumor cells *in vitro*.
The fractions directly modulated cytokine production, inducing production of the Th2 cytokines IL-4, IL-6, and IL-10, and inhibiting production of IL-2.
Both fractions further modulated mitogen-induced cytokine production in the following manner: Both fractions enhanced the PHA-induced production of IL-6 and reduced the PHA-induced production of TNF-alpha. Both fractions enhanced the PWM-induced production of TNF-alpha and IFN-gamma. In addition, MTB also enhanced both the PHA- and the PWM-induced expression of IL-10.

Conclusion: The data suggest that consumption of GanedenBC30TM may introduce both cell wall components and metabolites that modulate inflammatory processes in the gut. Both the cell wall and the supernatant possess strong immune modulating properties *in vitro*. The anti-inflammatory effects, combined with direct induction of IL-10, are of interest with respect to possible treatment of inflammatory bowel diseases as well as in support of a healthy immune system.

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Background

An intact and properly functioning gastrointestinal (GI) system is of paramount importance in the maintenance of optimal human health. The GI tract plays important roles in nutrient absorption and assimilation, providing a protective barrier against invasion by harmful organisms, and training the immune system to distinguish between harmful and harmless substances. To maximize the absorption of nutrients obtained from the diet, the luminal side of the GI tract is organized into finger-like projections called villi. The surface area of the average GI tract has been calculated to be 300 m^2 and is lined by a single layer of epithelial cells. As a protective barrier against abrasion and to minimize contact of this epithelium by harmful organisms the GI tract is lined by a mucus layer consisting of polysaccharides. Beneath the epithelial cell layer are highly structured and active anatomical areas of our immune system, including the lamina propria and Peyer's patches. In order for the immune system to provide meaningful protection, it needs to be able to carefully discriminate between a large number and variety of antigens (proteins capable of inducing an immune response) that the GI tract is exposed to [1,2].

An optimal state of health can be adversely affected when any of these functions are compromised and can result in deficiencies due to malabsorption, infection and inflammation due to invasion by opportunistic organisms, or even autoimmune disease due to inappropriate immune system response to self. These perturbations can lead to a state of chronic low-grade inflammation. Chronic inflammation and gut microbiota imbalances are thought to be contributing factors to a variety of common diseases that have become serious public health problems including, but not limited to cardiovascular disease, obesity, cancer, diabetes, arthritis, depression, and inflammatory bowel diseases [3-7].

The GI tract is populated by as many as 10^{14} microbes, which is many times greater than the entire number of cells comprising the human body. Since these organisms can be either commensal or pathogenic, the human body has had to develop effective strategies to maintain a balance such that the opportunistic pathogenic organisms are kept to a minimal number, thus limiting the inflammation and damage they can induce [2].

Probiotics are defined as viable microorganisms that can populate the GI tract in an active state and extend beneficial qualities to the host [8]. They play an important role in the health of the GI system by altering the environment, limiting the growth of pathogenic organisms, synthesizing nutrients, and increasing energy harvesting from the food we ingest. Species such as *Lactobacillus*, *Bifidobacteria*, and *Bacillus coagulans* are lactic acid producing bacteria, which can lower the pH, creating an environment that is not hospitable to many yeasts and bacterial species. Probiotic bacteria can also secrete antimicrobial compounds that are harmful to pathogenic organisms and thus limit their growth [9]. These bacteria also synthesize nutrients that are beneficial to the host and other beneficial gut organisms such as vitamin K2 and a variety of the B vitamins including folate and B12.

The immune system and the GI system are integrally connected [2]. It is at this interface between the lumen of the gut and the lamina propria that the immune system must make decisions regarding which substances are harmless and which are potentially deleterious. The foods we consume as well as opportunistic and beneficial microbes that occupy our gut are full of antigenic peptides and proteins capable of inducing immune responses. One function of the mucosal immune system is to mount an inflammatory immune response, when appropriate, to eliminate pathogens or to induce tolerance for those substances and organisms that are advantageous, such as food nutrients and probiotic bacteria [10]. Probiotic bacteria affect the adaptive and innate immune system by interacting with numerous cell types along the mucosa including B cells, T cells, regulatory T cells, monocytes, macrophages, NK cells and dendritic cells [9,11]. The immune response is determined by the environment, including cytokines, as well as the absence versus presence of inflammatory mediators.

Commonly consumed probiotic bacteria such as the *Lactobacillus* species are very sensitive to normal physiological conditions like the very low pH of the stomach, bile salts, and high temperatures. This creates challenges for the delivery of these bacteria to the intestines by oral consumption as well as challenges in manufacturing, shipping and storage conditions [12-14].

On the contrary, some strains of *Bacillus coagulans* are able to survive the extremes of heat, acidity of the stomach and bile acids. These characteristics make it an ideal probiotic due to the greater shelf-life stability and survivability to the intestines when consumed. *Bacillus coagulans* transiently occupies the gut for just a few days without repeated oral consumption. Spores of *Bacillus subtilis*, which is very closely related to Bacillus coagulans, have been shown to germinate and colonize the gastrointestinal tract in a murine model for a limited period [15]. The safety of long-term oral consumption of GanedenBC30TM (GBC30), was recently demonstrated [16].

The subject of this study is a proprietary strain of *Bacillus coagulans* (GBI-30, PTA-6086). In Asia, *Bacillus coagulans* is used in a number of commercially available

products including a digestive biscuit for children and in natto (a fermented soy food) along with *Bacillus subtilus* [16,17]. GBC30, supplied by Ganeden Biotech, Inc. (Mayfield Heights, OH, USA) is a lactic acid producing bacteria that has been used as an ingredient in functional foods, dietary supplements, and medical foods. GBC30 is a gram-positive spore-forming rod that is aerobic to microaerophilic in nature and is manufactured as a pure cell mass consisting solely of a proprietary *B. coagulans strain*. Recent clinical studies have shown the efficacy of oral consumption of GBC30 for alleviating symptoms of irritable bowel syndrome [18] and inducing an increased immune response to viral challenge [19].

One aspect of probiotic research that has been questioned is whether the entire effect is due to cell wall components, or whether there are further advantages provided by live, metabolically active probiotic bacteria [20]. Much has been documented on the interaction of specific bacterial cell wall components with Toll-like receptors (TLRs) on immune cells', typically inducing innate immune defense mechanisms [21,22]. Gram negative bacteria predominantly engage the TLR-4 receptor through interaction with lipopolysaccharide present in the bacterial cell wall. Conversely, gram positive bacteria engage the TLR-2 receptor via interaction with lipoteichoic acids present in the bacterial cell wall [23]. However, the mechanism of action for atypical cell wall components, as well as the metabolites secreted by these organisms needs further elucidation [24]. Bacillus coagulans is not a typical inhabitant of the human gastrointestinal tract. Limited, but emerging research has been published on *Bacillus coagulans* as a probiotic and to the best of our knowledge this is the first study to investigate the differential effects of the isolated cell wall components compared with the bacterial metabolites. A broad panel of *in vitro* bioassays was perfomed to explore potential effects of GBC30 on different aspects of the immune system. This involved the study of both peripheral blood mononuclear cells (PBMC) and polymorphonuclear (PMN) cells.

Methods

Reagents

The following buffers and reagents were obtained from Sigma-Aldrich (St. Louis, MO): The T-cell mitogen Phytohemagglutinin (PHA), phosphate-buffered saline (PBS), RPMI-1640 culture medium, hydrogen peroxide 30% solution, Histopaque 1077 and 1119, fibronectin 0.1% from bovine plasma, and dimethyl sulfoxide (DMSO) 99.9%. The following reagents were obtained from Molecular Probes (Eugene, OR): 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), FluoSpheres® carboxylate-modified microspheres 1.0 μm, and CyQuant®. The cytometric bead array for human Th1/Th2 cytokine kit II, CD69-FITC, CD25-FITC, CD107a-FITC, CD56-PE and CD3-PerCP were obtained from BD Biosciences (San Jose, CA). Sodium Azide (NaN_3) was obtained from LabChem Inc. (Pittsburgh, PA). Low-binding 200 μm zirconium beads were obtained from OPS Diagnostics (Lebanon, NJ) and 0.2 μm cellulose acetate filters from Whatman (Florham Park, NJ). Multiscreen plates with 3.0 μm pore size for cell migration were obtained from Millipore (Bedford, MA). The *Bacillus coagulans* strain (GanedenBC$^{30™}$) was obtained from Ganeden Biotech Inc. (Mayfield Height, OH).

Preparation of Bacillus Coagulans Supernatant and Cell Wall Fractions

A sample of 2.0 g of GBC30 spores was placed into 25 mL PBS and heated at 70°C for 30 minutes. Spores were then centrifuged at 2400 rpm for 5 minutes, PBS removed and spores re-suspended in RPMI-1640 culture medium. The culture was incubated at 37°C for 2 days.

Preparation of GBC^{30} culture supernatant (MTB) as a source of GBC30 metabolites: Following an initial spin at 3000 rpm for 15 minutes, the supernatant was removed and centrifuged further at 3500 rpm for 20 minutes. The supernatant was then filtered twice through a 0.2 μm cellulose acetate syringe filter and 250 μL aliquots stored at -20°C.

Preparation of GBC30 cell wall fragments (CW): The bacterial pellet from the initial centrifugation of the 2-day culture was processed through multiple bead milling and freeze/thaw cycles. In brief, the pellet was resuspended in 4 mL of PBS and 4 mL of 200 μm low-binding zirconium beads were added. One cycle of bead milling consisted of 60 one-second pulses of the bacteria/bead mixture on a vortex mixer. Five of these cycles were performed. The supernatant was removed from the beads and spun at high speed in 1.5 mL microcentrifuge tubes. The pellets were combined and re-suspended in 1 mL PBS and processed through 3 freeze/thaw cycles and placement in a sonication bath for an hour. The final solution was filtered through a 0.2 μm cellulose acetate filter and 250 μL aliquots stored at -20°C.

Purification of peripheral blood mononuclear cells (PBMC) and polymorphonuclear (PMN) cells

Healthy human volunteers between the ages of 20 and 50 years served as blood donors upon informed consent, as approved by the Sky Lakes Medical Center Institutional Review Board (FWA 2603). Freshly drawn peripheral venous blood samples in sodium heparin were layered onto a double-gradient of Histopaque 1119 and 1077, and centrifuged for 25 minutes at 2400 rpm. The upper, PBMC-rich and lower PMN interfaces were

harvested using sterile transfer pipettes into new vials, and washed twice with 10 mL PBS without calcium or magnesium by centrifugation at 2400 rpm for 10 minutes. The PBMC fraction contains the natural killer (NK) cell population and was used for testing of NK cell activity as well as lymphocyte proliferation and cytokine production.

Phagocytosis assay

Evaluation of phagocytic activity was performed using human PMN cells. The choice of particles for phagocytosis was carboxylated FluoroSpheres (Molecular Probes, Eugene OR), due to their surface treatment that decreases nonspecific binding and facilitates uptake by phagocytes. These beads fluoresce in the yellow-green spectrum (505/515 nm). An aliquot of 0.05 mL FluoroSpheres was removed from the stock bottle into a 1.5 mL microcentrifuge tube and washed twice in PBS. FluoroSpheres were then re-suspended in 7.5 mL RPMI 1640. PMN cells were plated into 96-well plates in RPMI-1640 at a concentration of 2×10^6 cells/mL. Ten microliters of 10-fold serial dilutions of MTB or CW were added to test wells in quadruplicate, and PBS was added to control wells in quadruplicate. The plate was immediately centrifuged, and the supernatant removed. The cells were re-suspended in RPMI-1640 containing FluoroSpheres, and then incubated for 2 minutes with FluoroSpheres with continuous pipetting. The phagocytic activity was stopped by adding PBS with 0.02% sodium azide. Cells were washed twice in PBS with sodium azide to remove beads not ingested by the cells. Samples were transferred into vials for flow cytometry, ensuring the continued presence of sodium azide. Samples were acquired by flow cytometry immediately (FacsCalibur, Becton-Dickinson San Jose, CA). The analysis was performed using the FlowJo software (TreeStar Inc., Ashland OR). During analysis, electronic gating for the PMN population was performed using the forward and side scatter properties. The relative amount of phagocytosis within the PMN population in each sample was evaluated by the mean fluorescence intensity (MFI) for the green fluorescence. The MFI (green) for the untreated samples showed the relative amount of phagocytosis in the absence of MTB and CW. The MFI (green) for the MTB and CW treated samples were compared to untreated samples. Phagocytosis samples were assayed in triplicates and experiments repeated three times with PMN cells from three different donors.

Reactive Oxygen Species (ROS) production by PMN cells

The production of ROS by PMN cells was tested as described previously [25]. Parallel samples of PMN cells were incubated at 37°C, 5% CO_2 for 20 minutes, either untreated or with test products over a range of 10-fold serial dilutions (1:10, 1:100, 1:1000). The precursor dye DCF-DA, which becomes brightly green fluorescent upon exposure to free radicals, was prepared by adding 0.18 mL DMSO to a 0.05 mg aliquot of DCF-DA. A working solution of DCF-DA was then prepared by adding 0.01 mL stock to 10 mL PBS. The PMN cells were washed three times in PBS and then re-suspended in the DCF-DA working solution and incubated for 1 hour at 37°C. All samples, except for the untreated control samples, were then exposed to 167 mM H_2O_2 for a period of 45 minutes to induce ROS production. Samples were washed twice in PBS to remove the peroxide, and transferred to vials for flow cytometry. The DCF-DA fluorescence intensity in untreated versus H_2O_2-challenged cells was analyzed by flow cytometry. Data was collected in quadruplicate and experiments performed 3 times using cells derived from 3 different donors. The relative amount of ROS formation in PMN cells was evaluated by green fluorescence intensity.

PMN cell random migration and chemotactic migration towards three chemo-attractants: f-MLP, IL-8 and Leukotriene B4

The PMN cell is a highly active and migratory cell type. The differential effect on PMN cell migration towards the bacterial peptide formyl-Met-Leu-Phe (f-MLP) and two different inflammatory chemo-attractants IL-8 and Leukotriene B4 (LTB4) were tested, as described previously [26]. The following experimental model was performed in quadruplicate in order to obtain data significance. Cells were incubated with 10-fold serial dilutions of GBC30 supernatant or cell wall fractions for 10 minutes in a polystyrene round-bottom tube before plating commenced. During this time the Millipore trans-well (3.0 μm pore size) migration plate was coated with 50 μg/mL Fibronectin for a period of 30 minutes. Chemoattractants and RPMI 1640 were then added to the appropriate bottom chamber wells of the trans-well migration plate in a volume of 150 μL: f-MLP (10 nM), Interleukin-8 (10 μg/mL), and Leukotriene B4 (10 nM). Fibronectin was removed from the top wells by aspiration before plating of cells. Fifty microliters of cells (1×10^6/mL) were plated in the top chambers, and the top chamber plate was then lowered into the bottom plate and allowed to incubate overnight at 37°C. Quantification of the relative amount of migrated cells was performed by fluorescent CyQuant® staining of the cells that had accumulated in the bottom chambers. Fluorescence intensity was quantified in a Tecan Spectrafluor fluorescence plate reader. Samples were assayed in triplicate or quadruplicate and experiments repeated at least 3 times with cells from different donors.

Immunostaining for Natural Killer cell activation markers

Freshly isolated PBMC were distributed in a sterile U-bottom 96-well culture plates (NUNC, Denmark) and treated with serial dilutions of test products [27,28]. For activation of natural killer (NK) and natural killer T (NKT) cells the incubation time was 18 hours. Cells were transferred to V-bottom 96-well plates (NUNC Denmark) and washed in IF buffer (PBS containing 1% bovine serum albumin and 0.02% sodium azide). Cells were re-suspended in 0.05 mL IF buffer and monoclonal antibodies were added in previously established optimal quantities (CD3-PerCP, CD56-PE, CD69-FITC, and CD25-FITC: 8 μL/sample), and incubated in the dark at room temperature for 10 minutes. The cells were washed twice with an additional 0.15 mL of PBS with 0.02% azide. Following centrifugation and aspiration of the supernatant, the cells were re-suspended in 0.05 mL PBS with 0.02% azide and transferred to 5 mL polystyrene round-bottom tubes each containing 0.4 mL of 1% formalin. Samples were stored in the dark and acquired by flow cytometry within 24 hours using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose CA). Analysis was performed using the FlowJo (Tree Star Inc., Ashland OR) software. Samples were assayed in duplicate and experiments repeated three times with cells derived from three different donors.

Externalization of CD107a on NK cells in response to K562 tumor cells

The CD107a marker is constitutively expressed on the interior of lysosomes, and transiently expressed onteh cell surface of NK cells that are actively engaged in the killing of transformed cells [29]. Freshly purified human peripheral blood mononuclear cells (PBMC) re-suspended in RPMI 1640 were used for this assay. The cells were plated at 2×10^5/well in round-bottomed 96-well micro-assay plates, and treated with serial dilutions of the test products in triplicate. Negative control wells in triplicate were left untreated. In addition, three wells containing PBMC alone and K562 cells alone served as negative controls for baseline CD107a expression. 1×10^6 K562 cells, an NK-cell sensitive tumor cell line widely used in NK cell cytotoxicity studies, were added to wells containing PBMC with product and untreated PBMC. The two cell types were loosely pelleted by a brief 30-second centrifugation at 2400 rpm followed by incubation at 37°C for 45 minutes. Cells were transferred to V-bottom microtiter plates for processing and staining. Cells were stained with CD3-PerCP, CD56-PE and CD107a-FITC. The expression of CD107a on the NK cells was determined by flow cytometry. The CD3 negative, CD56 positive NK cells were differentiated from the K562 cells based on forward and side scatter properties, and from other lymphocytes by electronic gating on $CD3^-$, $CD56^+$ cells, followed by evaluation of fluorescence intensity for CD107a. Samples were assayed in triplicate and experiments repeated three times using cells derived from three different donors.

Modulation of proliferation and cytokine production in response to PHA and PWM

Freshly purified PBMC re-suspended in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 mg/mL) were plated in a U-bottom cell culture plate at a volume of 180 μL at a concentration of 1×10^6/mL. Next, 20 μL of 10-fold serial dilutions of MTB and CW were added to the individual wells in triplicate. In a parallel set of wells, the combinatorial effect of MTB and CW with known mitogens was tested. Mitogens were added at a concentration of 5 μL of PWM (200 μg/mL) and 4 μL of PHA (2 μg/mL) to initiate proliferation. The plate was sealed with parafilm and was incubated at 37°C, 5% CO_2 for 5 days. After 5 days the cells were transferred to a flat-bottom black 96-well plate and the relative cell numbers in each culture well quantified by CyQuant® staining and a Tecan Spectrafluor fluorescence plate reader. Samples were assayed in triplicate and experiments repeated three times with cells derived from three different donors.

Supernatants from the 5-day lymphocyte proliferation cultures were harvested and relative levels of the 6 cytokines: IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ were measured using a flow cytometry-based bead array kit (CBA human Th1/Th2 cytokine kit II, BD Biosciences, San Jose, CA). Samples were assayed for the 1:100 dilution of MTB and CW. Samples were tested in duplicate according to the manufacturer's specifications, and data acquired immediately by flow cytometry, using a FacsCalibur flow cytometer (Becton-Dickinson San Jose, CA). The analysis was performed using the FlowJo software (TreeStar Inc., Ashland, OR).

Statistical analysis

Statistical significance was tested using Student's t-test with $p < 0.05$ indicating a statistically significant and $p < 0.01$ a highly statistically significant difference between two data sets. Analyses were performed using Microsoft Excel. Only statistically significant p values are reported.

Results

Inhibition of Formation of Reactive Oxygen Species (ROS) by PMN cells

The PMN cell is involved in innate immune defense mechanisms, including the formation of ROS as part of both anti-bacterial and pro-inflammatory reactions. Treatment with complex natural products can cause signaling of pro- as well as anti-inflammatory mechanisms,

leading to either enhancement or reduction of ROS formation.

Both MTB and CW showed a clear inhibition of the spontaneous formation of reactive oxygen species in PMN cells (Figure 1A). The effect of CW showed a consistent inhibition of ROS formation across all doses tested, whereas the effect of MTB showed stronger anti-inflammatory effect at the lowest doses tested. The presence of MTB (1:1000) reduced spontaneous ROS formation by 20% ($p < 0.004$). CW (1:1000) showed a similar effect on lowering ROS formation ($p < 0.005$). At the 1:10 dilution of CW this effect was even stronger, resulting in a nearly 30% reduction in spontaneous ROS formation ($p < 0.0008$).

Treatment of PMN cells with MTB and CW before exposing the cells to oxidative stress and resulting ROS formation showed that both MTB and CW inhibited the H_2O_2-induced ROS formation (Figure 1B). MTB (1:100) and CW (1:100) reduced ROS formation by 15% and this reduction was statistically significant for CW ($p < 0.03$).

Effect on PMN phagocytic activity

Phagocytosis of microbial particles is an important part of the innate immune response. It is a rapid process, and the effect of a test product on enhancing this cellular function can be almost immediate. Phagocytosis was measured by how well PMN cells engulfed carboxylated fluorospheres. The mean fluorescence intensity (MFI) of phagocytic cells was then evaluated by flow cytometry. Exposure of PMN cells to MTB at the 1:10 dilution increased phagocytosis by 40% (Figure 2A). Exposure of PMN cells to CW at the 1:10 dilution increased phagocytosis by 25% ($p < 0.02$). Figure 2A shows the overall increase for the entire population of PMN cells, where some cells are phagocytic and others remain non-phagocytic. Figures 2B and 2C show the comparison of an untreated PMN cell to a PMN cell exhibiting increased phagocytosis following exposure to CW. Further dilutions of both products resulted in reduced PMN phagocytosis ($p < 0.05$) (data not shown).

Differential effect on PMN cell random migration and chemotactic migration towards three chemo-attractants: f-MLP, IL-8, and Leukotriene B4 (LTB-4)

The PMN cell is a highly active and migratory cell type that plays a major role in immune surveillance. The migratory behavior of this cell type is divided into at least two types: a) random migration and b) directed migration. Random migration is part of normal immune surveillance, whereas directed migration happens towards specific chemoattractants.

The effects of MTB and CW on both types of migration were tested in parallel. Furthermore, the directed migration was tested towards three distinctly different chemotactic compounds: i) bacterial peptide f-Met-Leu-Phe (f-MLP); ii) the inflammatory cytokine Interleukin-8 (IL-8); and iii) Leukotriene B4 (LTB4).

Both fractions of GBC30 induced the random migration of PMN cells. MTB (1:10) increased the random migration by 300% ($p < 0.05$), and CW (1:10) increased the random migration by 25% ($p < 0.006$) (Figure 3A). A distinct dose-dependent effect was seen with treatment of cells with MTB but not CW.

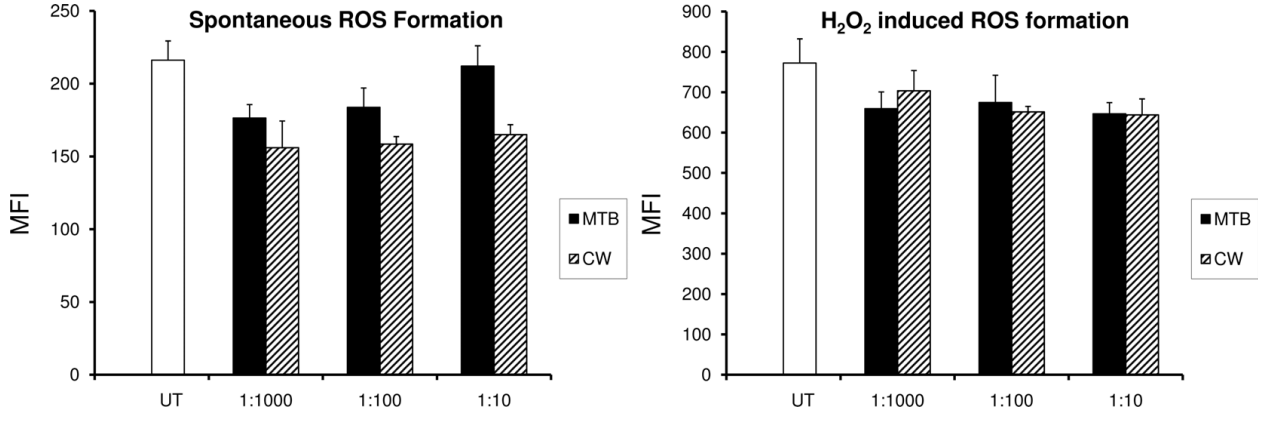

Figure 1 The inhibition of formation of Reactive Oxygen Species (ROS) was evaluated in human polymorphonuclear (PMN) cells. The DCF-DA mean fluorescence intensity in the cells was proportional to the oxidative damage. Untreated cells served as a baseline for oxidative activity in the absence of oxidative stress. The presence of BC fractions MTB and CW led to inhibition of the spontaneous ROS production in the cultures (**A**). H_2O_2 was used to induce oxidative stress in the cultures. Cultures treated with H_2O_2 in the absence of GBC30 fractions served as a positive control for ROS formation. Cultures treated with GBC30 fractions MTB and CW in the presence of oxidative stress showed reduced ROS formation compared to cultures treated with H_2O_2 alone (**B**). Data reflect averages of cultures performed in quadruplicate for each test condition. The data shown are representative of three independent experiments performed on PMN cells from different donors.

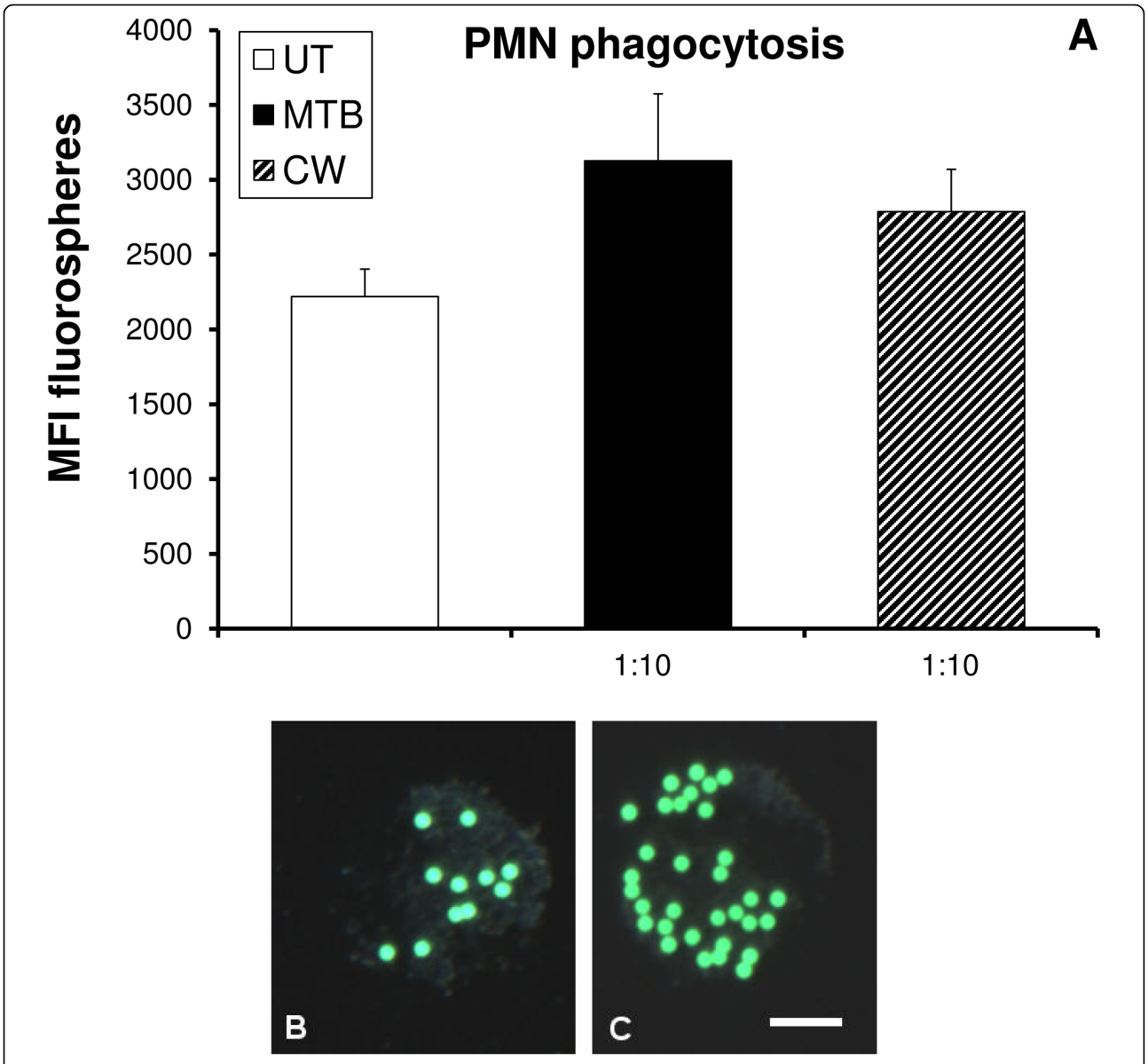

Figure 2 Human polymorphonuclear (PMN) cells were evaluated for phagocytic activity, measured by the uptake of green-fluorescent beads. (**A**) Flow cytometry analysis showed an increase in mean fluorescence intensity (MFI) of PMN cells treated with either MTB or CW. Microscopy showed that the level of bead uptake by an untreated PMN cell (**B**) was increased in PMN cells treated with CW (**C**). Photographs were taken at 600× magnification and the bar drawn in figure 2C represents 5 uM. The data shown are representative of PMN cells assayed in triplicate for each test condition.

Both MTB and CW increased the migration towards the bacterial peptide f-MLP, implicating a support of anti-bacterial defense mechanisms (Figure 3B). Once again a dose-dependent response was seen following MTB treatment of cells. At the 1:10 dilution, MTB showed a 200% enhancement of f-MLP directed migration that was highly statistically significant ($p < 0.0005$). A 25% increase following CW treatment (1:10) was also statistically significant ($p < 0.04$).

Both MTB and CW reduced the IL-8 directed migration. Because we saw a strong reduction in IL-8 directed migration of PMN cells treated with low doses of CW and also saw some reduction with low doses of MTB, a dose study of IL-8 directed PMN migration with much lower doses of both MTB and CW was performed. As shown in Figure 3C, a reduction in IL-8 directed PMN migration was demonstrated at all dilutions of CW. This effect of CW was strongest at the 10^{10} dilution, where

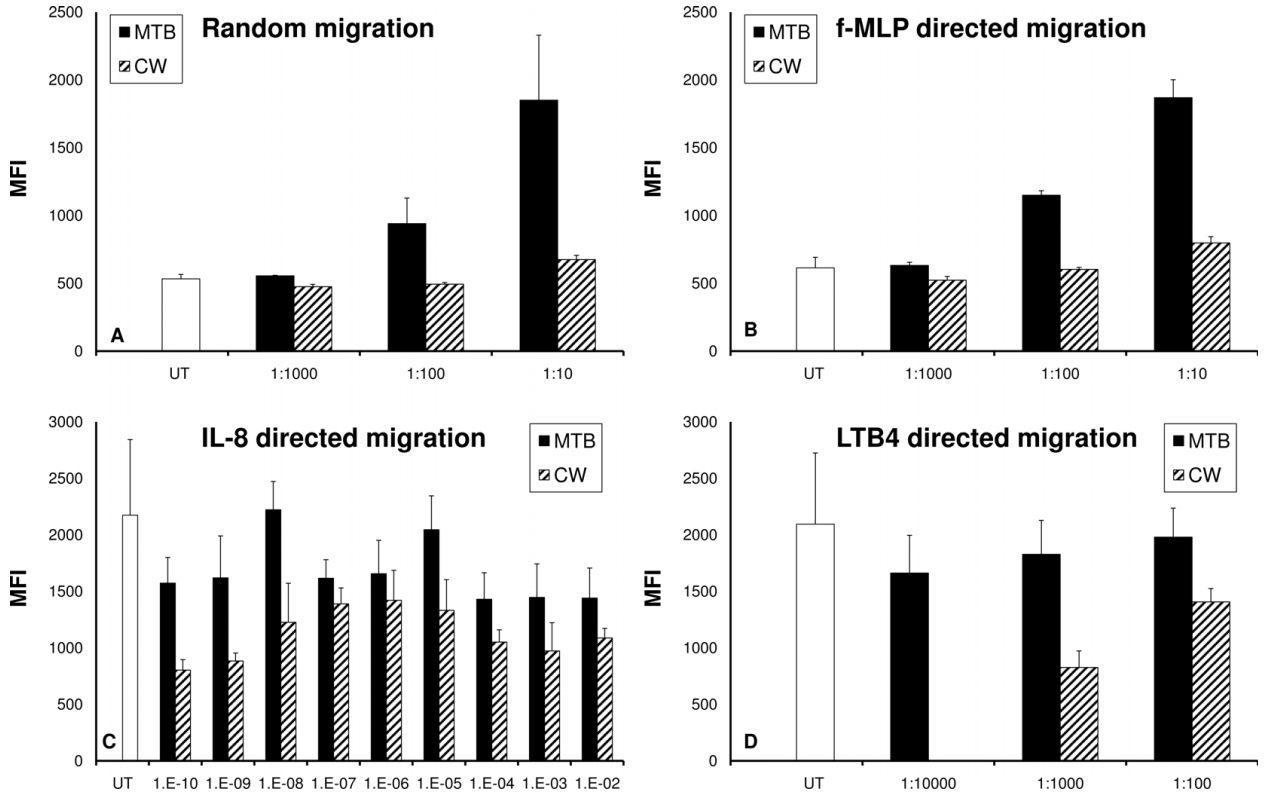

Figure 3 Effects of GBC30 fractions on human PMN migration were evaluated using Transwell migration plates. (**A**) Both MTB and CW treated PMN cells showed increased random migration behavior. (**B**) PMN cells treated with MTB or CW showed increased migratory behavior in response to the bacterial peptide f-MLP. (**C**) PMN chemotactic migration towards the cytokine IL-8 was decreased in the presence of MTB and CW. Extremely low doses of MTB and CW were tested for effects on IL-8 directed PMN cell migration. The cell wall fraction CW triggered inhibition of PMN cell migration towards IL-8 down to dilutions of 10^{-10}. The metabolite fraction MTB showed similar but weaker inhibition across the same dose range. (**D**) PMN chemotactic migration towards the inflammatory mediator LTB4 was decreased by both the cell wall fraction MTB and the metabolite fraction CW, indicating an anti-inflammatory effect. The graphs show the averages and standard deviations of each culture condition performed in triplicate. The data shown are representative of three experiments performed on cells from different donors.

migration was inhibited by over 60% ($p < 0.03$). MTB treatment of PMN cells at low doses also reduced IL-8 directed migration but not as much as CW. An interesting pattern of IL-8 directed PMN migration inhibition was seen with both MTB and CW. Neither product demonstrated a linear dose curve but rather intermediate doses (10^4 to 10^8) of both MTB and CW showed less inhibition of IL-8 directed migration compared to higher or lower doses.

Both MTB and CW demonstrated a dose-dependent reduction in PMN migration towards LTB4 (Figure 3D). The 1:1000 dilution of CW inhibited migration by 60%. Cells treated with 1:1000 and 1:10000 dilutions of MTB also showed anti-inflammatory effects but these were not statistically significant.

Induction of the CD69 activation marker on Natural Killer cells

Natural Killer (NK) cells are involved in our primary defense mechanisms against transformed cells and viruses. These cells travel in our blood stream in a state of rest, but can be immediately activated to a) kill cancer cells by either cell contact or secretion of cytotoxic compounds such as perforin and granzyme, b) proliferate, and c) secrete substances that attract other cells into the site. In order to investigate a possible effect of MTB and CW on NK cell activation, we examined changes in expression of the NK activation cell surface marker CD69. The increased expression of this marker has been associated with an increased cytotoxic activity of NK cells [30].

Both MTB and CW showed a clear dose-dependent induction of the expression of CD69 on NK cells (Figure 4A). This increase was statistically significant for the 1:400, 1:1600 and 1:6400 dilutions of both MTB and CW ($p < 0.05$). At the 1:400 dilution, CD69 expression was increased by 32% ($p < 0.05$) for MTB, and by 36% for CW ($p < 0.006$).

A mild increase in CD25 expression was seen on NK cells treated with a 1:100 dilution of MTB. No changes

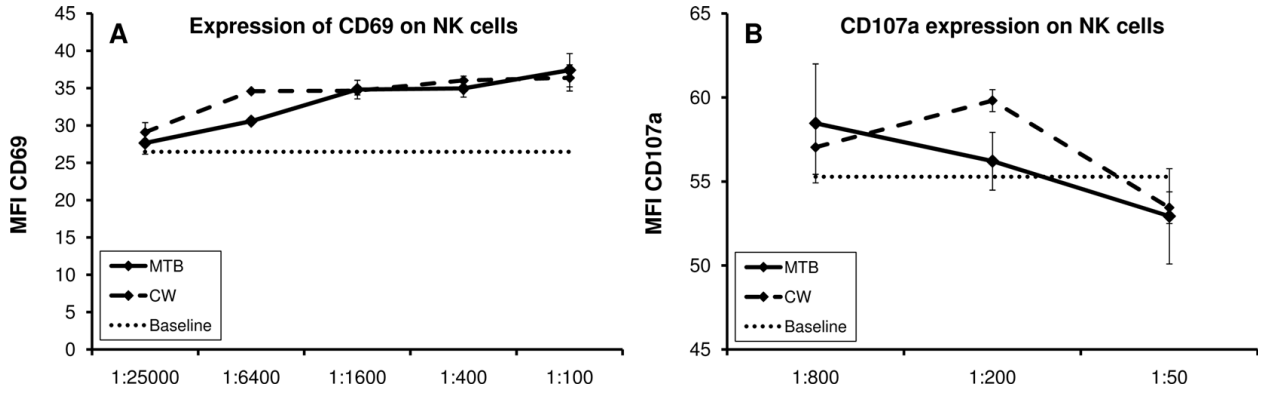

Figure 4 Effects of the metabolite fraction MTB and cell wall fraction CW on NK cell activation status and cytotoxic activity was evaluated using human PBMC. (**A**) Both MTB and CW fractions induced the expression of the activation marker CD69 on CD3 negative, CD56 positive NK cells in a dose-dependent manner. The effect was statistically significant for the 1:400 dilution of both MTB and CW. (**B**) Expression of CD107a on NK cells pretreated with MTB or CW showed a mild increase over baseline. The graphs show the averages and standard deviations of each culture condition performed in triplicate. The data shown are representative of three experiments performed on cells from different donors.

in CD25 expression on T cells were observed when comparing untreated cells to those treated for 18 hours with either MTB or CW (data not shown).

Externalization of CD107a on NK cells in the presence of K562 tumor cells

One of the functions of NK cells is to kill tumor cells and virus-infected cells via cell-cell contact and by secretion of substances such as perforin. During this process, the CD107a receptor expressed on the interior of granules in the cytoplasm of NK cells is transiently brought to the cell surface. Thus, the transient CD107a expression on NK cells is a measure of their cytotoxic activity by secretion of cytotoxic substances. Figure 4B shows the change in mean fluorescence intensity (MFI) of CD107a expression on natural killer cells that have been exposed to tumor cells, with or without the addition of MTB or CW. Both MTB and CW show a mild increase in CD107a cell surface expression, with CW having the strongest effect at the 1:200 dilution, however the effect did not reach statistical significance.

Effects on lymphocyte proliferation

With the question of immune support always comes the need to prove that a product would not on its own trigger exaggerated immune reactions. As part of a standard safety testing of natural products, we tested whether the test products had mitogenic potential, that is, whether they induce cell division in healthy human lymphocytes. Simultaneous to the test of mitogenic potential, we tested whether the test products had an effect on cells responsible for the adaptive immune defense, that is, T and B lymphocytes.

The GBC30 fractions were tested in serial dilutions in the absence and presence of mitogens. Two mitogens were tested in parallel: Phytohemagglutinin (PHA), which is a T cell mitogen that induces T cell proliferation, and Pokeweed Mitogen (PWM), which requires the collaboration of T cells, B cells and monocytes in the culture. Supernatants from the same cultures were processed for cytokine levels (see below).

Neither MTB nor CW had a mitogenic effect on lymphocyte proliferation following five days incubation at 37°C with product and culture media (data not shown). Both MTB and CW showed a reduction in lymphocyte proliferation in the presence of PHA and PWM. This reduction was statistically significant for both MTB and CW ($p < 0.02$) (data not shown).

Effects on cytokine production

A flow cytometry-based Th1/Th2 cytokine bead array for the 6 cytokines IL-2, IL-4, IL-6, IL-10, TNF-α and INF-γ was used to evaluate the levels of cytokines present in the supernatants from 5-day lymphocyte proliferation cultures. Phytohemagglutinin (PHA) was used to induce T cell proliferation, and Pokeweed Mitogen (PWM) was used to induce T and B lymphocyte proliferation in a process that requires the collaboration of T cells, B cells and monocytes in the culture. Comparisons were made between untreated PBMC versus PBMC cultured in the presence of 1:100 dilutions of either MTB or CW, with and without PHA and PWM (Figure 5A-F). Untreated PBMC cells were cultured under identical conditions as MTB and CW treated PBMC cultures and cytokine levels in the untreated cultures used as a baseline for comparison to MTB and CW treated cultures.

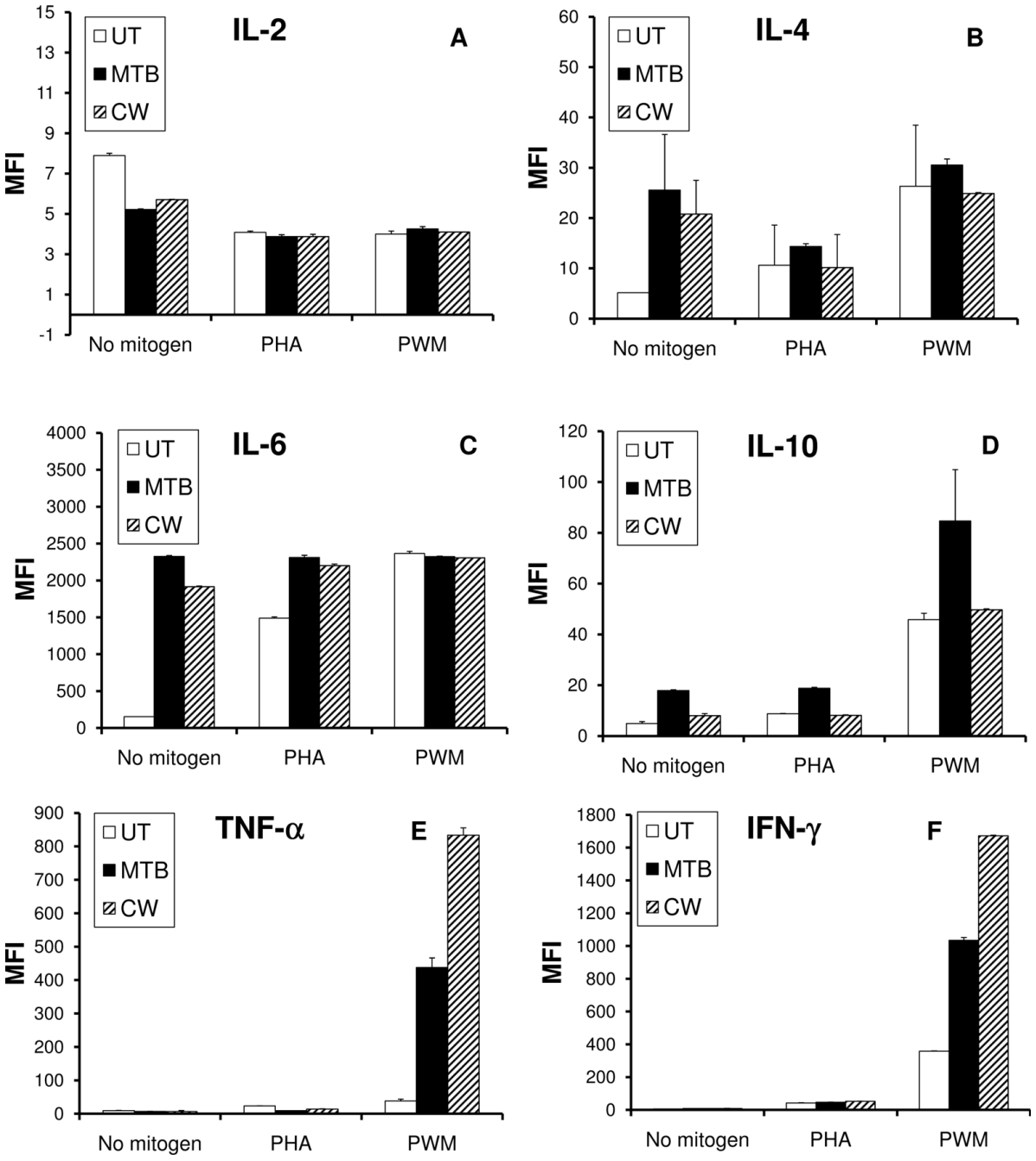

Figure 5 The effects of the metabolite fraction (MTB) and cell wall fraction (CW) on cytokine production were evaluated on human PBMC cultures. (**A**) Treatment of PBMC with MTB and CW resulted in reduced IL-2 production. There was no effect on mitogen-induced IL-2 production. (**B**) Treatment of PBMC with MTB and CW resulted in increased IL-4 production. There was no effect on mitogen-induced IL-4 production. (**C**) Treatment of PBMC with MTB and CW resulted in a strong increase in IL-6 production. Both MTB and CW further enhanced the PHA-induced IL-6 production. In contrast, neither MTB nor CW modulated the PWM-induced IL-6 production. (**D**) Treatment of PBMC with MTB, and to a lesser extent CW, resulted in an increased IL-10 production. MTB but not CW further enhanced the PHA-induced as well as the PWM-induced IL-10 production. (**E**) Both MTB and CW treatment of PBMC resulted in a mild reduction in TNF-α production, both in the absence of mitogens, and in the presence of PHA. In contrast, co-stimulation of PBMC with PWM and either MTB or CW resulted in a strong increase in TNF-α production. (**F**) Very slight increases in IFN-γ cytokine levels were seen following MTB or CW treatment in the absence of mitogens or presence of PHA. In contrast, co-stimulation of PBMC with PWM and either MTB or CW resulted in a strong increase in IFN-γ production. Cytokine production in PBMC cultures was assayed in duplicate. The concentration of MTB and CW in the assayed cell cultures was 1:100.

The relative changes of cytokines are shown, where "0" on the y axis indicates that treatment did not alter cytokine production from baseline.

In the absence of mitogens, both MTB and CW treatment of PBMC led to decreased IL-2 levels compared to untreated PBMC (Figure 5A). This reduction was statistically significant for MTB and CW ($p < 0.02$). No statistically significant changes in IL-2 levels were observed with MTB or CW treatment in the presence of either mitogen, compared to mitogen treatment alone.

In the absence of mitogens, both MTB and CW treatment of PBMC led to increased IL-4 levels compared to untreated PBMC (Figure 5B). This increase was statistically significant for MTB ($p < 0.03$). No statistically significant changes in IL-4 levels were observed with MTB or CW treatment in the presence of either mitogen, compared to mitogen treatment alone.

Both MTB and CW treatment of PBMC, in the absence of mitogens, led to massive induction of IL-6 production (Figure 5C). The increase was highly statistically significant ($p < 0.003$). No statistically significant changes in IL-6 levels were observed with MTB or CW treatment in the presence of Pokeweed mitogen, compared to Pokeweed mitogen treatment alone. The IL-6 induction by both MTB ($p < 0.003$) and CW ($p < 0.002$) in the presence of PHA was found to be highly statistically significant.

Both MTB and CW treatment of PBMC, in the absence of mitogens, led to induction of IL-10 production (Figure 5D). The increase was statistically significant ($p < 0.02$). PBMC treated with both MTB and PHA led to higher IL-10 production ($p < 0.008$) than if cells were treated with PHA alone. Treatment of PBMC with MTB and PWM also led to an increase in IL-10 production, however the data was not found to be statistically significant. No statistically significant changes in IL-10 levels were observed with CW treatment in the presence of either mitogen when compared to mitogen treatment alone.

In the absence of mitogens, TNF-α production was slightly lower than untreated PBMC in the presence of both MTB and CW (Figure 5E). This mild reduction was not statistically significant for either MTB or CW. Treatment of PBMC with either MTB or CW in the presence of PHA resulted in 2-fold decreases in TNF-α expression that were statistically significant for both MTB ($p < 0.03$) and CW ($p < 0.006$). In contrast, treatment of PBMC with MTB and CW in the presence of PWM resulted in strong increases in TNF-α levels. In the presence of PWM, MTB treatment produced an 11-fold increase ($p < 0.03$) and CW treatment a 22-fold increase ($p < 0.02$).

In the absence of mitogens, INF-γ levels increased in response to treatment with both MTB and CW (Figure 5F). MTB produced a 41% increase ($p < 0.02$) and CW resulted in a 54% increase ($p < 0.02$). Treatment of PBMC with either MTB or CW in the presence of PHA did not produce statistically significant changes in INF-γ expression. In contrast, treatment of PBMC with MTB and CW in the presence of PWM resulted in 3-fold (MTB) and 4-fold (CW) increases in INF-γ levels, both of which were statistically significant ($p < 0.01$).

Thus, when evaluating the overall effect on production of all six cytokines, MTB induced an increased production of the three Th2 cytokines IL-4, IL-6, and IL-10 (Figure 6A). Given the strong increase in IL-6 production when cells were treated with MTB, a second graph shows the data on the five cytokines where data on IL-6 were removed (Figure 6B). Simultaneously, a reduced production of the two Th1 cytokines IL-2 and TNF-α was seen. The increase in all three Th2 cytokines was also seen for the CW fraction. However, Th1 cytokines were not reduced, and IFN-γ showed an increase above untreated cells (Figure 6C-D).

Discussion

Inflammation, particularly chronic, low-grade inflammation, is an underlying phenomenon in many diseases, including cardiovascular disease, obesity, arthritic conditions, malignant transformation and progression of tumor growth, Alzheimer's disease, age-related cognitive decline, and depression. This has brought nutritional and nutraceutical anti-inflammatory products into focus, as people look for non-pharmacological ways to stay healthier longer [3].

Many types of bacterial cell wall components are known to interact with Toll-Like Receptors (TLR) 2 and 4 and thereby trigger pro-inflammatory immune defense reactions [31]. It was therefore particularly surprising that the GBC30 cell wall fraction, CW, showed inhibitory activity in several bioassays involving pro-inflammatory types of immune reactions. These included inhibition of ROS formation and reduced PMN cell chemotactic migration in response to IL-8 and LTB4.

The GBC30 culture supernatant, MTB, showed similar anti-inflammatory properties, although not quite as potent as CW. There were sufficient data sets showing distinctly different effects of MTB compared to CW. These included a more robust effect on increasing phagocytosis, enhancement of random and f-MLP directed PMN migration and induction of the cytokine IL-10 in 5 day PBMC cultures. Conversely, NK cell activation, demonstrated as an increase in CD69 expression, was nearly identical following MTB or CW treatment, across a broad dilution range. There were also multiple instances where MTB demonstrated a dose-dependent response that was not seen with CW, suggesting that with MTB treatment unique effects of the metabolites

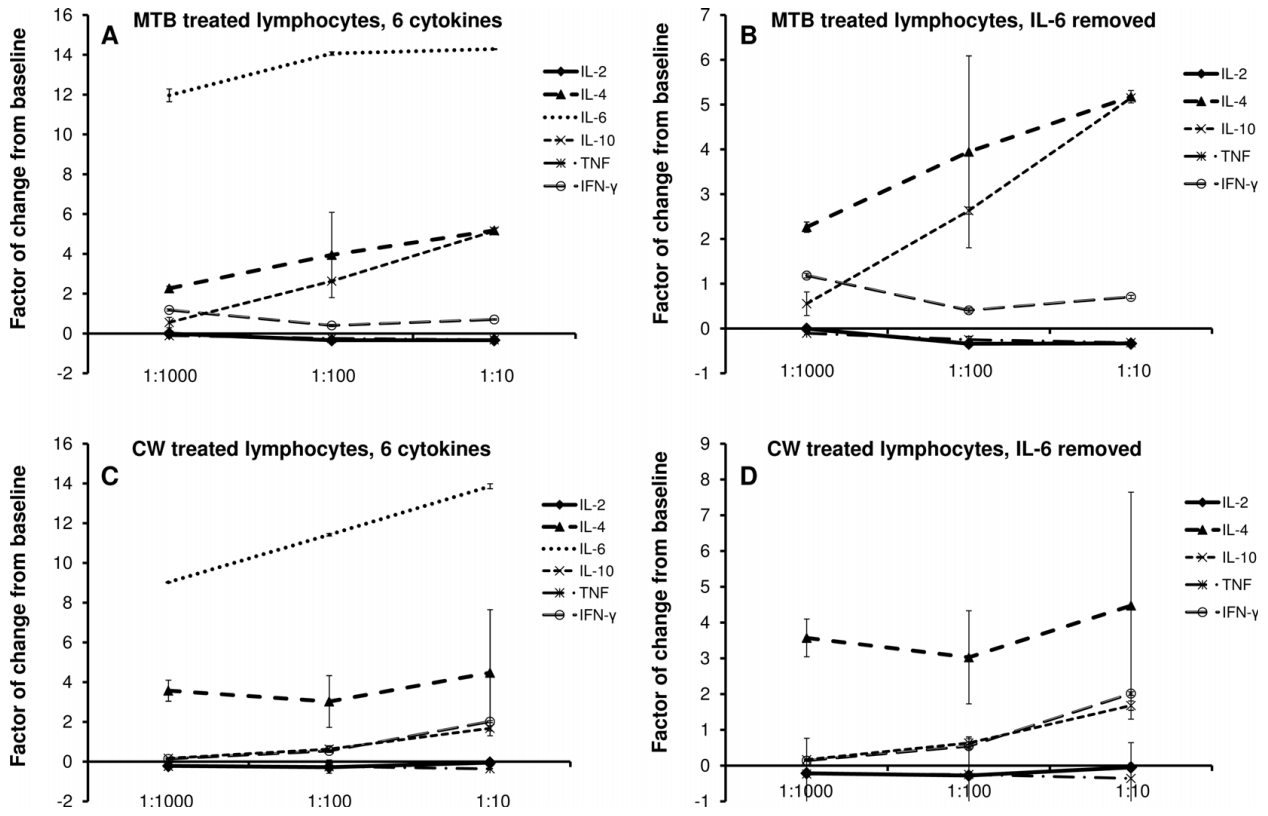

Figure 6 The direct and dose-dependent effect of GBC30 fractions on Th1/Th2 cytokine production, in the absence of other stimuli, is shown. The increase in production of the Th2 cytokines IL-4, IL-6, and IL-10, and composite effect on Th1 cytokines IL-2, IFN-γ, and TNF-α, is shown for the metabolite-rich fraction MTB (**A, B**) and the cell wall fraction CW (**C, D**). In the graphs **B** and **D**, the data from IL-6 were removed to better show the changes in production of the other 5 cytokines.

and not residual activity from contaminating CW was seen. Therefore, we conclude that even though some trace amount of cell wall components may be present in MTB, they do not account for the overall biological activity of MTB.

The effects of both GBC30 fractions were highly complex, and were not limited to a simple anti-inflammatory action. Both GBC30 fractions enhanced PMN cell random migration, which is an important aspect of immune surveillance. Both fractions also enhanced PMN cell migration in response to the bacterial peptide f-MLP, when used at higher doses. MTB treatment of cells had a greater effect on enhancing random and f-MLP directed PMN migration than CW treatment and this effect was dose dependent.

Both fractions induced the CD69 activation marker on human CD3-negative, CD56-positive NK cells, and also enhanced the transient expression of CD107a as an indication of increased NK cell cytotoxicity in the presence of transformed cells. The expression of CD107a as a consequence of degranulation of NK cells in the presence of NK sensitive tumor cells is transient, as CD107a is rapidly recycled by re-uptake into cytoplasmic granules containing cytotoxic substances. Thus, even though the CD107a data did not reach statistical significance, it supports the evidence of increased cytotoxic activity as indicated by increased expression of CD69. CD69 expression on NK cells is the only assay where MTB and CW performed nearly identical.

Such observations are normally associated with a more general activation of innate immune defense mechanisms, such as production of ROS, which was clearly not the case for the GBC30 fractions. Both MTB and CW inhibited spontaneous and H_2O_2-induced ROS formation. In the case of MTB, inhibition of spontaeous ROS formation was strongest at the lowest dilution tested. This interesting concentration-dependent effect of responses was also seen for both MTB and CW with regard to the migratory behavior of PMN cells in response to 3 different chemotactic stimuli. This suggests a complex immune response dependent on the concentration of the different BC30 fractions assayed. At high concentrations, a pro-inflammatory response is

seen while at low concentrations an anti-inflammatory response predominates.

The effects on cytokine profile were tested in several ways, where both the direct effect, as well as the synergistic effects with other known lymphocyte activating compounds was studied. The products directly affected the cytokine levels *in vitro* by increasing the production of the Th2 cytokines IL-4 and IL-10. The shift towards Th2 cytokine production reflects support of adaptive immunity and antibody production, including sIgA secretion, which may enhance immune defense mechanisms along the gut mucosa [32]. The products may also affect important regulatory mechanisms affecting how the immune system reacts to food-borne microbes and antigens, such as induction of oral tolerance [33]. The products may have direct immune modulating effects on immune cells in lamina propria and Peyer's patches, affecting local and systemic cytokine levels and thus potentially affecting inflammatory conditions at locations unrelated to the gut environment.

A greater increase in IL-10 production occurred in cultures treated with MTB compared to cultures treated with CW. In the presence of the mitogens PHA or PWM, only MTB treatment produced an increase in IL-10 above baseline. IL-10 has an anti-inflammatory role in the gut and the consumption of IL-10 producing transgenic bacteria has shown efficacy in the treatment of Crohn's disease [34]. This suggests that consumption of GBC30 may also have a beneficial effect on conditions of chronic intestinal inflammation such as Crohn's disease and ulcerative colitis.

Both GBC30 fractions strongly induced production of IL-6 in PBMC cultures. IL-6 is not a simple pro- or anti-inflammatory cytokine, but helps to regulate inflammation. This regulatory cytokine has pleiotropic functions that are tissue-specific and dependent upon the physiological context [3]. The effects of IL-6 are influenced by whether it is present acutely or chronically. Thus, further evaluation of IL-6 levels in people consuming GBC30 is needed, in healthy individuals, individuals experiencing acute infection, in those with chronic inflammatory conditions, insulin resistance, and endothelial dysfunction. Based on the complexity of the *in vitro* effects of both GBC30 fractions, we suggest that consumption of the probiotic strain may show protective effects in both acute and chronic conditions.

A mild reduction of IL-2 and TNF-α was seen, along with an increase in IFN-γ production. The pro-inflammatory functions of TNF-α and IFN-γ are undisputed; however IL-2 has complex and opposing roles during the induction and termination of inflammatory responses. In contrast to the direct anti-inflammatory effect on cytokine profile, GBC30 strongly enhanced responses to the known stimulus Pokeweed mitogen (PWM), which requires the collaboration of macrophages and different lymphocyte subsets. The increase in IFN-γ in PWM-treated cultures following treatment with MTB and CW is of interest regarding the role of IFN-γ producing dendritic cells in mucosal immunity against orally acquired pathogens [35]. This suggests that GBC30 compounds may enhance the response to invading pathogens in, for example, Peyer's Patches, where different cell types including dendritic cells collaborate on producing innate and adaptive immune defense reactions.

Conclusions

The complex actions of both GBC30 fractions include nti-inflammatory effects, while also supporting key aspects of innate immune defense mechanisms. If some or all of the effects observed *in vitro* can also potentially take place when GBC30 replicates in the intestinal environment, this could have important implications, not only for protection of the host from potentially pathogenic bacteria, but even more importantly, for controlling local inflammatory processes and protecting epithelial integrity, which then allows proper separation of gut lumen, and proper nutrient assimilation and antigen presentation to the immune system. The data suggest that further clinical work should be initiated to evaluate the effect of consumption of GBC30 as well as the MTB and CW fractions on the control of inflammatory reactions *in vivo*, while simultaneously supporting anti-bacterial, anti-viral, and anti-tumor defenses *in vivo*.

Abbreviations

DCF-CA: Dichlorofluorescein Diacetate; DMSO: Dimethylsulfoxide; f-MLP: formyl-met-leu-phe; GBC30: GanedenBC$^{30™}$ *Bacillus coagulans*: GBI-30, PTA-6086; IL: Interleukin; LTB4: Leukotriene B4; MFI: Mean Fluorescence Intensity; NF-κB: Nuclear Factor κB; NK: Natural Killer Cells; NKT: Natural Killer T Cells; PBMC: Peripheral Blood Mononuclear Cells; PBS: Phosphate Buffered Saline; PHA: Phytohemagglutinin; PMN: Polymorphonuclear Cells; PWM: Poke Weed Mitogen; ROS: Reactive Oxygen Species; RPMI: Roswell Park Memorial Institute Culture Medium; TLR: Toll-like Receptors; TNF: Tumor Necrosis Factor.

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Authors' contributions

GSJ and JRE conceived of the idea to test and compare the bioactivity of bacterial cell walls and metabolites. GSJ and KFB planned the procedure for generating the two test fractions. GSJ designed the study and supervised the lab work and data analysis. KFB performed the production of the two fractions. KFB and SGC performed the in vitro testing, analysis, and contributed to the writing of the manuscript. KFB did the statistical analysis. GSJ and JRE finalized the manuscript writing. All authors have read and have approved the final manuscript.

Competing interests

Ganeden Biotech, Inc. sponsored the study that is being reported. AIBMR Life Sciences and NIS Labs were paid for the conception, design and execution of the *in vitro* study, as well as for the preparation of the

manuscript. Neither organization has any financial interest in Ganeden Biotech, Inc.

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Research article

A prospective, randomized, double-blind, placebo-controlled parallel-group dual site trial to evaluate the effects of a *Bacillus coagulans*-based product on functional intestinal gas symptoms

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Abstract

Background: This randomized double blind placebo controlled dual site clinical trial compared a probiotic dietary supplement to placebo regarding effects on gastrointestinal symptoms in adults with post-prandial intestinal gas-related symptoms (abdominal pain, distention, flatulence) but no gastrointestinal (GI) diagnoses to explain the symptoms.

Methods: Sixty-one adults were enrolled (age 36.5 ± 12.6 years; height 165.1 ± 9.2 cm; weight 75.4 ± 17.3 kg) and randomized to either Digestive Advantage™ Gas Defense Formula - (GanedenBC^{30} *Bacillus coagulans* GBI-30, 6086): n = 30; or Placebo: n = 31. Study subjects were evaluated every two weeks over a four-week period using validated questionnaires and standard biochemical safety testing. Outcome criteria of interest included change from baseline in Gastrointestinal Symptom Rating Scale (GSRS) abdominal pain, abdominal distention, flatus, and the Severity of Dyspepsia Assessment (SODA) bloating and gas subscores over four weeks of product use.

Results: Measured against the placebo, subjects in the probiotic group achieved significant improvements in GSRS abdominal pain subscore (p = 0.046) and the GSRS total score (p = 0.048), with a strong trend for improvement on the GSRS abdominal distension subscore (p = 0.061). A strong placebo effect was evident which could explain the lack of statistical significant differences between the groups for many of the efficacy variables.

Conclusion: In conclusion, the *Bacillus coagulans*-based product was effective in improving the quality of life and reducing gastrointestinal symptoms in adults with post prandial intestinal gas-related symptoms and no GI diagnoses.

Trial Registration: ClinicalTrials.gov Identifier: NCT00881322

Background

It is estimated that only 10% of the 10^{14} cells in the human body actually belong to the body itself. The overwhelming majority of cells consist of a diverse ecology of nonpathogenic bacteria, and 1-2 kg of them live in the gut alone, mainly in the large intestine [1]. Bengmark suggests that human beings should indeed be considered to have two separate, equally vital digestive systems: one being the organs of the gastrointestinal tract; the other being the bacteria that colonize them [2]. The bacteria have defined an ecological niche for themselves in the intestines, fermenting non-digestible dietary residue and endogenous mucus from the epithelia [3]. Though the colon contains over 500 strains of bacteria, it is generally dominated by 35-40 different types of microbes. Many disorders of the gut have been associated with a disturbance in this distribution of species. Inflammatory bowel disease, diarrhea, and even multisystem organ failure [4] are believed to be correlated with an imbalance in gut ecology favoring the growth of pathogenic strains [5].

Probiotics are nutritional supplements designed to target pathogenic microbial species distribution by augmenting the growth of nonpathogenic bacteria. A commonly accepted definition of probiotic is "a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects on the host [6]." There is strong evidence that probiotics work by helping non-pathogenic bacteria to compete with their pathogenic counterparts for nutrient availability as well as for adhesion sites along the intestinal lining, preventing both the overgrowth of pathogenic bacteria as well as their translocation through the epithelial mucosa into the rest of the body [7]. There is also evidence to suggest that intestinal flora play an important role in immune system response. Studies in humans and rodents have shown that probiotic treatment is directly correlated with an increase of salivary immunoglobin A (sIgA) production. Furthermore, exposure to luminal microbes instantly increases the number of intraepithelial lymphocytes.

In addition to overwhelming evidence in support of the effectiveness of probiotics, their lack of detrimental side effects is further reason for their growing popularity. In fact in a recent review paper, Levri et. al. suggest that physicians' advice to patients regarding a given probiotic should be a cavalier "try it [8]." It is no surprise then that there is great interest in investigating their use as an inexpensive treatment for a variety of causes of gastrointestinal discomfort.

Digestive Advantage™ Gas Defense Formula (Ganeden Biotech, Mayfield Heights, Ohio) is a probiotic supplement containing *Bacillus coagulans* as well as an enzyme blend of cellulases from *Trichoderma longibrachiatum* and *Aspergillus niger*. Studies suggest that the probiotic Bacillus coagulans decreases the symptoms of abdominal pain and bloating in subjects with inflammatory bowel disease [9]. With this in mind, we undertook a randomized, double-blind, placebo-controlled clinical trial to evaluate Gas Defense (GD). The purpose of the study was to compare its effect versus placebo on gastrointestinal quality of life in adults with intestinal symptoms but no GI diagnoses.

Methods

Experimental Design

This double-blind, placebo-controlled clinical study randomized 61 subjects at two investigative sites (Miami and the Dominican Republic). Subjects provided written informed consent prior to participating in any study procedures. Subjects were then randomized within each site in a 1:1 manner into intervention (GD) or placebo groups. Investigators and subjects were blinded to product assignment. Subjects were seen at three visits over the course of four weeks - a screening/randomization visit at Day 0, and two follow-up visits at Days 14 and 29. On Day 0, the participants were instructed to begin taking one capsule daily, at approximately the same time of day, and to continue doing so for the duration of the study. Participants were provided sufficient product at visits 1 and 2 to cover the time between visits. Compliance with product use was measured via the pill counting method. During each visit, the participants were evaluated with a series of questionnaires in addition to hemodynamics and adverse event monitoring. The research was in compliance with the Helsinki Declaration and approved by the Aspire Independent Review Board San Diego, California (approved May 13, 2008) and Consejo Nacional de Bioetica en Salud (Conabios), Santo Domingo, Dominican Republic (approved June 23, 2008).

Subject Population

Subjects were drawn from the Greater Miami area and the Dominican Republic. All were between 20-68 years of age and had self-reported post-meal intestinal gas-related symptoms including abdominal pain, cramps, distended feeling/bloating, and flatulence. Out of a total of 98 subjects interviewed by phone, 64 attended the screening evaluation. Three of those subjects did not meet entry criteria. In the final study population, seven subjects came from Miami and 54 came from the Dominican Republic. Sixty subjects began the study but one was terminated at the discretion of the investigator after a single dose. An additional subject was subsequently enrolled with IRB notification and approval. All subjects were in otherwise good health, willing and able to comply with the protocol, and, if female, neither pregnant nor lactating and willing to use a reliable method of birth control. All subjects

signed the IRB-approved Informed Consent prior to any procedures being conducted.

Exclusion criteria for entering this study included; active heart disease, uncontrolled high blood pressure, renal or hepatic impairment, Type I or II diabetes, psychiatric and immune disorders, unstable thyroid disease, Parkinson's disease, a history of cancer, previous stomach or intestinal surgery, the consumption of medication or supplements that would interfere with the natural flora of the gut such as antibiotics, probiotics, or prebiotics within the last 30 days prior to screening. Subjects with gastrointestinal disorders or other digestive problems such as Crohn's disease, short bowel, ulcerative colitis, Irritable Bowel Syndrome, constipation, or lactose intolerance were also excluded. Lactose intolerance was excluded as per subject profession or previous diagnosis. Similarly, subjects using GI medications to control the function of the gut, such as anti-spasmodics, motility agents, pro-kinetic agents, or laxatives were excluded. Subjects were only permitted to use over-the-counter gas relief products as rescue treatment during the study. Only one subject reported having done so. Subjects allergic to wheat, fish, or any other ingredients in GD or the placebo were excluded.

Intervention

The active product tested is a probiotic supplement containing *Bacillus coagulans* (specifically *Bacillus coagulans* GBI-30, 6086, also known as GanedenBC[30]). The product specifically contained *B. coagulans*, Enzyme Blend (cellulase - *Trichoderma longibrachiatum*, cellulase - *Aspergillus niger*, hemicellulase, α-galactosidase, invertase) with the inactive ingredients of a vegetarian capsule, magnesium stearate, silicon dioxide, and maltodextrin. There were 2.0×10^9 colony forming units per capsule.

The placebo was provided by the manufacturer and matched in size and color to the active product. Independent product analysis for content was carried out to confirm label content claim (ULTRAtab Labs, Highland, New York). All subjects were instructed to take one tablet daily for the duration of the study.

Assessment

During the study, subjects were asked to complete several questionnaires, each targeting a different symptom. Distension, pain, and flatus were tracked using the corresponding subsections of the GI Symptoms Rating Scale (GSRS) [10]. Bloating and gas were measured with the Severity of Dyspepsia Assessment (SODA) [11]. Other assessments included the GSRS overall score and the SODA Non-Pain Symptoms (NPS) subscore, as well as the SODA subscore for satisfaction with dyspepsia-related health, SF-36v2 quality of life physical and mental component summaries, and 7-point anchored Visual Analog Scale (VAS-Gas) assessment of gas symptoms.

All questionnaires were completed by the study subjects at every visit, except for VAS-Gas, which was administered only at the second and third visits because it asks for a consideration of relative change from baseline. Blood pressure and heart rate were measured at each visit, and study compliance was monitored by the pill count method.

Statistical Methods

The two primary endpoints for analysis in this study were the GSRS subscores for abdominal pain, distension, and flatus; and the SODA subscores for bloating and gas. Other endpoints included the GSRS overall score, SODA-NPS score, SODA subscore for satisfaction with dyspepsia-related health, the SF-36v2 summaries, and the VAS-Gas assessment.

The formal efficacy analysis consisted of a set of analyses of covariance (ANCOVAs), one for each efficacy endpoint. The value of the efficacy variable at Visit 3 (end of study) was the dependent variable, the product group (GD or placebo) was the variable of interest, and the value of the efficacy variable at Visit 1 (baseline) was a covariate. Investigative site (US or DR) was also included in the model. Only p-values less than or equal to 0.05 were considered significant.

Other descriptive (non-inferential) summaries and comparisons were carried out - mean changes from baseline to each subsequent time point were tested by the paired Student t test or Wilcoxon signed-ranks test, and mean differences between product groups were tested by the unpaired Student t test or Mann-Whitney U test. Differences in the distribution of categorical variables between the product groups were tested by the Fisher Exact test.

Sample size was determined on the basis of time, cost, and the ability to detect a clinically important effect size. It was determined that 25 analyzable subjects per group would provide 80% power to obtain a significant result for a 0.8-sigma effect size. To allow for a possible 15% attrition from the study, 30 subjects were enrolled per group. No adjustment for multiple testing was applied in the analysis of data from this study. Each test was evaluated at the 0.05 alpha level ($p \leq 0.05$ considered significant).

Results and Discussion

Most subject characteristics at baseline (the screening/randomization) were evenly matched between the two product groups (Table 1). The placebo group was, on average, four years older and eight kilograms heavier than the GD

Table 1: Baseline and Descriptive Characteristics

Group	Gas Defense (n = 30)	Placebo (n = 31)
Site		
Dominican Republic	27 (90%)	27 (87%)
Miami, FL	3 (10%)	4 (4%)
Age, Years	34.8 ± 12.5	38.2 ± 12.6
Gender		
Female	16 (53%)	17 (55%)
Male	14 (47%)	14 (45%)
Ethnicity		
Hispanic	27 (90%)	28 (90%)
Non-Hispanic	3 (10%)	3 (10%)
Race		
Black/AA	8 (27%)	7 (23%)
Caucasian	9 (30%)	9 (29%)
Other	12 (43%)	15 (48%)
Height, cm	164.2 ± 8.6	165.8 ± 9.8
Weight, kg	71.4 ± 14.1	79.2 ± 19.3
Status		
Completed Protocol	30 (100%)	30 (97%)
Early Termination	0 (0%)	1 (3%)
Heart Rate, beats/minute	69.9 ± 12.1	70.7 ± 10.3
Systolic Blood Pressure, mm Hg	121.2 ± 17.0	122.2 ± 10.9
Diastolic Blood Pressure, mm Hg	75.1 ± 9.0	76.0 ± 7.2
GSRS - Abdominal Pain Subscore	3.17 ± 1.85	3.14 ± 1.48
GSRS - Abdominal Distension Subscore	3.38 ± 2.13	4.14 ± 1.43
GSRS - Increased Flatus Subscore	3.86 ± 1.92	4.07 ± 1.53
GSRS - Total GI Symptom Score	40.8 ± 19.8	39.4 ± 12.1
SODA - Bloating Subscore	2.52 ± 1.48	2.93 ± 1.25
SODA - Gas Subscore	3.28 ± 0.96	3.28 ± 0.84
SODA - Non-pain Symptoms Score	16.83 ± 3.35	17.00 ± 2.09
SODA - Satisfaction Score	8.3 ± 3.4	9.2 ± 3.5
SF-36v2 - Physical Component Summary	49.9 ± 8.5	49.0 ± 9.9
SF-36v2 - Mental Component Summary	51.3 ± 10.1	51.3 ± 9.9

Values are expressed as mean ± standard deviation.

group. Most of the endpoints tracked did not show a significantly different response between GD and placebo. These included the GSRS increased flatus subscore, the SODA bloating subscore, the SODA non-pain symptoms and satisfaction scores, and the SF-36v2 physical and mental component summaries. However, all but the SF-36v2 MCS showed differences in the direction that indicated a larger beneficial effect for GD than the placebo.

Table 2 shows the ANCOVA coefficient of the product group - an estimate of the amount by which the four-week improvement in the GD group exceeds that of the placebo group, along with its standard error and p-value indicating whether or not there is a statistically significant difference between the product and placebo. GD performed significantly or nearly significantly better than placebo for the following endpoints (Tables 3, 4 and 5): GSRS: abdominal pain subscore (p = 0.046), GSRS: abdominal distension subscore (p = 0.061), and GSRS total score (p = 0.048).

While other efficacy endpoints do not indicate statistical significance for GD relative to placebo, all but the SF-36v2 MCS and the VAS-Gas score showed differences in the direction that indicates a larger beneficial effect for GD than for placebo.

The lack of significance for many of the efficacy endpoints can be attributed to several factors. First, as seen in the descriptive summary tables for each endpoint, a very strong placebo effect was evident in this study. Subjects generally liked the product they were taking and tended to report substantial improvement regardless of which product they were taking. All endpoints showed large four-week improvement in both product groups. This may be partly cultural, with people wanting to demonstrate what they considered to be the "expected" improvement, although this cannot be established from the available data. Whatever the cause, this kind of phenomenon is quite common in studies involving subjective endpoints (especially discomfort-related endpoints). With the pla-

Table 2: Efficacy Analysis (ANCOVA)

Endpoint	Coefficient ± Std Err	p-value
GSRS: Abdominal Pain Subscore (lower is better)	-0.627 ± 0.307	0.046 †
GSRS: Abdominal Distension Subscore (lower is better)	-0.572 ± 0.299	0.061 ‡
GSRS: Increased Flatus Subscore (lower is better)	-0.511 ± 0.353	0.154
GSRS: Total Score (lower is better)	-4.806 ± 2.381	0.048 †
SODA: Bloating Subscore (lower is better)	-0.229 ± 0.216	0.294
SODA: Gas Subscore (lower is better)	-0.348 ± 0.219	0.118
SODA: Non-Pain Symptoms Score (lower is better)	-1.025 ± 0.870	0.244
SODA: Satisfaction Score (lower is better)	-0.058 ± 1.358	0.966
SF-36v2: Physical Component Summary (higher is better)	0.941 ± 1.118	0.403
SF-36v2: Mental Component Summary (higher is better)	-2.400 ± 2.010	0.238

† Significant ($p \leq 0.05$)
‡Approaches significance ($p \sim 0.05$)

Table 3: Gastrointestinal Symptom Rating Scale Abdominal Pain Subscore

Visit	Gas Defense	Placebo
Day 0 Screen/Rand	3.17 ± 1.85 (29)	3.14 ± 1.48 (29)
Day 14 Mid-Study	2.10 ± 1.29 (29)	2.28 ± 1.51 (29)
Day 29 End-of-Study	1.59 ± 0.95 (29)	2.21 ± 1.45 (29)
Change from Day 0 to Day 14	-1.07 ± 1.70 (29)	-0.86 ± 1.81 (29)
Change from Day 0 to Day 29	-1.59 ± 1.70 (29)	-0.93 ± 1.67 (29)

Values are expressed as mean ± standard deviation.

cebo group showing such a large improvement, there was not much "room for improvement" for the GD group over placebo.

Also, there was a considerable amount of random variability in most of the efficacy endpoints. That is, most of the efficacy variables had large within-group standard deviations for four-week changes from baseline. This is quite common with subjective, semi-quantitative endpoints like the GSRS and SODA questionnaire scales, and it has the effect of reducing the power to detect significance. This study was powered to provide a good chance of getting a significant result for an endpoint if the average amount of improvement (for GD, compared to Placebo) was at least 4/5 as large as the within-group standard deviation for that endpoint. In this study, the magnitude of the improvements tended to be less than that.

Table 4: Gastrointestinal Symptom Rating Scale Abdominal Distension Subscore

Visit	Gas Defense	Placebo
Day 0 Screen/Rand	3.38 ± 2.13 (29)	4.14 ± 1.43 (29)
Day 14 Mid-Study	1.83 ± 1.04 (29)	2.48 ± 1.38 (29)
Day 29 End-of-Study	1.66 ± 1.08 (29)	2.38 ± 1.21 (29)
Change from Day 0 to Day 14	-1.55 ± 1.88 (29)	-1.66 ± 1.70 (29)
Change from Day 0 to Day 29	-1.72 ± 2.02 (29)	-1.74 ± 1.68 (29)

Values are expressed as mean ± standard deviation.

Table 5: Gastrointestinal Symptom Rating Scale Total Score

Visit	Gas Defense	Placebo
Day 0 Screen/Rand	40.8 ± 19.8 (29)	39.4 ± 12.1 (29)
Day 14 Mid-Study	29.0 ± 8.7 (29)	31.6 ± 11.4 (29)
Day 29 End-of-Study	25.2 ± 10.0 (29)	29.4 ± 9.7 (29)
Change from Day 0 to Day 14	-11.9 ± 16.4 (29)	-7.8 ± 11.7 (29)
Change from Day 0 to Day 29	-15.6 ± 17.4 (29)	-9.9 ± 12.3 (29)

Values are expressed as mean ± standard deviation.

Conclusion

The *Bacillus coagulans*-based probiotic product showed superior numerical scores to placebo in 10 of 12 efficacy variables, and the differences were significant or nearly significant in three of the 12 variables. Within this study population, the *Bacillus coagulans*-based probiotic product was effective and safe for abating symptoms of gastrointestinal distress, particularly abdominal pain and distention in the post-prandial period.

Competing interests

The authors received funding for this study from Ganeden Biotech.

Authors' contributions

DSK participated in the design of the study and drafting of the manuscript. HIS participated in the design of the study and served as a Sub Investigator. PA participated in the design of the study, served as co-Principal Investigator and contributed to the manuscript. SF participated in the design of the study, data collection and manuscript preparation. JCP participated in the study design, data collection and performed the statistical analysis. DRK participated in the design of the study, served as co-Principal Investigator and contributed to the manuscript. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

A CONTROLLED CLINICAL TRIAL TO EVALUATE THE EFFECT OF GANEDENBC^{30} ON IMMUNOLOGICAL MARKERS

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SUMMARY

GanedenBC^{30}, a probiotic, has been shown to significantly increase T-cell production of TNF-α after ex vivo exposure to a strain of adenovirus (AdenoVI) or influenza A (H3N2 Texas strain [FluTex]). The current controlled study was designed to further evaluate the effect of GanedenBC^{30} on immunological marker levels following viral exposure. Ten healthy subjects' baseline immunological marker levels were analyzed. Subjects consumed 1 capsule/day of GanedenBC^{30} for 28 days and returned for post-treatment immunological marker evaluation. Subjects' baseline measurements served as their own control. All subjects completed the study with no adverse events; however, one subject was excluded from the final analysis based on a reasonable consideration as an outlier. CD3$^+$CD69$^+$ cells, IL-6, IL-8, interferon-γ (IFN-γ) and TNF-α levels were increased after exposure to AdenoVI and FluTex. IL-1β levels also increased after exposure to AdenoVI but were decreased after ex vivo exposure to FluTex. CD3$^+$CD69$^+$ cells increased significantly ($P = 0.023$) after exposure to both viral strains. Differences in IL-8 levels after FluTex exposure achieved statistical significance ($P = 0.039$) as did IFN-γ levels after AdenoVI exposure ($P = 0.039$). A regimen of one capsule per day containing 500 million CFU of GanedenBC^{30} may be a safe and effective option for enhancing the immunological response to common viral respiratory tract infections.

INTRODUCTION

The relationship of normal microbial flora and their host have long been known to promote health (1), while fluctuations in the equilibrium of this relationship can often lead to disease (2). Probiotics are live bacteria that show a documented beneficial effect on the host (3). Probiotics work through a variety of mechanisms that increase the systemic immune response and mucosal immunity (4-8). GanedenBC^{30} (Ganeden Biotech, Inc., Mayfield Heights, OH) is a patented strain of probiotic bacteria, *Bacillus coagulans* GBI-30 6086, that can potentially boost the immune response (9) and help regulate digestion (10, 11). It is a Gram-positive, spore-forming, lactic acid-producing bacterium that is able to withstand the harsh acidic environment of the stomach and colonize the intestine (12). A subchronic oral toxicology study performed in rats in doses ranging from 3,173 to 95,200 times greater than the suggested human dose, demonstrated that it is considered safe for chronic human consumption (13). Recently, GanedenBC^{30} was shown to significantly increase T-cell production of TNF-α after exposure to adenovirus or influenza A (9). The current study was designed to further evaluate the effect of GanedenBC^{30} on immunological marker levels following antigen challenge in healthy adults.

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SUBJECTS AND METHODS

A cohort of 10 healthy subjects between the ages of 18 and 43 years with no known chronic or current medical conditions were enrolled in this open-label, single-site study. All participants gave signed informed consent at screening. The study was conducted according to principles set forth in The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research of the U.S. National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research (April 18, 1979) and codified in 45 CFR Part 46 and/or the ICH E6 Good Clinical Practice Guidelines (GCP) (14). The study was approved by an independent institutional review board (IRB), Aspire IRB, La Mesa, CA. Subjects were excluded on the basis of chronic or current illness, history of psychosis or any other condition that would render the individual unable to comply with the requirements of the protocol, known allergic reactions to the investigational product, pregnancy or breast-feeding, habituating drug and/or alcohol abuse, receipt of a vaccine for influenza within the concurrent influenza season, receipt of antibiotics, and/or any immunization or other investigational drug within 30 days prior to enrollment. Subjects were instructed to maintain their usual diets and avoid consuming any products containing other probiotics.

Study design and laboratory evaluations

Subjects enrolled in the study presented for a blood sample at screening for a baseline immunological cytokine panel evaluation.

They were instructed to consume 1 capsule a day of GanedenBC[30] with water, at approximately the same time each day, regardless of meals. Each capsule contained 500 million colony-forming units (CFU) of *B. coagulans*. After 28 days, subjects returned for a second blood draw for a post-immunological cytokine panel evaluation. Subjects' baseline measurements served as their own control. Compliance with the consumption of the investigational product was determined by capsule count.

Immunological cytokine IL-1β, IL-6, IL-8, IfN-γ and TNF-α levels were measured by an electrochemiluminescent assay. Briefly, aliquots of whole blood were stimulated with adenovirus 10 µg/mL (Fitzgerald, 30-AA02), influenza A Texas 1/77 (H3N2) 50 µg/mL (Fitzgerald, 30-AI50), lipopolysaccharides (LPS) 50 ng/mL (Sigma, L4391) or sterile saline. After a 24-h incubation at 37 °C and 5% carbon dioxide, supernatants were collected and cytokine concentrations were measured using Meso Scale Discovery (MSD) Assay Kits according to the manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD).

$CD3^+CD69^+$ cells were analyzed by flow cytometry. Briefly, CD3-FITC (Beckman-Coulter, Fullerton, CA; IM1281U), CD69-PC5 (Beckman-Coulter, IM2656U) and CD45-ECD (Beckman-Coulter, IM2710U) were added to each stimulated sample and incubated for 10 min at room temperature. Stained samples were then lysed and fixed (Beckman-Coulter, Immunoprep lysing kit 7546999) for acquisition.

Statistical analysis

A Shapiro-Wilk statistical test was used to calculate the difference between pre- and post-immunological marker measurements to assess the normality of the measurements. Log transformations were applied to measurements in which a departure from normality was evident and then reassessed. To determine possible outliers, each subject was assessed in the context of a regression model (post-treatment regressed on pretreatment measurements). An RSTUDENT (or studentized residual) and the COVRATIO (which assesses each subject as a possible outlier relative to baseline measurements; considered as a covariate) were calculated based on outcome and baseline immunological marker measurements, respectively. An absolute value of COVRATIO −1 greater than 3 × (number of parameters)/(number of subjects) was considered worthy of investigation. Exact Wilcoxon signed-rank tests (for paired measurements) were performed to evaluate whether marker levels were higher overall post-treatment compared to pretreatment. Analyses were performed using SAS Version 8.02 (SAS System Institute) and StatXact Version 4.0 (Cytel Software Corporation, Cambridge, MA).

RESULTS

Baseline demographics for the 10 healthy adult subjects who were enrolled in the study are presented in Table I. All of the subjects completed the study; however, 1 was excluded from the final analysis based on a reasonable consideration as an outlier. Subject ID#7 was flagged most frequently (7 times) as a covariate (baseline) outlier. Furthermore, 3 of the COVRATIO values for Subject ID#7 were greater than 7 and thus out of the normal range. Based on these baseline differences, Subject ID#7 was considered an outlier and removed from the final analyses.

Table I. *Subject demographics (N = 10).*

Race		Gender		Age (years)	
White	Asian	Male	Female	Average	Range
9	1	5	5	27	18-43

A statistically significant departure from normality ($\alpha = 0.05$) was found for IL-6 and IL-8 after exposure to adenovirus. Thus, a log transformation was applied to both pretreatment and post-treatment IL-6/adenovirus and IL-8/adenovirus responses. Applying the Shapiro-Wilk statistical test to the resulting difference scores did not indicate a departure from normality for either response (data not shown). The differences between pretreatment and post-treatment changes in marker levels along with the *P* values for the Wilcoxon signed-rank tests are presented in Table II. Results before and after removal of Subject ID#7, the one outlier, are shown for comparison. The immunological markers ($CD3^+CD69^+$, IL-1β, IL-6, IL-8, IFN-γ and TNF-α) were increased after exposure to AdenoVI, but only $CD3^+CD69^+$ reached significance ($P = 0.023$). All of these markers were also increased in response to FluTex exposure except for IL-1β; however, only IL-8 achieved significance ($P = 0.039$). Significant differences not found for the complete data analysis were found for $CD3^+CD69^+$/FluTex ($P = 0.023$) and IFN-γ/AdenoVI ($P = 0.039$) after removal of the outlier. The saline control and LPS showed no differences pretreatment and post-treatment as indicated by cytokine and cytometric analysis (data not shown). Figure 1 represents the differential analysis of $CD3^+CD69^+$ cells before and after treatment with GanedenBC[30]. All statistically significant differences from the complete data analysis (at $\alpha = 0.05$) were retained. No serious adverse events were reported throughout the study.

DISCUSSION

B. coagulans has been safely used for over 50 years (15) and is safe for chronic human consumption. Preclinical studies have not resulted in evidence of any mutagenic or genotoxic effects (13), and it has been well tolerated in clinical trials with no reported serious adverse events (9, 16). In fact, *B. coagulans* has not been implicated as a primary causative organism of any disease (15).

The mechanisms by which the equilibrium between normal human microbial flora and the host promote health and help prevent disease are largely unknown. However, studies on the composition, competition and intracooperation of the microbiome as well as on the host–microbe relationship to inflammation and disease have revealed parts of a complex and dynamic symbiotic environment (2). Both immunological (17) and nonimmunological (18) mechanisms have been identified. Although probiotics are effective in a number of gastrointestinal disorders such as viral diarrheal disease and inflammatory bowel disease, the efficacy of one probiotic in a specific condition should not be generalized to other probiotics or conditions (19).

Capsules containing 2 billion CFU of GanedenBC[30] have been shown to significantly increase the T-cell production of TNF-α in response to adenovirus and influenza A (H3N2 Texas strain) exposure (9). The

Table II. *Post-treatment minus pretreatment difference scores.*

Complete data set	Removal of one outlier (ID#7)			
Immunological marker	Mean (SE)	*P* value (Wilcoxon)	Mean (SE)	*P* value (Wilcoxon)
CD3CD69 AdenoVI	0.64 (0.27)	0.039	0.76 (0.28)	0.023⁺
CD3CD69 FluTex	0.39 (0.31)	0.20	0.63 (0.20)	0.023⁺
IL-1β AdenoVI	465.0 (512.4)	0.43	437.0 (580.1)	0.55
IL-1β FluTex	−570.7 (901.3)	0.57	−997.3 (900.2)	0.38
IL-6 AdenoVI*	15,878.8 (15,628.5)	0.50	15,979.6 (17,720.7)	0.64
IL-6 FluTex*	21,237.1 (13,312.6)	0.16	14,493.4 (13,015)	0.31
IL-8 AdenoVI*	205,082 (115,071)	0.20	220,536 (129,296)	0.31
IL-8 FluTex*	413,158 (132,109)	0.020	382,661 (145,751)	0.039⁺
IFN-γ AdenoVI	48.8 (38.4)	0.20	72.7 (34.0)	0.039⁺
IFN-γ FluTex	45.0 (19.6)	0.074	45.1 (22.2)	0.11
TNF-α AdenoVI	665.7 (370.8)	0.13	524.8 (388.9)	0.25
TNF-α FluTex	805.0 (417.0)	0.13	536.0 (361.2)	0.25

*Log-transformed data. ⁺Significant ($P \leq 0.05$)

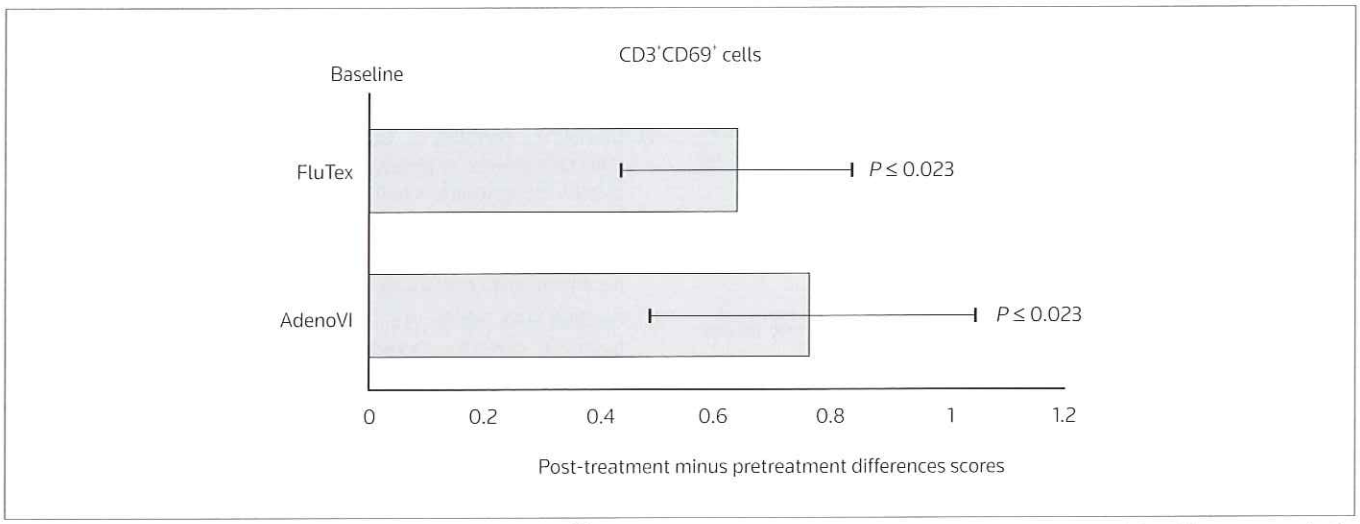

Figure 1. CD3⁺CD69⁺ cell differences before and after GanedenBC[30]. Samples of peripheral blood stimulated with either FluTex or AdenoVI were stained with anti-CD3 and anti-CD69 antibodies. Difference scores were calculated by subtracting the pretreatment level from the post-treatment level. Baseline represents no change after treatment.

current study further explored the effects of lower doses of GanedenBC[30] (500 million CFU/capsule) on immunological markers in response to viral exposure. Analyses of IL-6 and IL-8 after exposure to adenovirus were found to depart from normality and were recalculated after a log transformation of the measurements. However, this did not substantially alter the results since the Wilcoxon test is based on rank and is distribution-free. Furthermore, the calculated values for the two outlier regression diagnostics, RSTUDENT and COVRATIO, suggested that it would be reasonable to consider Subject ID#7 as an outlier. The significant increases in CD3⁺CD69⁺ and IL-8 that were observed after exposure to FluTex and the significant increases in CD3⁺CD69⁺ and IFN-γ that were observed after AdenoVI, suggest that GanedenBC[30] may elicit a heightened immunological response to viral infection. Viral respiratory tract infections are common among humans and have profound health and economic consequences (20). Data on the use of probiotics to prevent or treat viral infection are limited, and the results are often conflicting (21, 22) and appear to be strain-specific (19). GanedenBC[30] has been shown to increase immunological markers in response to viral infection. Thus, future studies are warranted to evaluate whether this increased immunological response confers protection from the incidence or decreases the duration or severity of the infection. One of the potential advantages of GanedenBC[30] is its spore-forming ability, which makes it highly resistant to environmental, industrial and physiological hazards such as the acidity of the stomach and heating during industrial manufacturing (23).

Additionally, spore formation allows for increased viability over long periods of time, limits the need for refrigeration and extends the shelf life of the product (9).

In conclusion, a 4-fold reduction in the CFU dose from a previous study (9) of GanedenBC^{30} was able to boost the immune response to a similar extent. The immunological markers, IL-8 and IFN-γ, were elevated in response to a strain of influenza A or adenovirus, respectively. $CD3^+CD69^+$ cells were increased under both types of infection. At a dose of 500 million CFU per day, GanedenBC^{30} may be considered a safe and effective option for enhancing the immunological state in response to common viral respiratory tract infections.

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DISCLOSURES

David Keller and Sean Farmer are employees of Ganeden Biotech, Inc. Murray Kimmel and Dominic Warrino have no conflicts of interest to disclose.

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Survival and metabolic activity of the GanedenBC^{30} strain of *Bacillus coagulans* in a dynamic *in vitro* model of the stomach and small intestine

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Abstract

We have investigated the survival and activity of GanedenBC^{30} during passage through the upper gastro-intestinal tract. GanedenBC^{30} was tested in a dynamic, validated, *in vitro* model of the stomach and small intestine (TIM-1) on survival and its potential to aid in digestion of milk protein, lactose and fructose. The survival of GanedenBC^{30} was high (70%), although germination of the spores was minimal (<10%) under the conditions tested. Survival of the strain in the presence of lactose and fructose was markedly lower (56-59%) than in the absence of the sugars. The amount of digested milk protein available for absorption was somewhat higher (+0.2 g) when GanedenBC^{30} was added to the milk. When GanedenBC^{30} was tested with lactose or fructose added to the meal, the cumulative amount of lactate produced was slightly higher (+0.12-0.18 mmol) compared to the GanedenBC^{30} alone. In conclusion, although the differences in survival of GanedenBC^{30} are small, these results show the potential of GanedenBC^{30} to aid in protein digestion and in the digestion of lactose and fructose. If a larger fraction of the *Bacillus coagulans* cells had germinated, the influence on protein and carbohydrate digestion would probably have been much greater. Importance of the findings: the potential of GanedenBC^{30} to aid in the digestion of lactose and fructose could be used to prevent occurrence of intestinal symptoms in individuals sensitive to these carbohydrates.

Keywords: probiotic, *Bacillus coagulans*, GanedenBC^{30}, survival, metabolic activity, fructose intolerance, lactose intolerance

1. Introduction

Probiotics are live bacteria that show a documented beneficial effect to the host (Sanders, 2008). Probiotics exhibit a variety of effects: they may be used to prevent and treat antibiotic-associated diarrhoea and acute infectious diarrhoea; they also may be effective in relieving symptoms of irritable bowel syndrome, inflammatory bowel disease, and in treating atopic dermatitis in children. Other documented effects include such overlapping mechanisms as the regulation of intestinal microbial homeostasis, the stimulation of local and systemic immune responses, the prevention of pathogens infecting the mucosa, the stabilisation or maintenance of the gastrointestinal barrier function, the inhibition of procarcinogenic enzymatic activity, and the competition for limited nutrients (Boirivant and Strober, 2007; Rolfe 2000). However, probiotic effects appear to be strain specific (De Vecchi and Drago, 2006). Regardless of the strain and its potential effect, probiotics intended for the gastro-intestinal tract must survive gastric and bile acids (De Vecchi and Drago, 2006) in order to reach that intestinal tract, colonise the host epithelium, and exhibit a beneficial effect (Hyronimus *et al*., 2000). *Bacillus coagulans* is a Gram-positive spore-forming rod, which is aerobic to microaerophilic. Due to the formation of spores, these bacilli can withstand the acidic environment of the stomach to reach the intestine where they germinate and proliferate, producing the favoured L (+) optical isomer of lactic acid (Breed *et al*., 1957). *B. coagulans* GBI-30, PTA 6086 (GanedenBC^{30}) is a strain of lactic-acid producing bacteria, with self-affirmed GRAS (generally recognised

as safe) status, that can sustain the low pH of stomach acid (De Vecchi and Drago, 2006; Hyronimus *et al*., 2000).

Once active in the small intestine after germination, *B. coagulans* may aid in digestion of proteins and sugars from the diet. This may be beneficial to the host, especially in the case of lactose and fructose intolerance: once the sugars are digested in the upper gastro-intestinal (GI) tract, they will no longer cause the symptoms associated with the intolerance in the lower part of the gut, such as bloating, diarrhoea, etc.

As mentioned above, an important criterion for the selection of probiotic bacteria to be used in (functional and medical) food products or formulations in relation to health promotion is the survival and activity of these micro-organisms in the gastro-intestinal tract of the consumer. In order to investigate the survival of probiotic bacteria in the stomach and small intestine in humans, intubation methods are available. However, these *in vivo* methods are laborious, expensive and meet ethical constraints. Instead, validated *in vitro* methods should be performed, at least for the first selection of strains. The TNO *in vitro* gastro-Intestinal Models (nick-named TIM) are computer controlled, dynamic models that simulate to a large degree the successive dynamic processes in the stomach, the small intestine (TIM-1; Minekus *et al*., 1995; Havenaar and Minekus, 1996) and in the large intestine (TIM-2; Minekus *et al*., 1999; Venema *et al*., 2000). During the experiments samples from different sites of the GI tract can be taken in time. These models are unique tools and give good insight into the stability and activity of functional ingredients, such as probiotics. The results obtained in these models were validated and showed a very good resemblance to the results obtained in studies with humans and animals with respect to the digestibility and the availability for absorption of a broad scope of nutrients (e.g. proteins/amino acids, carbohydrates, minerals, vitamins, nutrients; Minekus, 1998; Larsson *et al*., 1997; Smeets-Peeters, 2000), the release, absorption and function of bioactive food compounds (e.g. functional proteins, anti-mutagenic compounds; Krul *et al*., 2002) and the survival of lactic acid producing bacteria (Marteau *et al*., 1997).

The aim of the study was to determine the survival and activity of a particular strain of *B. coagulans* known as GanedenBC^{30}, a commercial product, in the upper GI tract. This was tested in TIM-1, the model for the stomach and small intestine. Survival was evaluated on the basis of production of L-lactic acid, a metabolite produced by *B. coagulans* (Breed *et al*., 1957). In addition, the effect of the addition of GanedenBC^{30} to milk was studied, to evaluate whether the probiotic would aid in digestion of lactose and milk protein. Similarly, the probiotic was added to a fructose solution (mimicking apple-juice) to evaluate whether the strain would aid in the digestion of fructose.

2. Materials and methods

Test products

GanedenBC^{30} was provided by Ganeden Biotech (Mayfield Heights, OH, USA). This *B. coagulans* containing product contained 2.15×10^{10} colony forming units per gram powder (cfu/g). Lactose and fructose were obtained from Sigma (Zwijndrecht, the Netherlands) and low-fat, pasteurised milk was bought fresh in the local supermarket the day before the experiment was carried out.

Test system

The study was performed in the TNO dynamic, multi-compartmental system of the stomach and small intestine (TIM-1) as described in detail by Minekus *et al*. (1999) and schematically presented in Figure 1. Briefly, the model comprises four connected compartments, representing the stomach, duodenum, jejunum and ileum. Each compartment is made of a glass outer wall with a flexible silicone inner wall. The space between the inner and outer walls is filled with water at body temperature (37 °C). By periodically pumping the water, the flexible inner walls are squeezed, which simulates peristaltic movements of the GI tract. In the four compartments the pH is measured continuously and regulated by 'secretion' of hydrochloric acid in the gastric compartment and sodium bicarbonate in the intestinal compartments. Hollow fibre membrane systems continuously dialyse the digested and dissolved low-molecular weight compounds from the jejunum and ileum compartments, which simulates absorption of nutrients in the body. The set-points of pH, gastric emptying and intestinal transit time were controlled by a computer and are displayed in Figure 2. As a result, over the course of 3 hours, approximately 95% of the gastric contents were gradually delivered into the small intestine through the 'pyloric sphincter' (at B in Figure 1). Over the course of 6 hours, approximately 90% of the small-intestinal contents were gradually delivered into the 'large intestine' (sampling bottle) through the 'ileo-caecal sphincter' (at H in Figure 1). The gastric pH decreased from 6.5 to 2.0 in 120 minutes. In the small-intestinal compartments the pH was set at 6.5 in the duodenum compartment, 6.8 in the jejunum compartment and 7.2 in the ileum compartment (Minekus *et al*., 1999).

Study design

Throughout the study, the experiments in TIM-1 were performed under the average physiological conditions of healthy human adults (Figure 2). Duplicate experiments were performed to determine the survival of GanedenBC^{30}. For this, 2×10^{9} cfu of the *B. coagulans* spores were introduced into the gastric compartment in a mixture of

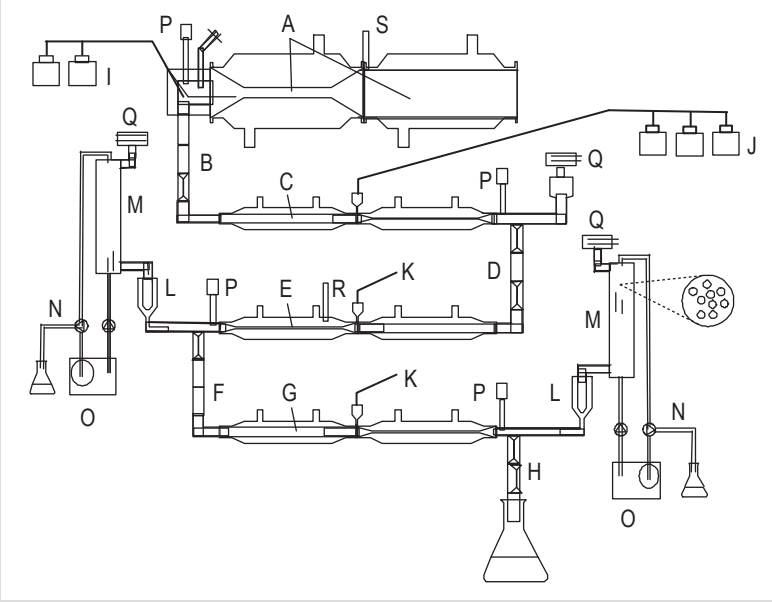

Figure 1. Schematic diagram of the dynamic, multi-compartmental model of the stomach and small intestine (TIM-1): A. stomach compartment; B. pyloric sphincter; C. duodenum compartment; D. peristaltic valve; E. jejunum compartment; F. peristaltic valve; G. ileum compartment; H. ileo-caecal sphincter; I. stomach secretion; J. duodenum secretion; K. jejunum/ileum secretion; L. pre-filter; M. semi-permeable membrane; N. water absorption; O. collected dialysate; P. pH electrodes; Q. level sensors; R. temperature sensor; S. pressure sensor.

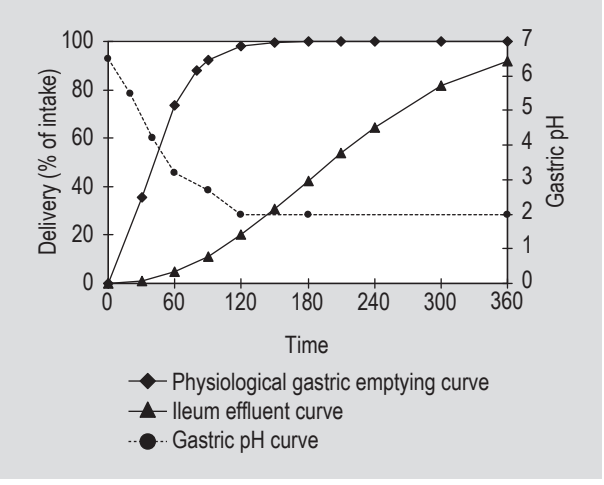

Figure 2. Physiological gastric emptying curve, ileum effluent curve and gastric pH curve for human adults ingesting milk.

200 ml of sterile demi-water and 100 ml artificial saliva (Minekus *et al*., 1999). In addition, duplicate experiments were performed by adding the same amount of spores to 200 ml of a lactose solution (5% w/v in water), a fructose solution (5% w/v in water), or milk. The outcome of these latter experiments revealed information on the effect of GanedenBC^{30} on the digestion of lactose, fructose and milk protein, respectively. It should be noted that in these experiments, there was no full complement of substrates that bacteria need for their growth, such as carbon and nitrogen sources, due to reasons of interference with the analysis on survival and more importantly lactose, fructose and milk protein utilisation. For each experiment the secretion products (i.e. saliva and gastric juice with enzymes and electrolytes, dialysis liquids, bile, pancreatin) were freshly prepared, the pH electrodes calibrated and the dialysis membranes (hollow fibre units) replaced (Minekus *et al*., 1999).

Sampling

The 'ileo-caecal sphincter' delivered the intestinal contents according to a computer-controlled ileal effluent curve (Figure 2). Over the course of 6 hours the ileal effluent was collected on ice in three 2 hourly aliquots by changing the sampling bottle (at H in Figure 1) at 2, 4 and 6 hours, except for evaluation of survival of the strain, where hourly fractions were taken. For each sample the volume was measured and samples were stored at -18 °C prior to analysis. At the same time intervals the dialysed and absorbed liquids of the jejunum and ileum compartments (at O in Figure 1) were collected. The total volumes were measured and individual samples were taken in time and stored as above.

Analyses

Total Kjeldahl nitrogen (Isaac and Johnson, 1976) was determined in the samples collected in the milk experiments. Lactate was measured as a parameter for fermentation of lactose and fructose. Lactate was measured using a Cobas Mira plus autoanalyser (Roche, Almere, the Netherlands) as previously described (Venema *et al.*, 2005).

Samples collected at the ileal-caecal sphincter (at H in Figure 1) were evaluated for survival. *B. coagulans* in the samples was cultured on Glucose Yeast Extract Agar Medium (Tritium Microbiology, Veldhoven, the Netherlands) at 37 °C for 48 hours, under aerobic conditions. For this, samples were serially diluted 10-fold in sterile physiological saline solution (9 g/l; pH 7.0). The dilutions were plated immediately for viable *B. coagulans* (germinated cells and spores) and after heating (spores only). During the heating step, the dilutions were heated for 30 minutes in a 70 °C water bath, and then cooled immediately to approximately 45 °C before pipetting. Survival was determined in duplicate experiments under three conditions: GanedenBC^{30} only; GanedenBC^{30} + lactose; GanedenBC^{30} + fructose. Survival was expressed as a percentage of intake.

3. Results and discussion

Survival

GanedenBC^{30} spores are protected by a hardened coating primarily consisting of proteins, called the integument, that can withstand gastric acid and bile salts for delivery to the small and large intestines (Ara *et al.*, 2002). Figure 3 shows the cumulative survival of GanedenBC^{30} after passage through TIM-1 for the spores. After 6 hours, the mean cumulative survival of the GanedenBC^{30} (spores only) as a percentage of the intake without a sugar added was 70.4%±9.4%. With lactose added to the meal it was 56.0%±2.5% and with fructose added it was 59.7±12.05%. The theoretical survival (assuming no changes occurred during passage, i.e. no death and no growth) after 6 hours was 87.1% (Figure 3). This indicated that a large number of spores were still present at the terminal ileum (approximately 81% of the theoretical amount). Germinated cells (data not shown) could not be counted in the 4-6 hour sample due to overgrowth of the endogenous microbiota in the system. However, after 4 hours 13.9% more viable cells were present in the samples that were not heated (65.6% versus 51.7% respectively). Assuming that after this time-point no more viable germinated cells reached the colon (sampling bottle), a minimum of approximately 85% (70.4% in terms of spores + 13.9% as germinated cells) of the cells introduced in the gastric compartment survived passage through the stomach and small intestine.

Survival of the strain in the presence of lactose and fructose was markedly lower than in the absence of the sugars. This may mean that more spores germinated in the presence of these sugars, and since germinated cells are more sensitive to the GI conditions, survival was lower.

Kjeldahl nitrogen

The amount of milk protein available for absorption during the experiment with milk only was 3.5 g. The addition of GanedenBC^{30} to the milk gave a somewhat higher result of 3.7 g (Figure 4). Although the difference is small, this result shows the potential of GanedenBC^{30} to aid in protein digestion. This is even more likely given the low germination of the spores (as discussed above). If more spores had germinated, the influence on protein digestion would probably have been greater.

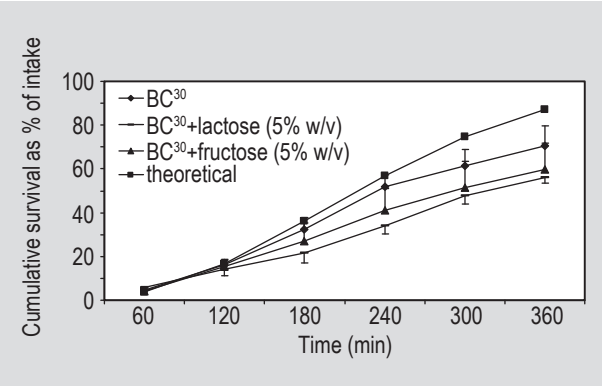

Figure 3. Cumulative survival of *Bacillus coagulans* spores (BC^{30}) under the three different conditions, as a percentage of the intake, after passage through TIM-1. The theoretical survival is the survival with the assumption that there would not be any growth or death of bacteria during passage through TIM-1.

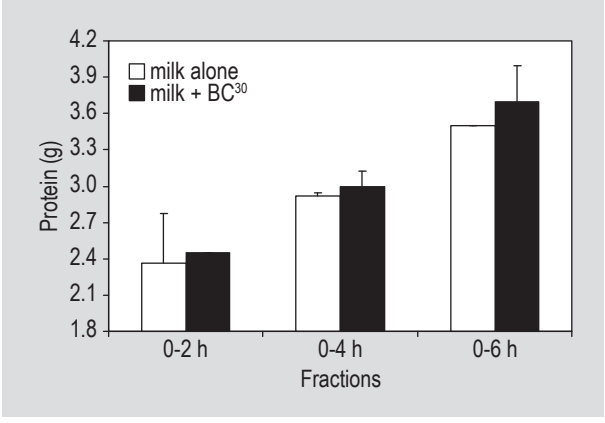

Figure 4. Cumulative amount of the bioaccessible fraction of protein in TIM-1 with and without the addition of GanedenBC^{30}.

Lactose and fructose digestion

Lactose and fructose digestion were measured by analysing the production of lactate, produced after fermentation of the sugars by *B. coagulans*. Lactate is only produced by vegetative cells, and thus is a measure of germination. In addition, the amount of lactate produced in comparison to an experiment without the addition of sugars was an indication of the digestion of the sugars, and therefore indicated that GanedenBC^{30} aided in digestion of the sugars. Figure 5 shows the cumulative amount of lactate produced under the various conditions. Lactate was measured in the dialysis liquid collected from the jejunum and ileum compartments (at O in Figure 1). Normally there is no lactate present in the dialysates of TIM-1. In the TIM-1 trial with GanedenBC^{30} alone there is already lactate present. This indicated that the germinated *B. coagulans* cells already produced lactic acid from endogenous materials such as bile and/or pancreatic juice or from the maltodextrin that was used as an excipient in the powder. In the TIM-1 trials with lactose or fructose added to the meal, the cumulative amount of lactate produced was slightly higher compared to the GanedenBC^{30} alone (0.12 and 0.18 mmol more for lactose and fructose, respectively). Although only minimally higher, the slightly higher lactate production in TIM-1 with lactose and fructose added implied that GanedenBC^{30} has the potential to aid in the digestion of these sugars and to prevent the occurrence of symptoms in individuals sensitive to these carbohydrates. As discussed above, most of the cells were recovered in the form of spores. If a larger fraction of the *B. coagulans* cells had germinated, the effect on lactose and fructose digestion would probably have been much greater. It should be realised that *in vivo* the interaction between *B. coagulans* and the intestinal microbiota may be much more complex than in the *in vitro* model, including various competitions, mutual inhibition, synergies *et cetera*. The model has a certain level of approximation to the *in vivo* conditions. However, the biggest advantage of TIM-1 is that individual parameters can be studied separately to determine their influence on the survival and/or activity of *B. coagulans*. For instance, in its real use, GanedenBC^{30} would be consumed as part of a standard diet. Due to reasons of interference with the analysis on survival and more importantly lactose, fructose and milk protein utilisation, no meal was added to TIM-1 besides the test product. This meant that the substrates available in the model (endogenous secretion fluids) were not sufficient for optimal bacterial growth. If the bacteria were added in the matrix of a complete meal, greater vegetative growth and metabolism of the strain would be expected. In that case, the assumed increase in germination would give an increased utilisation of lactose, fructose and protein. Furthermore, germination triggers such as L-alanine or inosine could be added to increase germination of the cells in GanedenBC^{30} (Hornstra *et al*., 2005), which will be investigated in future experiments.

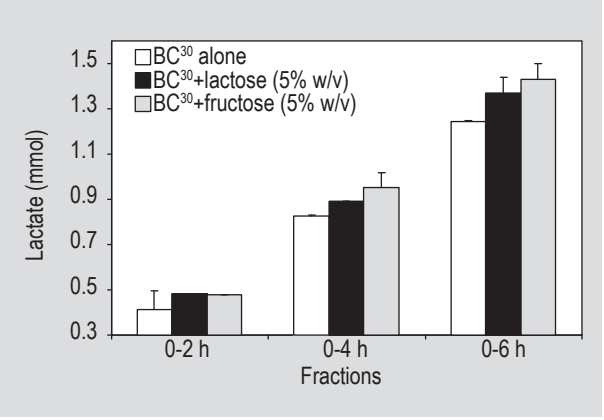

Figure 5. Cumulative amount of lactate produced in TIM-1 under the three different conditions.

The TIM-1 system simulates to a large degree the successive dynamic physiological conditions in the stomach and the small intestine. The model offers the possibility to simulate very closely the pH curves and the concentrations of enzymes in the stomach and small intestine, the concentrations of bile salts in the different parts of the gut, and the kinetics of passage of food through the stomach and intestine. In this model survival studies were performed with *Bifidobacterium* spp., *Lactobacillus* spp., *Streptococcus thermophilus*, *Lactococcus* strains and several pathogenic and food-borne bacteria under different gastro-intestinal conditions. The *in vitro* results showed good resemblance with the results obtained in studies with human volunteers (Marteau *et al*., 1997). This means that the results can be used to predict the survival of probiotic bacteria in the human intestinal tract. Therefore, the results obtained in the current study point into the direction of a good survival of GanedenBC^{30} in the GI tract of the human adult, as well as the potential to aid in digestion of protein carbohydrates. Specifically for lactose and fructose, this would be beneficial for those individuals that experience symptoms when ingesting these sugars due to a genetic or acquired intolerance. The benefit of ingestion of GanedenBC^{30} would be significant. Especially if the cells could be triggered into germination at the beginning of the small intestine, e.g. by ingesting them together with a diet containing L-alanine and/or inosine, or by including these in the powder formulation. In addition, in patients with pancreatic exocrine deficiency disorders such as chronic pancreatitis and cystic fibrosis, in which only small amounts of the normal amount of pancreatic enzymes are excreted (Ferrone *et al*., 2007), GanedenBC^{30} could contribute to the digestion of the protein and carbohydrates in their meal.

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Bacillus coagulans: a viable adjunct therapy for relieving symptoms of rheumatoid arthritis according to a randomized, controlled trial

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Abstract

Background: Lactic acid-producing bacteria (LAB) probiotics demonstrate immunomodulating and anti-inflammatory effects and the ability to lessen the symptoms of arthritis in both animals and humans. This randomized, double-blind, placebo-controlled, parallel-design, clinical pilot trial was conducted to evaluate the effects of the LAB probiotic preparation, *Bacillus coagulans* GBI-30, 6086, on symptoms and measures of functional capacity in patients with rheumatoid arthritis (RA) in combination with pharmacological anti-arthritic medications.

Methods: Forty-five adult men and women with symptoms of RA were randomly assigned to receive *Bacillus coagulans* GBI-30, 6086 or placebo once a day in a double-blind fashion for 60 days in addition to their standard anti-arthritic medications. Arthritis activity was evaluated by clinical examination, the American College of Rheumatology (ACR) criteria, the Stanford Health Assessment Questionnaire Disability Index (HAQ-DI), and laboratory tests for erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP).

Results: Subjects who received *Bacillus coagulans* GBI-30, 6086 experienced borderline statistically significant improvement in the Patient Pain Assessment score ($P = .052$) and statistically significant improvement in Pain Scale ($P = .046$) vs placebo. Compared with placebo, *Bacillus coagulans* GBI-30, 6086 treatment resulted in greater improvement in patient global assessment and self-assessed disability; reduction in CRP; as well as the ability to walk 2 miles, reach, and participate in daily activities. There were no treatment-related adverse events reported throughout this study.

Conclusions: Results of this pilot study suggest that adjunctive treatment with *Bacillus coagulans* GBI-30, 6086 LAB probiotic appeared to be a safe and effective for patients suffering from RA. Because of the low study population size, larger trials are needed to verify these results.

Trial registration: ACTRN12609000435280

Background

Probiotics have been used to treat and prevent a wide range of infectious and inflammatory diseases [1,2]. Of particular interest are lactic acid bacteria (LAB) probiotics and their immunomodulating and anti-inflammatory effects, which have been shown to lessen the symptoms of arthritis [3-8].

Rheumatoid arthritis (RA) affects more than 1.3 million American adults [9]. It commonly leads to significant disability and compromises quality of life. Pharmacological treatments for arthritis target the inflammatory process by suppressing the host reaction. Despite the number of effective pharmacological agents available today, a substantial proportion of patients will experience persistent, low-level disease activity [10]. This underscores the need for adjunctive therapies that are safe and can help relieve the painful symptoms of arthritis.

RA is an autoimmune disorder in which unchecked immune and inflammatory responses cause articular pain and eventually cartilage degradation and bone destruction [11]. Disease develops when there is an imbalance in the cytokine network, either from excess production of pro-inflammatory cytokines or from

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inadequate natural anti-inflammatory mechanisms [12]. Evidence has shown that there is a relationship between the gastrointestinal microbiota, the mucosal and systemic immune responses, and the development of arthritis [5,13,14]. LAB have been shown to significantly downregulate proinflammatory cytokines (eg, IFN-γ, IL-12, TNF-α) without altering regulatory cytokines (eg, IL-10, TGF-β) to cause anti-inflammatory effects that alleviate RA symptoms [3,14-19]. Therefore, it might be speculated that therapeutic strategies that aim to normalize the gut microflora in order to maintain proper gastrointestinal and immune system function may downregulate the abnormal inflammatory response and alleviate symptoms of RA.

GanedenBC^{30}*(Bacillus coagulans* GBI-30, 6086, Ganeden Biotech, Inc., Mayfield Heights, OH) is a strain of LAB that can withstand the low pH of stomach acid is activated in the intestines to modulate the gut microflora and the immune response [18-20]. The objective of this pilot study was to evaluate the effects of *Bacillus coagulans* GBI-30, 6086 on RA symptoms and the functional ability of patients with RA when used in combination with pharmacological anti-rheumatic medications.

Methods

Study design

This was a randomized, double-blind, placebo-controlled, parallel-design study of *Bacillus coagulans* GBI-30, 6086 LAB probiotic as an adjunctive therapy for the relief of symptoms of RA. The objective was to evaluate the effects of *Bacillus coagulans* GBI-30, 6086 on RA symptoms compared with placebo.

The treatment allocation scheme was generated and assigned by a third party who did not have direct patient contact. The treatment assignments were in sealed, tamper-proof, blinded envelopes and were handed out in sequential fashion according to a computer-generated randomization list. Study personnel did not have access to the randomization scheme or blinding process. Collected data were concealed in a pouch that remained unopened until all results were submitted.

This study was registered with the Australian New Zealand Clinical Trials Registry.

Patient population

Forty-five adult men and women with symptoms of RA were included in the study. Sample size was determined by power analysis. Subjects were recruited by the primary investigator from his daily practice and follow-up visits were conducted at the practice.

Inclusion criteria included men and women with RA for at least 1 year and up to 80 years of age. The criteria for the diagnosis of RA included four or more of the following symptoms: 1) morning stiffness lasting at least 1 hour; 2) soft tissue swelling in 3 or more joint areas observed by a physician; 3) swelling of the proximal interphalangeal, metacarpophalangeal, or wrist joints; 4) symmetric swelling; 5) rheumatoid nodules; 6) the presence of rheumatoid factor; and 7) radiographic erosions and/or periarticular osteopenia in hand and/or wrist joints. The first 4 criteria must have been present for at least 6 weeks for inclusion in the study.

The exclusion criteria were pregnancy, chronic inflammatory bowel disease, kidney disease, liver disease, exposure to > 10 mg/day of prednisolone, or treatment with other probiotic products.

Patients read and signed an approved consent form prior to enrollment in the study. The study protocol and test product(s) information were approved by an Institutional Review Board (IRB; Schulman Associates, Cincinnati, OH) prior to the beginning of the study.

Study treatment

Patients in each group underwent a baseline physical assessment and then were randomized in a double-blind manner to receive either placebo or 1 caplet of *Bacillus coagulans* GBI-30, 6086 (2 billion CFU) daily at approximately the same time each day, regardless of meals, for a period of 60 days. The probiotic preparation includes *Bacillus coagulans* GBI-30, 6086, green tea extract, methylsulfonylmethane, and vitamins and minerals (including vitamins A, B, C, D, E, folic acid, and selenium). The matching placebo contained microcrystalline cellulose. Adherence to the study protocol was determined by caplet count at each examination.

Patient assessment

The 45 men and women enrolled in the study were randomly assigned to receive *Bacillus coagulans* GBI-30, 6086 or placebo once a day for 60 days. Patients were examined at the primary investigator's practice at baseline, at 30 days, and at 60 days to assess symptoms and measures of disease activity. Blood draws were performed at each visit. The primary outcome was change from baseline compared with the end of the study period obtained by the American College of Rheumatology (ACR) [21] criteria questionnaire and the Stanford Health Assessment Questionnaire Disability Index (HAQ-DI) [22]. Outcomes were classified as "global" (sensitive to change in clinical trials) and "individual" (relatively insensitive to change in clinical trials). The global outcomes included patients' global assessment, pain assessment, and disability assessment, as well as physicians' global assessment, assessment of total painful joints out of 68 joints assessed, total swollen joints out of 66 joints assessed, and erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels from ACR and pain from the HAQ-DI. Individual outcomes were assessed based on hygiene (ability to wash and dry the body, take a tub bath, get on and off the toilet) and the ability to dress and groom, arise, eat, walk 2 miles,

reach, grip, and participate in daily activities. Serious adverse events were reported directly to the sponsor and IRB by each investigator.

Statistical analysis

For each outcome, summary measures based on the change from baseline to 60 days were obtained for each group and group differences were tested. For global variables, each response was normalized (using Blom scores) and the difference (end minus baseline) was obtained to provide a change score. A Student's t test was performed to test for a difference in the mean change score between subjects randomized to *Bacillus coagulans* GBI-30, 6086 vs placebo. The effectiveness of the normalization was checked via the Shapiro-Wilk test for normality and the folded form F statistic to test for equality of group variances. For individual outcomes, due to their discrete nature, we utilized the binary indicator of a difference score of less than zero, indicating some improvement in the score for the item. Barnard's exact unconditional test was used to compare the proportions who improved in the *Bacillus coagulans* GBI-30, 6086 group vs placebo for each individual scale. Two-sided $P < .05$ was considered statistically significant. Ninety-five percent confidence intervals were computed for the group difference for each outcome. For continuous outcomes, confidence intervals for mean differences were obtained based on a t test for normalized scores. For binary outcomes, exact confidence intervals for the difference in proportions were computed.

Analyses were performed using SAS Version 8.0 (SAS System, SAS Institute, Cary, NC) and StatXact Version 4.0 (Cytel Software Corporation, Cambridge, MA).

Results

Forty-five men and women who had RA for at least 1 year were enrolled in the study and randomly assigned to receive *Bacillus coagulans* GBI-30, 6086 or placebo once a day for 60 days (Figure 1). The majority of subjects were female (81.8%) and all were Caucasian. Subjects included in the study were between 36 and 82 years of age. The average age was 62.5 years. Although subjects over the age of 80 were excluded from the study because many subjects past this age have multiple health issues that might impact their participation or end results, 2 patients over the age of 80 were included because they met all other study criteria and were in good overall health. The subjects' standard of care for RA was not altered during the course of the study. Most subjects were taking and continued to take disease modifying antirheumatic drugs (DMARDs) (17 in the placebo group and 18 in the study treatment group) and 5 subjects continued to take non-steroidal anti-inflammatory drugs (NSAIDs) (3 in the placebo group and 2 in the study treatment group). Four subjects were not taking medication for RA (3 in the placebo group and 1 in the treatment group). One subject developed an upper respiratory infection (URI) and was started on antibiotics, so the study treatment was discontinued. This subject did not return for further visits and was, therefore, excluded from analyses. Although this was not an intent-to-treat analysis due to the 1 subject who discontinued treatment, each study group included 22 patients for analysis.

Efficacy

Subjects who received *Bacillus coagulans* GBI-30, 6086 experienced borderline statistically significant improvement from baseline in the Patient Pain Assessment score ($P = .052$) and statistically significant improvement from baseline in the Pain Scale ($P = .046$) compared with subjects randomized to receive placebo. *Bacillus coagulans* GBI-30, 6086 treatment resulted in greater improvement in patient global assessment, patient self-assessed disability, and reduction in total CRP (Table 1). There were no significant differences in the physician global assessment or physician assessment of painful and swollen joints. For the HAQ individual disability scores, the ability to walk 2 miles was marginally significant ($P = .072$) and the ability to reach was not quite significant ($P = .11$) (Table 2). *Bacillus coagulans* GBI-30, 6086 also outperformed placebo for improvement in the ability to participate in daily activities.

Eight subjects in the *Bacillus coagulans* GBI-30, 6086 group met ACR 20 criteria vs 6 subjects in the placebo group. See Table 3 for an analysis of each group.

Safety

There were no serious adverse reactions reported throughout this study. The treatment group reported 4 adverse events including shingles, poison ivy, a cold, and leg edema, all of which were deemed unrelated to the study treatment. The placebo group reported 3 adverse events including gastrointestinal reflux, URI, and urinary tract infection (UTI).

Discussion

The potential role of the intestinal microflora in modulating immune responses has led to an interest in using probiotics as preventive and therapeutic interventions. For example, it has been shown that the enteric microflora impact intestinal inflammatory responses and may contribute to the articular inflammatory characteristics of arthritis [13].

Rheumatoid arthritis is an autoimmune disease characterized by a loss of tolerance to autoantigens (self antigens), which triggers an inflammatory immune response that causes joint damage and functional impairment [23]. It has been speculated that infection by microbial pathogens may trigger autoimmune reactions through cross-reactivity, as environmental

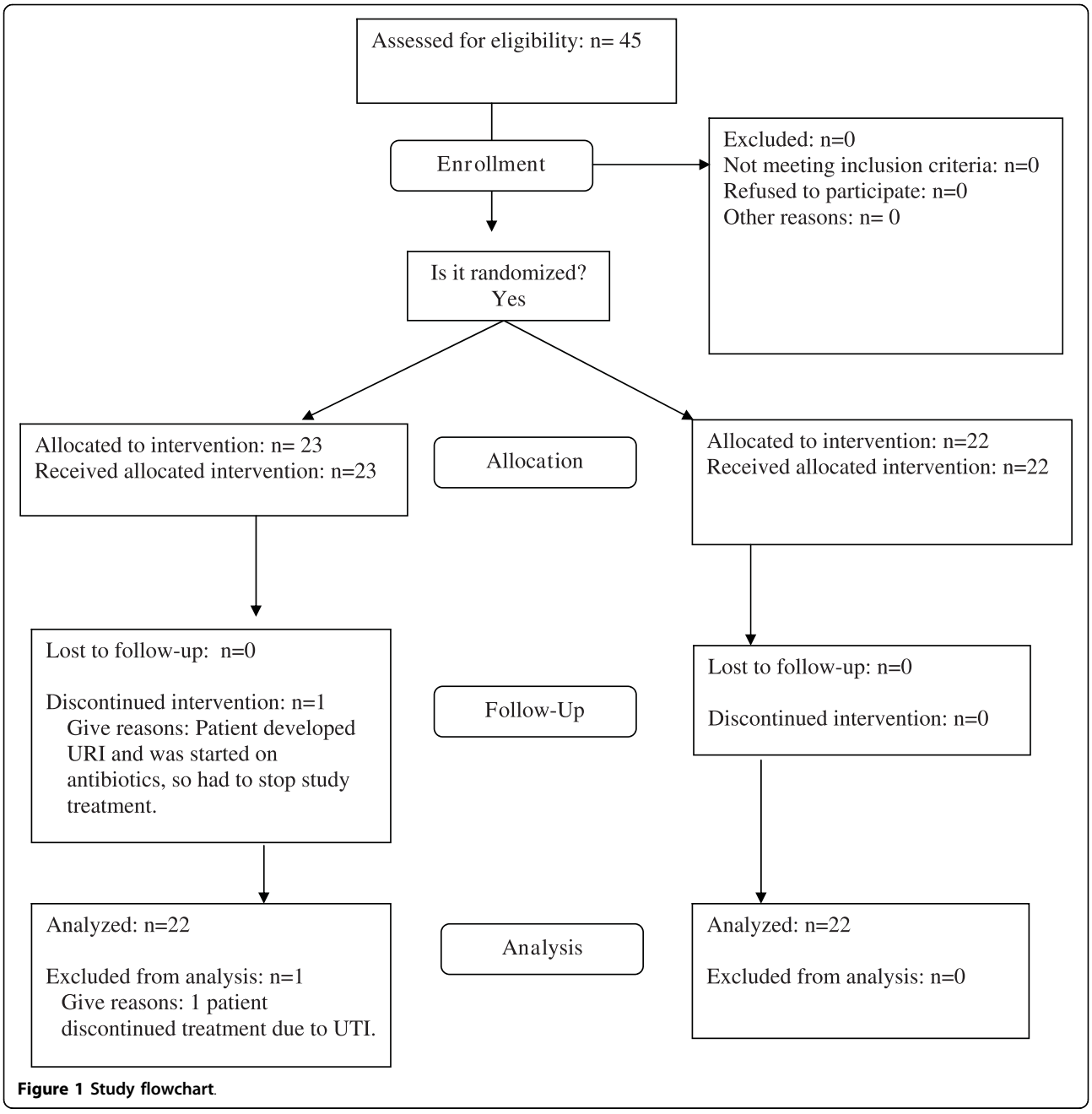

Figure 1 Study flowchart.

pathogens present antigens that mimic autoantigens [23]. Changes to the normal gastrointestinal microflora and dysregulation of the mucosal immune response to these pathogens may contribute to the development of autoimmune diseases such as RA [23]. This possibility has led investigators to evaluate the efficacy of probiotics for alleviating RA symptoms through modulation of the aberrant inflammatory autoimmune response.

Several preclinical studies have evaluated the effects of various strains of LAB probiotics on symptoms and clinical markers of arthritis. In one study [3], there was a statistically significant decrease in inflammation over 1 month in rats fed yogurt containing *Lactobacillus* GG (LGG) compared with rats fed plain yogurt or milk ($P < .05$). An earlier study [18] found that *Lactobacillus casei* reduced the incidence and development of collagen-induced arthritis in mice and downregulated the cellular and humoral immune responses to collagen in a dose-dependent manner. More recently, a study in rats with collagen-induced arthritis demonstrated that oral administration of *L casei* for 12 weeks reduced signs of arthritis, lymphocyte infiltration into the joint, and

Table 1 Mean change in global outcomes from baseline to 60 days

	Placebo/*Bacillus coagulans* GBI-30, 6086	Difference in Means (95% CI)	*P* Value[a]
Score	22/22	0.006 (-0.33, 0.35)	.97
Pain	21/22	0.46 (0.01, 0.91)	.046
Patient global	22/22	0.047 (-0.38, 0.47)	.83
Patient pain	22/22	0.41 (-0.004, 0.82)	.052
Patient disability	22/22	0.19 (-0.16, 0.54)	.28
Physician global	22/22	0.019 (-0.62, 0.66)	.95
Painful joints	22/22	-0.074 (-0.81, 0.66)	.84
Swollen joints	22/22	0.011 (-0.62, 0.64)	.97
ESR	21/21	-0.054 (-0.49, 0.38)	.80
CRP	22/22	0.008 (-0.52, 0.53)	.98

Score = the mean of the eight category scores in the HAQ.
ESR = erythrocyte sedimentation rate.
CRP = C-reactive protein.
[a]*P* values calculated using Student's *t* test for group differences in the mean change scores from baseline to 60 days (95% confidence interval).

degradation of cartilage when compared with control animals [15]. In addition, rats receiving *L casei* had lower levels of proinflammatory cytokines and reduced T cell proliferation, as well as increased production of the anti-inflammatory cytokine IL-10. Other studies have reported similar improvements in measures of arthritis after administration of *Lactobacillus fermentum* [16] and *Lactobacillus delbrueckii* [17].

A pilot clinical study [5] evaluated the long-term effects of LGG on symptoms of RA. In this double-blind study, 21 patients with RA were randomized to receive 2 capsules of LGG or placebo twice daily for 12 months. The mean number of tender and swollen joints decreased from 8.3 to 4.6 in the LGG group and from 5.5 to 4.8 in the placebo group (P = .41). However, considering only the long-term effects from baseline to 12 months, the mean number of tender and swollen joints decreased from 8.3 to 4.4 in the LGG group and increased from 5.5 to 5.6 in the placebo group (P = .09). RA activity was reduced in 71% of patients in the LGG group vs 30% of patients in the placebo group (P = .15). Although there were no statistical differences in clinical or biochemical parameters, more patients in the LGG group reported a greater feeling of well-being.

In the present study, patients with RA who received *Bacillus coagulans* GBI-30, 6086 experienced borderline statistically significant improvement from baseline in the Patient Pain Assessment score (P = .052) and statistically significant improvement from baseline in the Pain Scale (P = .046) vs placebo. Treatment with *Bacillus coagulans* GBI-30, 6086 resulted in greater improvement in patient global assessment, patient self-assessed

Table 2 Proportion of patients who improved in individual outcomes from baseline to 60 days

	Placebo		*Bacillus coagulans* GBI-30, 6086			
	Number Improved/n	Proportion	Number Improved/n	Proportion	Difference in Proportions (95% CI)	*P* Value[a]
Arising	3/22	0.14	4/21	0.19	-0.054 (-0.36, 0.24)	.71
Walking 2 miles	2/22	0.091	7/22	0.32	-0.23 (-0.51, 0.09)	.072
Daily activities	2/22	0.091	4/22	0.18	-0.091 (-0.38, 0.21)	.53
Dressing and grooming	4/22	0.18	4/22	0.18	0 (-0.30, 0.30)	1.0
Eating	6/22	0.27	4/22	0.18	0.09 (-0.22, 0.39)	.53
Hygiene	2/22	0.091	2/22	0.091	0 (-0.28, 0.28)	1.0
Reach	4/22	0.18	9/22	0.41	-0.23 (-0.52, 0.09)	.11
Grip	5/22	0.23	4/22	0.18	0.045 (-0.26, 0.35)	.81

[a]*P* values calculated using Barnard's exact unconditional test to compare the proportion who improved in both groups for each individual scale.

Table 3 Outcomes meeting ACR 20 criteria by treatment group

	Bacillus coagulans GBI-30, 6086		Placebo	
	% Change	Met ACR 20 Criteria?	% Change	Met ACR 20 Criteria?
Total painful joints	-29.9	Yes	-29.3	Yes
Total swollen joints	-17.0	No	-19.3	No
Patient global	-11.7	No	-1.5	No
Patient pain	-19.8	No	-1.6	No
Patient disability	-22.5	Yes	-2.2	No
Physician global	-21.6	Yes	-22.3	Yes
CRP/ESR	-46.0/+6.5	Yes	+4.8/+9.1	No

ACR = American College of Rheumatology.
ESR = erythrocyte sedimentation rate.
CRP = C-reactive protein.

disability, and reduction in total CRP. In addition, the ability to walk 2 miles, reach, and participate in daily activities steadily improved over the 60 days; however, these results did not reach statistical significance. The ability to arise, dress and groom, eat, grip, and maintain hygiene were similar between the 2 treatment groups. The overall results of this pilot study support a role for *Bacillus coagulans* GBI-30, 6086 as adjunctive therapy for inflammatory diseases, as well as affirm the pathophysiological connection between the gut microflora, the mucosal immune system, and arthritic diseases.

Bacillus coagulans GBI-30, 6086 is a gram-positive, spore-forming, aerobic to microaerophilic LAB bacillus [24] that has a demonstrated ability to improve gastrointestinal health. To be effective, probiotics must survive gastric and bile acids [25] in order to reach the intestinal tract, colonize the host epithelium, and exert a beneficial effect [26]. Most LAB probiotics are inactivated by bile and low gastric pH, whereas *Bacillus coagulans* GBI-30, 6086 cultures are protected by a hardened layer of organic spore coating that can withstand low gastric pH for delivery to the more favorable environment of the small intestine [20,25,27].

Although still controversial, evidence has suggested a possible causal link between gut microbes and systemic inflammatory disorders [5]. Once in the intestines, *Bacillus coagulans* GBI-30, 6086 is activated and releases anti-inflammatory molecules or acts indirectly to eradicate organisms in the gut responsible for the inflammatory immune response. Activated *Bacillus coagulans* GBI-30, 6086 produces bacteriocins [28] and lowers local pH by producing L(+) lactic acid that, along with competition for sites of mucosal adherence, works to dislodge and eliminate any antagonizing microbes that may be contributing to an inflammatory response. *Bacillus coagulans* GBI-30, 6086 also produces short-chain fatty acids such as butyric acid, a compound known to support the health and healing of cells in the small and large intestines and to contribute to modulation of the mucosal immune system.

While some RA studies follow subjects for a longer period of time, it was the feeling of the investigators that this study could be completed in a shorter timeframe. Many patients show relief from symptoms within 60 days when treated with other traditional anti-inflammatory agents such as NSAIDs, and the same would be expected of the study treatment. Therefore, subjects in this pilot study were treated and evaluated for 60 days.

Conclusions

These study data suggest that *Bacillus coagulans* GBI-30, 6086 LAB probiotic may be a safe and effective adjunct therapy for the relief of symptoms of RA. Because of the low study population size, large-scale, controlled clinical trials are needed to confirm these results.

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Authors' contributions

DM conceived of the study and participated in the study design, patient evaluations, and manuscript development. KE and JH participated in patient evaluations and coordination, and helped draft the manuscript. All authors have read and approved the final manuscript.

Competing interests

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A review of probiotics studies in HIV research suggests improved immunological presentation and preservation of viral host restrictive factors of TH17 in HIV patients

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Background

Recovery of gut mucosal immune system is slow and incomplete during HAART therapy, leading to elevated inflammation rates, increased mitochondrial damage and the pathogenesis of replicatively competent escape mutations. A recent study of Ganeden BC30, Bacillus coagulans GBI-30, demonstrated safety, increased CD4+ counts in patients on HARRT, and a significant increase in CD69 and maturation of dendritic cells in vitro. Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14 demonstrate efficacy in treating naïve HIV patients against gastrointestinal and urogenital infections . Restoration of viral host restrictive factors of Th17 cell lines in GALT may prevent the evolving viral diversity, provoking increased CD4 presentation and regulation of inflammatory cytokine levels responsible for increased viral replication.

Methods

Metagenomic sequencing analysis has shown alternations in intestinal microbiota in HIV patients. Measurements of immunological parameters including Serum cytokine levels and total serum IgE levels (CD4 lymphocyte count, CBC, levels of TNFÎ±, NFAT, IL-12, IL-10, and G-CSF [Kim, et.al. 2006]) were measured in studies reviewed here.

Results

A recent study of Ganeden BC30, bacillus coagulans GBI-30, 6086 demonstrated safety, and an increase in % CD4, a significant increase in CD69 activation and maturation of dendritic cells in HIV patients on HARRT and improvement in GI function. Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14 have demonstrated efficacy treating naïve HIV patients gastrointestinal and urogenital infections and prevent diarrhea and increased CD4 T-lymphocyte percentages in HIV patients.

Conclusions

Intestinal microbiota are integral to the homeostasis and functioning of immune cells. The loss of intestinal flora by HIV infection is severely detrimental to the recombination of CD4 cells. Restoring proper biodiversity in the gut by safe, efficacious probiotics demonstrates promise in increasing Th17 cells by restoring GALT. GanedenBC30 may down regulate TNF-a and other inflammatory cytokines and mitigate ARV-related SAEs.

Data suggests that replenishment of Th17 CD4 cells in the gut mucosa during HAART correlates with improved function of the gut mucosal immune system and its function.. Studies by NIH of probiotics to lower microbial translocation and immune activation in HIV-infected adolescents.

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